Thioalkalivibrio sulfidiphilus sp. nov., a haloalkaliphilic, sulfur-oxidizing gammaproteobacterium from alkaline habitats

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A moderately salt-tolerant and obligately alkaliphilic, chemolithoautotrophic sulfur-oxidizing bacterium, strain HL-EbGr7T, was isolated from a full-scale bioreactor removing H2S from biogas under oxygen-limited conditions. Another strain, ALJ17, closely related to HL-EbGr7T, was isolated from a Kenyan soda lake. Cells of the isolates were relatively long, slender rods, motile by a polar flagellum. Although both strains were obligately aerobic, micro-oxic conditions were preferred, especially at the beginning of growth. Chemolithoautotrophic growth was observed with sulfide and thiosulfate in a pH range of 8.0–10.5 (optimum at pH 10.0) and a salinity range of 0.2–1.5 M total Na\(^+\) (optimum at 0.4 M). The genome sequence of strain HL-EbGr7T demonstrated the presence of genes encoding the reverse Dsr pathway and a truncated Sox pathway for sulfur oxidation and enzymes of the Calvin–Benson cycle of autotrophic CO\(_2\) assimilation with ribulose-bisphosphate carboxylase/oxygenase (RuBisCO) type I. The dominant cellular fatty acids were C\(_{18} : 1\)\,v\(_7\), C\(_{16} : 0\) and C\(_{19} : 0\)cyclo. Based on 16S rRNA gene sequencing, the two strains belonged to a single phylotype within the genus Thioalkalivibrio in the Gammaproteobacteria. Despite being related most closely to Thioalkalivibrio denitrificans, the isolates were unable to grow by denitrification. On the basis of phenotypic and phylogenetic analysis, the novel isolates are proposed to represent a novel species, Thioalkalivibrio sulfidiphilus sp. nov., with the type strain HL-EbGr7T (=NCCB 100376\(^T\) =UNIQEM U246\(^T\)).

To date, chemolithoautotrophic, haloalkaliphilic, sulfur-oxidizing bacteria (SOB) have been isolated only from soda lakes, naturally occurring extremely alkaline and saline habitats. They are represented by four genera in the Gammaproteobacteria: Thioalkalimicrobium (related to Thiomicrospira pelophila), Thioalkalibacter (related to Halothiobacillus), Thioalkalivibrio (a member of the family Ectothiorhodospiraceae) and Thioalkalispira (a member of the family Thioalkalispiraceae) (Sorokin et al., 2006; Banciu et al., 2008). The main feature of these organisms that differentiates them from other SOB is their ability to grow optimally at pH 9.5–10.0. Representatives of the genus Thioalkalivibrio are the most diverse in their metabolic capacities, including denitrifying and thiocyanate-utilizing species, and most of the strains can grow under soda-saturating conditions. At the time of writing, this genