

## *Thiohalomonas denitrificans* gen. nov., sp. nov. and *Thiohalomonas nitratireducens* sp. nov., novel obligately chemolithoautotrophic, moderately halophilic, thiodenitrifying *Gammaproteobacteria* from hypersaline habitats

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A novel group of moderately halophilic, obligately chemolithoautotrophic, sulfur-oxidizing *Gammaproteobacteria* was found in sediments of various inland hypersaline lakes and a solar saltern. These bacteria were enriched and isolated with thiosulfate as electron donor and nitrate as electron acceptor at 2 M NaCl. Ten isolates (HLD strains) were long non-motile rods. They grew anaerobically as complete denitrifiers, and aerobically under micro-oxic conditions. Sulfate was the final product of thiosulfate and sulfide oxidation, and nitrite and N<sub>2</sub>O were intermediates of nitrate reduction to N<sub>2</sub>. The HLD strains grew optimally at pH 7.3–7.8, and at NaCl concentrations of 1.5–2.0 M. On the basis of phenotypic and genetic analysis, the moderately halophilic, thiodenitrifying isolates are proposed to be assigned to a new genus and species, *Thiohalomonas denitrificans* gen. nov., sp. nov. The type strain is HLD 2<sup>T</sup> (= DSM 15841<sup>T</sup> = UNIQEM U222<sup>T</sup>). A single strain, HRhD 3sp<sup>T</sup>, with vibrio-shaped cells, was obtained from a co-culture capable of complete denitrification of nitrate in the presence of either thiocyanate or thiosulfate as electron donor. It grew anaerobically with thiosulfate, reducing nitrate to nitrite, or under micro-oxic conditions at 1.0–2.5 M NaCl with an optimum at 1.0 M. Strain HRhD 3sp<sup>T</sup> was genetically related to the HLD strains at the level of a separate species and is described as *Thiohalomonas nitratireducens* sp. nov. The type strain is HRhD 3sp<sup>T</sup> (= DSM 16925<sup>T</sup> = UNIQEM U248<sup>T</sup>).

Hypersaline habitats, such as inland salt lakes and solar salterns, are traditionally regarded as low-diversity environments, dominated by heterotrophic haloarchaea that grow optimally in saturated NaCl brines (Oren, 2002). Some of the moderately halophilic/extremely salt-tolerant *Gammaproteobacteria*, such as *Halomonas* and *Marinobacter*, better

adapted to lower salinity, have an advantage over less flexible haloarchaea at fluctuating salinity (Ventosa *et al.*, 1998). Recent studies have demonstrated that moderately and extremely halophilic bacteria represent an important component of prokaryotic communities, even under extremely high-salt conditions (Antón *et al.*, 2002; Sorokin *et al.*, 2006a). Furthermore, even chemolithoautotrophic bacteria can be abundant and active under such conditions. In particular, chemolithoautotrophic sulfur-oxidizing bacteria (SOB) have the best chance to adapt to extremely high-salt conditions because of a very high energy yield available during complete oxidation of sulfide/thiosulfate to sulfate (Oren, 1999).

Previously, we demonstrated the potential of haloalkaliphilic SOB belonging to the genus *Thioalkalivibrio* for growth in saturated soda brines (Sorokin & Kuenen, 2005). In addition, a large diversity of moderately and even

Abbreviation: SOB, sulfur-oxidizing bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences obtained in this study are DQ469580–DQ469581, DQ836238, EF117909–EF117913 and EF455919, and for the *nirS* gene from strain HLD 2<sup>T</sup>, AM492191.

Cytochrome difference spectra of cell-free extract obtained from strain HLD 2<sup>T</sup> cells grown anaerobically with nitrate and thiosulfate, and graphs showing the influence of NaCl on anaerobic growth of HLD strains with nitrate and thiosulfate, and on the activity of thiosulfate oxidation by washed cells of strain HLD 2<sup>T</sup> grown anaerobically are available with the online version of this paper.

extremely halophilic SOB has been discovered in hypersaline habitats with neutral pH (Sorokin *et al.*, 2006b). Among the six different types of halophilic SOB discovered, two groups of aerobic moderate halophiles belong to the known genera *Thiomicrospira* (Sorokin *et al.*, 2006c) and *Halothiobacillus*. The latter contains the only SOB species, *Halothiobacillus halophilus*, which was known previously as being capable of growing at NaCl concentrations up to 4 M (Wood & Kelly, 1991). The other four novel groups belong to previously unknown lineages within the *Gammaproteobacteria*. They include two groups of moderately halophilic thiodenitrifiers and two groups of extreme halophiles.

Here we describe the properties of a novel group of moderately halophilic, obligately chemolithoautotrophic and facultatively anaerobic, sulfur-oxidizing *Gammaproteobacteria*, consisting of 11 isolates from sediments of hypersaline lakes and a solar saltern. These bacteria dominated the anaerobic enrichment cultures with thiosulfate as electron donor and nitrate as electron acceptor at 2 M NaCl.

Sediments from hypersaline lakes in north-east Mongolia, the Kulunda Steppe (south-west Siberia, Altai, Russia) and the Crimea (Ukraine), Lake Baskunchak (South Russia) and the Sečovlje Adriatic Sea solar saltern were used as inoculum in this work (Sorokin *et al.*, 2006b). In general, the salt content of the brines varied from 10 to 38 % (w/v) and the pH ranged from 6.5 to 8.2; the dominant ions were Na<sup>+</sup>, Mg<sup>2+</sup>, Cl<sup>-</sup> and SO<sub>4</sub><sup>2-</sup>.

The following mineral base medium was used for enrichment and growth experiments: NaCl, 0.5–3.0 M; K<sub>2</sub>HPO<sub>4</sub>, 10 mM; NH<sub>4</sub>Cl, 5 mM. The pH was adjusted to 7.2–7.5 with 10 % KH<sub>2</sub>PO<sub>4</sub>. After sterilization, the medium was supplemented with 2 mM MgCl<sub>2</sub>·6H<sub>2</sub>O and 1 ml trace metal solution l<sup>-1</sup> (Pfennig & Lippert, 1966). Thiosulfate (5–20 mM) or thiocyanate (5–10 mM) were added as electron donors (the range is for aerobic and anaerobic conditions, respectively), and KNO<sub>3</sub> (20 mM), KNO<sub>2</sub> (5 mM), N<sub>2</sub>O (10 % in the gas phase) or O<sub>2</sub> (2–20 % in the gas phase) were used as electron acceptors. Filter-sterilized NaHCO<sub>3</sub> (10–40 mM, pH 8.0; the range depending on the amount of thiosulfate added) was used as the carbon source and as the pH buffer. Anaerobic cultivation was routinely performed in 100 ml serum bottles with 80 ml medium, which was made anoxic by five sequential cycles of evacuation–flushing with argon. In the case of aerobic incubations, the liquid/gas ratio was 10 : 90. Cultivation was performed at 30 °C. Growth was monitored by following the optical density at 600 nm and by qualitative tests for nitrate/nitrite (Merck). Positive enrichment cultures were stabilized by several 1 : 100 transfers and, finally, pure cultures were obtained after several dilution to extinction series. The purity of the cultures was checked by the absence of growth on medium where thiosulfate was replaced by yeast extract (1 g l<sup>-1</sup>) and further confirmed by sequencing. Dialysis cultivation and use of sucrose gradient centrifugation to separate a nitrate-reducing strain from the mixed denitrifying enrichment were performed as described previously (Sorokin *et al.*, 2006b).

Nitrogen (nitrate, nitrite, N<sub>2</sub>O) and sulfur (sulfide, sulfur, thiosulfate, sulfate, thiocyanate, tetrathionate) compounds in growth experiments were analysed as described previously (Sorokin *et al.*, 2001a, b, 2004). Protein concentrations were measured by the Lowry method after removal of interfering sulfur compounds either by washing (thiosulfate, thiocyanate) or by overnight extraction with acetone (sulfur). Cellular fatty acids were extracted with a mixture of methanol/chloroform and analysed by GC-MS according to Zhilina *et al.* (1997). Phase-contrast micrographs were obtained using an Axioplan Imaging 2 microscope (Zeiss). For electron microscopy, the cells were subjected to cryo-fixation in glutaraldehyde (final concn 3 %, v/v) at pH 7.0 in the presence of 0.5 M NaCl, then post-fixed in 1 % (w/v) OsO<sub>4</sub>/0.5 M NaCl for 3 h at room temperature, washed and stained overnight with 1 % (w/v) uranyl acetate, dehydrated in an ethanol series and embedded in Epon resin. Thin sections were contrasted with 1 % (w/v) lead citrate.

The isolation of genomic DNA and subsequent determination of the G+C content of the DNA were carried out according to Marmur (1961). DNA–DNA hybridization was performed by the thermal denaturation/reassociation technique (De Ley *et al.*, 1970). Genomic DNA for PCR amplification was extracted from the cells using the Ultra-Clean Soil DNA Extraction Kit (MolBio Laboratories) following the manufacturer's instructions. The nearly complete 16S rRNA gene was obtained from pure cultures using bacterial primers GM3F and GM4R (Schäfer & Muyzer, 2001). PCR products were purified from low-melting-point agarose using the Wizard PCR-Prep kit (Promega) according to the manufacturer's instructions. Sequencing was performed using the BigDye Terminator v.3.1 Cycle Sequencing Kit with an ABI 3730 DNA automatic sequencer (Applied Biosystems). The sequences were aligned with those from GenBank using CLUSTAL W. Phylogenetic trees were reconstructed with four different algorithms using the TREECONW software package (Van de Peer & De Wachter, 1994). Pairwise evolutionary distances (expressed as estimated changes per 100 nt) were computed by using the method of Jukes & Cantor (1969). The resulting phylogenetic tree was constructed by the neighbour-joining method. Bootstrap analysis (100 replications) was used to validate the reproducibility of the branching patterns of the trees.

Cytochrome *cd*<sub>1</sub>-containing nitrite reductase genes (*nirS*) were amplified from 50 ng genomic DNA from strain HLD 2<sup>T</sup> using primer pair nirS1F and nirS6R and protocols described previously by Braker *et al.* (1998). PCR products of the expected size (approx. 890 bp) were purified with the QIAquick PCR purification kit (Qiagen). Both strands were sequenced directly from the PCR product on an ABI 3100 automated DNA sequencer (Applied Biosystems) using gene-specific primers nirS1F and nirS6R and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Forward and reverse strand sequences were assembled by using Seqman II software (DNASTAR) and aligned to

sequences from the EMBL database with the ARB fast aligner feature ([www.arb-home.de](http://www.arb-home.de)). For phylogenetic analysis a filter was calculated to omit insertions or deletions (indels) due to ambiguous positional homology and included 241 aa positions. Analyses were performed with the PHYLIP software package version 3.66 (Felsenstein, 1989). Trees were reconstructed by using the distance-matrix-based neighbour-joining method with the Jones–Taylor–Thornton amino acid replacement model, the parsimony method PROTPARS and the maximum-likelihood method PROTML. Statistical evaluation of tree topologies was performed with 1000 resamplings for each method.

Only nitrate supported thiosulfate oxidation in anaerobic enrichments at 2 M NaCl with thiosulfate as electron donor and  $\text{NO}_x$  as electron acceptors. Positive enrichments were obtained from various lake sediments and from the solar saltern. In all cases nitrite and  $\text{N}_2\text{O}$  were observed as major intermediates of nitrate reduction with  $\text{N}_2$  as the final product. Thiosulfate was oxidized to sulfate with occasional formation of elemental sulfur (at high levels of nitrite production) as an intermediate. Despite vigorous growth in liquid culture, colony formation could not be achieved, neither on the agar plates in anaerobic jars nor in agar shake tubes. The isolation of pure cultures was only possible after several rounds of serial dilution to extinction with a reduced concentration of nitrate (i.e. 5 instead of 20 mM) to avoid inhibition by nitrite. Finally, 10 strains (abbreviated as HLD) were isolated in pure culture (Table 1).

All HLD isolates had long, non-motile, rod-shaped cells (Fig. 1a, c). Under unfavourable growth conditions, such as the substitution of  $\text{Cl}^-$  by  $\text{SO}_4^{2-}$  or  $\text{Na}^+$  by  $\text{K}^+$  (above 50%), the formation of cyst-like round cells (Fig. 1b) covered with an extracellular matrix layer (Fig. 1e) was observed. Thin sectioning of one of the strains revealed two interesting ultrastructural features inside the cells. The cytoplasm in the cell tips had a distinct grainy texture, which was clearly different from the more homogeneous middle zone containing the genomic DNA (Fig. 1d). It might be that the growing tips of the cells contain a high number of ribosomes in contrast to the 'old' middle part. Furthermore, in many cells a single- or double-ring structure could be seen (Fig. 1f), which might be mesosomes – a cell membrane extension binding to the chromosome (Silva *et al.*, 1976).

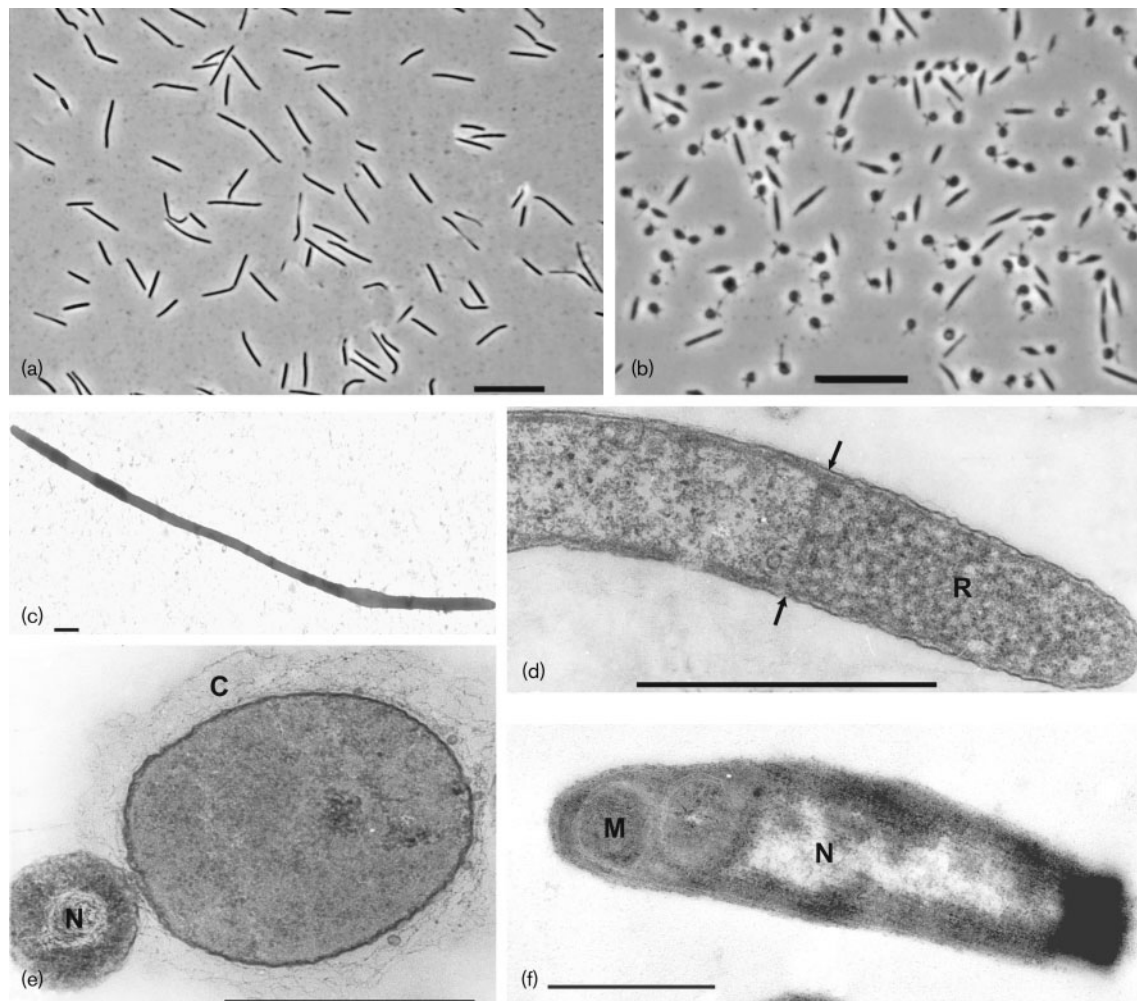
Despite different geographical origins, the HLD isolates consisted of a single gene species according to their G + C content (58–60 mol%) and the results of DNA–DNA hybridization (>65% homology) (Wayne *et al.*, 1987). Phylogenetic analysis of seven isolates from different geographical locations revealed that they formed a separate lineage within the *Gammaproteobacteria* with '*Thiobacillus prosperus*' as the closest culturable relative (92% sequence homology) (Fig. 2a). Analysis of the fatty acid composition of the polar lipids in strain HLD 2<sup>T</sup> demonstrated a pattern typical for the moderately halophilic *Gammaproteobacteria* (such as *Halomonas*), with  $\text{C}_{16:0}$ ,  $\text{C}_{16:1}$  and  $\text{C}_{18:1}$  dominant (Table 2).

Cytochrome spectra obtained from cells of HLD 2<sup>T</sup> grown anaerobically with nitrate showed a profile indicative of the presence of cytochrome *cd*<sub>1</sub>-type nitrite reductase (supplementary Fig. S1, available with the online version of this paper). This was further supported by detection of the *nirS* gene (encoding the *cd*<sub>1</sub> nitrite reductase) in strain HLD 2<sup>T</sup> by PCR. The obtained sequence fell into the radiation of gammaproteobacterial *nirS* genes with highest similarity to the enzyme from heterotrophic, halophilic denitrifiers of the genus *Marinobacter* (Fig. 2b).

The HLD strains grew best anaerobically with thiosulfate and nitrate. Growth was not observed with either  $\text{H}_2$  or organic compounds (i.e. acetate, pyruvate and yeast extract) used as electron donors instead of thiosulfate. Sulfate was the final product of thiosulfate oxidation. However, accumulation of nitrite at a high initial nitrate concentration (i.e. 10–20 mM) was a common problem for all the strains. Nitrite started to inhibit growth at concentrations above 4–5 mM and caused incomplete oxidation of thiosulfate to sulfur and growth inhibition. This can be avoided by providing the nitrate in small portions at low concentrations (3–5 mM) after complete reduction of the produced nitrite to  $\text{N}_2$ . In this way the HLD cultures had a relatively high growth yield [4–5 mg protein  $(\text{mmol S}_2\text{O}_3^{2-})^{-1}$ ] and a maximum growth rate of 0.03–0.04 h<sup>-1</sup> at 2 M NaCl. Despite the fact that nitrite and  $\text{N}_2\text{O}$  were produced as intermediates during anaerobic growth with nitrate, and that washed cells, grown with nitrate, could reduce both intermediates in the presence of thiosulfate, anaerobic growth could be initiated only with nitrate. Growth with

**Table 1.** Pure cultures of moderately halophilic denitrifying SOB from hypersaline habitats

Region	Type	Strains	DNA G + C (mol%)
Kulunda Steppe (south-west Siberia, Russia)	Hypersaline lakes	HLD 1–HLD 5, HLD 12	58.0–60.0
North-east Mongolia	Hypersaline lakes	HLD 13	59.0
Lake Baskunchak (South Russia)	Hypersaline lakes	HLD 14	58.8
Crimea (Ukraine)	Hypersaline lakes	HLD 15	60.1
Slovenia	Sea saltern	HLD 16	59.5

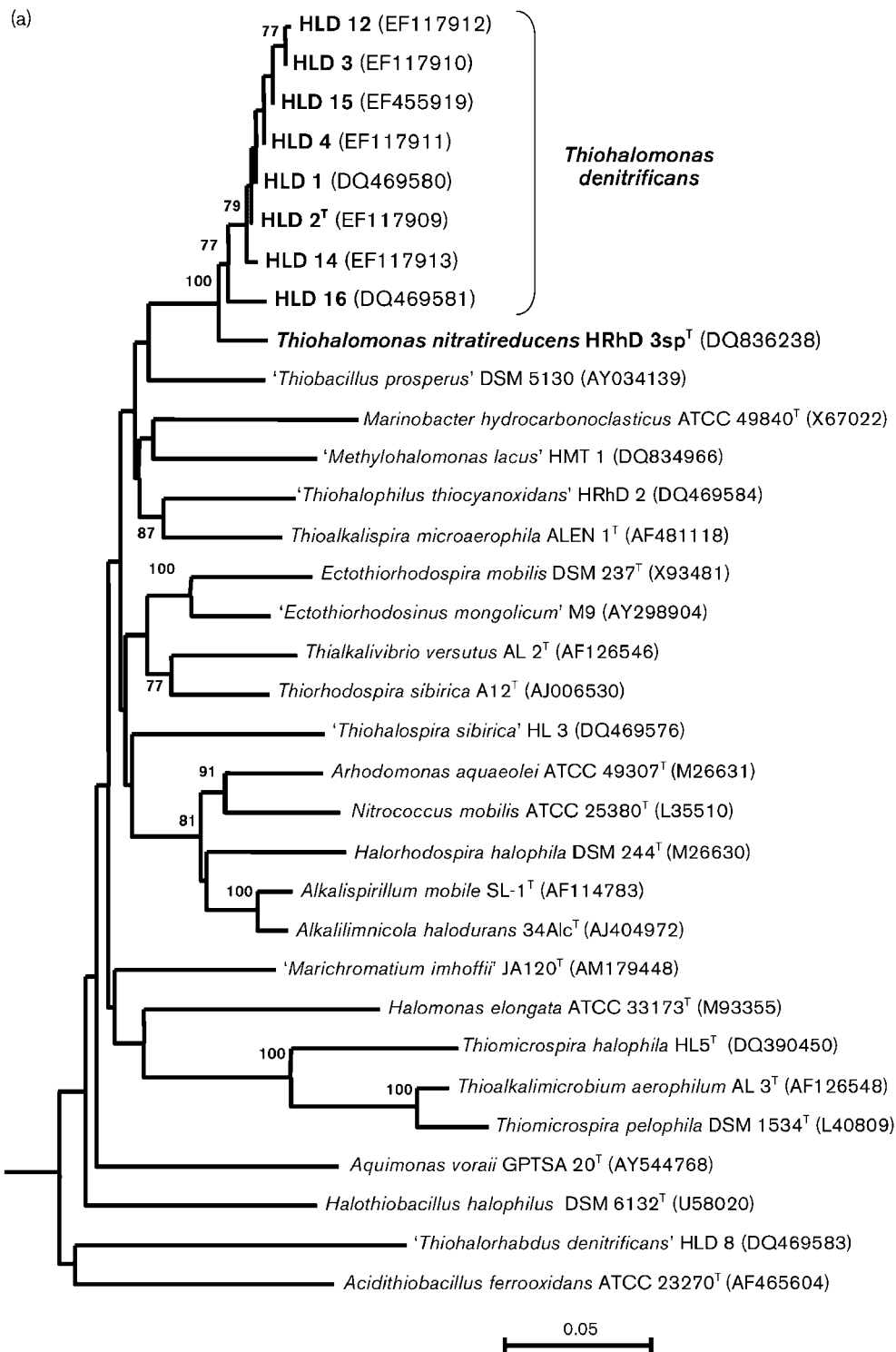


**Fig. 1.** Cell morphology of moderately halophilic, thiodenitrifying strain HLD 2<sup>T</sup> grown anaerobically with 2 M NaCl. (a, b) Phase-contrast micrographs (bars, 10  $\mu$ m); (c–f), electron micrographs of total preparation (c) and thin sections (d–f) (bars, 1  $\mu$ m). (a, c, d, f) Cells grown under normal conditions (2 M NaCl); (b, e), cyst formation under unfavourable conditions (i.e. replacement of 75% Cl<sup>-</sup> by SO<sub>4</sub><sup>2-</sup>). N, Nucleoid; R, ribosome-like structures; C, capsule; M, mesosome-like structures; arrows indicate separation of the cells into two different parts.

oxygen instead of nitrate as electron acceptor was less regular. In the case of a few successful incubations, growth was observed under micro-oxic conditions at O<sub>2</sub> concentrations between 2 and 5% (v/v) in the gas phase. The HLD strains grew within a relatively narrow salt range from 1.0 to 3.0 M NaCl, with an optimal concentration of 1.5–2 M (supplementary Fig. S2a, available with the online version of this paper). Some of them still oxidized thiosulfate at lower salinity with the production of nitrite and elemental sulfur, but without substantial biomass growth. Washed cells, grown anaerobically with nitrate at 2 M NaCl, oxidized thiosulfate most actively with N<sub>2</sub>O as electron acceptor. With all four different electron acceptors (O<sub>2</sub>, NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup> and N<sub>2</sub>O) washed cells exhibited maximum activity at very high salt concentrations, but were lysed rapidly at 0.5 M NaCl (supplementary Fig. S2b, available with the online version of this paper). The pH values suitable for growth

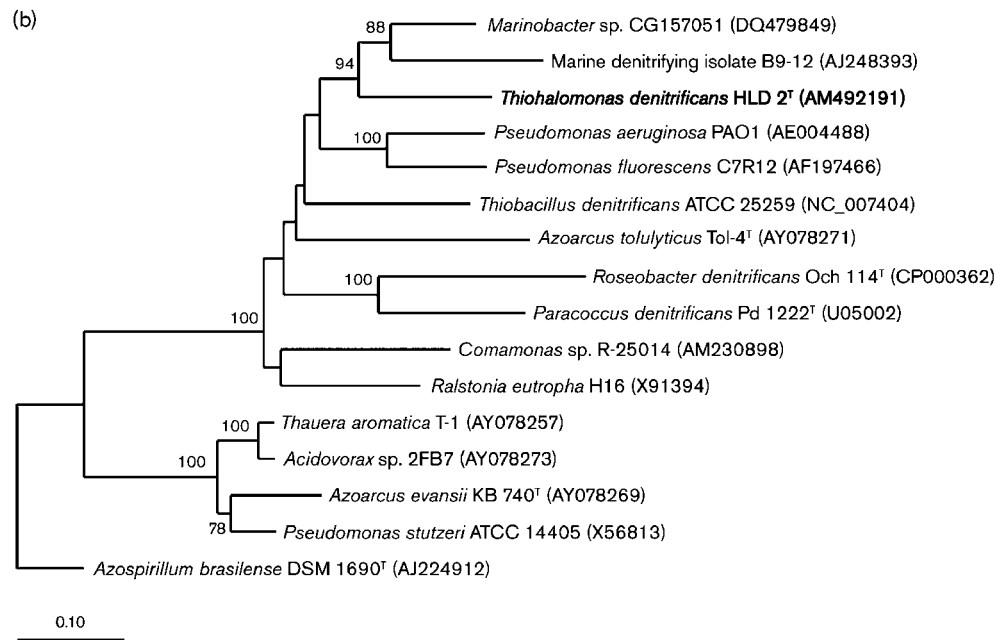
tested under aerobic conditions for strains HLD 2<sup>T</sup> and HLD 16 were limited to a range from 6.5 to 8.2, with an optimum between 7.3 and 7.8.

When thiosulfate was replaced by thiocyanate (NCS<sup>-</sup>), only a single anaerobic enrichment (from a mixture of sediment samples from the Kulunda Steppe lakes) gave a positive result at 1–2 M NaCl, in which the oxidation of thiocyanate to sulfate was linked to a complete reduction of nitrate to N<sub>2</sub> gas. Serial dilutions were positive only up to 10<sup>-4</sup> with four to five different morphotypes still present. However, replacement of thiocyanate by thiosulfate allowed selection of a binary co-culture, which reduced nitrate to N<sub>2</sub> without intermediate nitrite accumulation at 1–2 M NaCl. In this case the co-culture was more efficient than single HLD strains. The association consisted of thin rods as the dominant morphotype, and thick vibrios as a minor component.



The co-culture was very stable and very difficult to separate into individual species. Final resolution of the co-culture resulted in the isolation of a pure culture of the nitrate-reducing, vibrio-shaped strain HRhD 3sp<sup>T</sup> (by sucrose gradient centrifugation) and the rod-shaped, nitrite-reducing strain HRhD 2. The latter has recently been described as a new genus and species 'Thiohalophilus thio-

*cyanoxidans*' (Sorokin *et al.*, 2007). Strain HRhD 3sp<sup>T</sup> had vibrio-shaped, non-motile cells, the length of which depended on the growth conditions (Fig. 3). It had a capacity for anaerobic growth with thiosulfate (but not with thiocyanate) and nitrate, which was limited by high sensitivity to accumulating nitrite and an inability to reduce it further. Accumulation of 2.5–3.0 mM nitrite completely



**Fig. 2.** Phylogenetic position of moderately halophilic, thiodenitrifying bacteria from hypersaline habitats within the Gammaproteobacteria based on 16S rRNA gene sequence analysis (a) and based on inferred amino acids (partial amplicons, 241 aa) of the *nirS* gene encoding a subunit of the cytochrome *cd<sub>1</sub>* type of dissimilatory nitrite reductase (b). The trees were reconstructed from evolutionary distances by using the neighbour-joining method. The scale bar represents five inferred nucleotide changes per 100 nt (a) and 10 inferred amino acids changes per 100 aa (b). The percentage of bootstraps was derived from 100 (a) or 1000 (b) resamplings; values greater than 70% were considered as significant.

arrested growth and resulted in an incomplete oxidation of thiosulfate to elemental sulfur. This was probably the main reason for this bacterium growing in association with the nitrite-consuming thiodenitrifier. This conclusion was confirmed by growing HRhD 3sp<sup>T</sup> in a dialysis culture in which nitrite diffused out of the culture vessel; the culture reached a five times higher cell density than in ordinary batch mode. Growth in the presence of oxygen was irregular and only possible at oxygen concentrations below 5% in the gas phase. The NaCl tolerance range and optimum concentration of the nitrate-reducing isolate were lower than for the HLD strains (1.0–2.5 and 1.0 M, respectively). Phylogenetic analysis demonstrated that strain HRhD 3sp<sup>T</sup> is a member of the HLD cluster with a separate species status (97% 16S rRNA gene sequence homology; see Fig. 2a), which was also confirmed by DNA–DNA hybridization with HLD 2<sup>T</sup> (40% homology).

Overall, the data presented demonstrate for the first time that the process of autotrophic thiodenitrification can be carried out at high salt concentrations in NaCl brines and that a novel, highly specialized group of Gammaproteobacteria is responsible for it. On the basis of phenotypic and genetic properties, the rod-shaped HLD strains capable of complete reduction of nitrate to N<sub>2</sub> gas are proposed to be assigned to a new genus and species *Thiohalomonas denitrificans* gen. nov., sp. nov., while the vibrio-shaped, nitrate-

reducing strain HRhD 3sp<sup>T</sup> is proposed as a second species of the new genus with the name *Thiohalomonas nitrati-reducens* sp. nov.

#### Description of *Thiohalomonas* gen. nov.

*Thiohalomonas* [Thi.o.ha.lo.mo'nas. Gr. n. *thion* sulfur; Gr. n. *hals*, *halos* salt; Gr. n. *monas* a unit, monad; N.L. fem. n. *Thiohalomonas* salt (-tolerant), sulfur-utilizing monad].

Cells are non-motile rods or vibrios with a Gram-negative cell wall. Obligately chemolithoautotrophic and facultatively anaerobic. Utilizes reduced sulfur compounds as electron donors with oxygen or nitrate as electron acceptors. Moderately halophilic and neutrophilic. Member of the Gammaproteobacteria. Habitat is hypersaline lakes and salterns. Type species is *Thiohalomonas denitrificans*.

#### Description of *Thiohalomonas denitrificans* sp. nov.

*Thiohalomonas denitrificans* (de.ni.tri'fi.cans N.L. v. *denitrifico* to denitrify; N.L. part. adj. *denitrificans* denitrifying).

In addition to the genus description, cells are long, non-motile rods of variable size (0.4–0.5 × 2–6 μm) with a Gram-negative type of cell wall. Obligately chemolithoautotrophic. Utilizes sulfide and thiosulfate as electron donor

**Table 2.** Fatty acid profile in polar lipids of strain HLD 2<sup>T</sup> in comparison with a moderately halophilic heterotrophic Gammaproteobacterium

Dominant fatty acids are in bold type. ND, Not determined. Data for *Halomonas salina* are from Valderrama *et al.* (1998).

Fatty acid	Percentage of total	
	HLD 2 <sup>T</sup>	<i>Halomonas salina</i>
i10	0.42	ND
10:0	0.33	ND
3h10	0.29	ND
12:1	0.67	ND
12:0	3.90	0
14:0	0.53	0
15:0	0.64	0.8
i16:1	0	5–10
16:1 $\omega$ 7	<b>27.27</b>	ND
16:1 $\omega$ 5	2.97	ND
16:0	<b>25.41</b>	42
10Me16	1.26	0
9Me16:1	0	ND
11Me17:1	2.53	2.5
i17:1 $\omega$ 5	0	0.5
i17	0	ND
17:1 $\omega$ 8	0.91	ND
17cyc	<b>8.38</b>	ND
17:0	0.86	ND
18:1 $\omega$ 9	5.10	<b>25</b>
18:1 $\omega$ 7	<b>11.84</b>	ND
18:1 $\omega$ 5	1.64	ND
18:0	0.49	5
10Me18	0	ND
11Me18:1	1.29	ND
19cyc	3.26	5–10

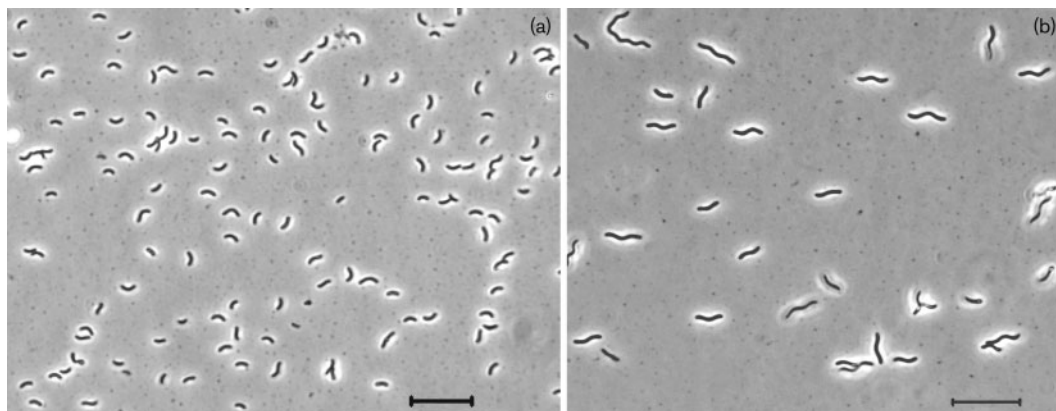
with sulfate as the final oxidation product. Grows best anaerobically with nitrate as electron acceptor. Reduces nitrate completely to N<sub>2</sub> with intermediate production of nitrite and N<sub>2</sub>O. Can grow with oxygen as electron acceptor under micro-oxic conditions. Moderately halophilic with a salinity range for growth from 1.0 to 3.0 M NaCl, and an optimum at 1.5–2.0 M. The pH range for growth is between 6.5 and 8.2 with an optimum between 7.3 and 8.2. The dominant cellular fatty acids are 16:0, 16:1 $\omega$ 7 and 18:1 $\omega$ 7. The G+C content of the DNA is 58–60 mol% (T<sub>m</sub>). Includes ten strains isolated from the sediments of hypersaline lakes and a solar saltern in Eurasia.

The type strain is HLD 2<sup>T</sup> (=DSM 15841<sup>T</sup>=UNIQEM U222<sup>T</sup>). Isolated from sediments of hypersaline lakes in south-west Siberia. 16S rRNA gene sequence accession number is EF117909.

### Description of *Thiohalomonas nitratireducens* sp. nov.

*Thiohalomonas nitratireducens* (ni.trat.i.re.du'cens. N.L. n. *nitras* nitrate; L. part. adj. *reducens* converting to a different state; N.L. part. adj. *nitratireducens* reducing nitrate).

In addition to the genus description, cells are non-motile vibrios of variable length (0.5  $\times$  1–5  $\mu$ m) with a Gram-negative type of cell wall. Obligately chemolithoautotrophic. Utilizes thiosulfate as electron donor with sulfate as the final oxidation product. Grows anaerobically with nitrate as electron acceptor, reducing it incompletely to nitrite. Anaerobic growth is inhibited by nitrite at concentrations above 2.5 mM. Can grow with oxygen as electron acceptor under micro-oxic conditions. Moderately halophilic with a salinity range for growth from 1.0 to 2.5 M NaCl and an optimum at 1.0 M. The pH optimum for anaerobic growth



**Fig. 3.** Phase-contrast micrographs of moderately halophilic, nitrate-reducing strain HRhD 3sp<sup>T</sup> grown anaerobically with 2 M NaCl in batch (a) or dialysis (b) culture with thiosulfate and nitrate. Bars, 10  $\mu$ m.

is 7.8–8.0. The G+C content of the DNA is 62.9 mol% ( $T_m$ ).

The type strain is HRhD 3sp<sup>T</sup> (=DSM 16925<sup>T</sup>=UNIQEM U248<sup>T</sup>). Isolated from sediments of hypersaline lakes in south-west Siberia. 16S rRNA gene sequence accession number is DQ836238.

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## References

- Antón, J., Oren, A., Benlloch, S., Rodríguez-Valera, F., Amann, R. & Rosselló-Mora, R. (2002). *Salimibacter ruber* gen. nov., sp. nov., a new species of extremely halophilic bacteria from saltern crystallizer ponds. *Int J Syst Evol Microbiol* **52**, 485–491.
- Braker, G., Fesefeldt, A. & Witzel, K.-P. (1998). Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Appl Environ Microbiol* **64**, 3769–3775.
- De Ley, J., Caffon, H. & Reinaerts, A. (1970). The quantitative measurements of hybridisation DNA from renaturation rates. *Eur J Biochem* **12**, 133–140.
- Felsenstein, J. (1989). PHYLIP – Phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. H. Munro. New York: Academic Press.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Oren, A. (1999). Bioenergetic aspects of halophilism. *Microbiol Mol Biol Rev* **63**, 334–348.
- Oren, A. (2002). *Halophilic Microorganisms and Their Environments*. Dordrecht, The Netherlands: Kluwer.
- Pfennig, N. & Lippert, K. D. (1966). Über das Vitamin B<sub>12</sub>-Bedürfnis phototropher Schwefelbakterien. *Arch Microbiol* **55**, 245–256.
- Schäfer, H. & Muyzer, G. (2001). Denaturing gradient gel electrophoresis in marine microbial ecology. *Methods Microbiol* **30**, 425–468.
- Silva, M. T., Sousa, J. C. F., Polonia, J. J., Macedo, M. A. E. & Parente, A. M. (1976). Bacterial mesosomes. Real structures or artifacts? *Biochim Biophys Acta* **443**, 92–105.
- Sorokin, D. Yu. & Kuenen, J. G. (2005). Haloalkaliphilic sulfur-oxidizing bacteria in soda lakes. *FEMS Microbiol Rev* **29**, 685–702.
- Sorokin, D. Yu., Kuenen, J. G. & Jetten, M. (2001a). Denitrification at extremely alkaline conditions in obligately autotrophic alkaliphilic sulfur-oxidizing bacterium *Thioalkalivibrio denitrificans*. *Arch Microbiol* **175**, 94–101.
- Sorokin, D. Yu., Tourova, T. P., Lysenko, A. M. & Kuenen, J. G. (2001b). Microbial thiocyanate utilization under highly alkaline conditions. *Appl Environ Microbiol* **67**, 528–538.
- Sorokin, D. Yu., Tourova, T. P., Antipov, A. N., Muyzer, G. & Kuenen, J. G. (2004). Anaerobic growth of the haloalkaliphilic denitrifying sulphur-oxidising bacterium *Thioalkalivibrio thiocyanodenitrificans* sp. nov. with thiocyanate. *Microbiology* **150**, 2435–2442.
- Sorokin, D. Yu., Tourova, T. P., Galinski, E. A., Belloch, C. & Tindall, B. J. (2006a). Extremely halophilic denitrifying bacteria from hypersaline inland lakes *Halovibrio denitrificans* sp. nov. and *Halospina denitrificans* gen. nov., sp. nov., and evidence that the genus name *Halovibrio* (Fendrich 1989) with the type species *H. variabilis* should be associated with DSM 3050. *Int J Syst Evol Microbiol* **56**, 379–388.
- Sorokin, D. Yu., Tourova, T. P., Lysenko, A. M. & Muyzer, G. (2006b). Culturable diversity of halophilic sulfur-oxidizing bacteria in hypersaline habitats. *Microbiology* **152**, 3013–3023.
- Sorokin, D. Yu., Tourova, T. P., Kolganova, T. V., Spiridonova, E. M., Berg, I. A. & Muyzer, G. (2006c). *Thiomicrospira halophila* sp. nov., a novel, moderately halophilic, obligately chemolithoautotrophic sulfur-oxidizing bacterium from hypersaline lakes. *Int J Syst Evol Microbiol* **56**, 2375–2380.
- Sorokin, D. Yu., Tourova, T. P., Bezoudnova, E. Yu., Pol, A. & Muyzer, G. (2007). Denitrification in a binary culture and thiocyanate metabolism in *Thiohalophilus thiocyanoxidans* gen. nov. sp. nov. – a moderately halophilic chemolithoautotrophic sulfur-oxidizing *Gammaproteobacterium* from hypersaline lakes. *Arch Microbiol* **187**, 441–450.
- Valderrama, M. J., Monteoliva-Sanchez, M., Quesada, E. & Ramos-Cormenzana, A. (1998). Influence of salt concentration on the cellular fatty acid composition of the moderately halophilic bacterium *Halomonas salina*. *Res Microbiol* **149**, 675–679.
- Van de Peer, Y. & De Wachter, R. (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* **10**, 569–570.
- Ventosa, A., Nieto, J. J. & Oren, A. (1998). Biology of aerobic moderately halophilic bacteria. *Microbiol Mol Biol Rev* **62**, 504–544.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Wood, A. P. & Kelly, D. P. (1991). Isolation and characterisation of *Thiobacillus halophilus* sp. nov., a sulphur-oxidizing autotrophic eubacterium from a Western Australian hypersaline lake. *Arch Microbiol* **156**, 277–280.
- Zhilina, T. N., Zavarzin, G. A., Rainey, F. A., Pikuta, E. N., Osipov, G. A. & Kostrikina, N. A. (1997). *Desulfonatronovibrio hydrogenovorans* gen. nov., sp. nov., an alkaliphilic, sulfate-reducing bacterium. *Int J Syst Bacteriol* **47**, 144–149.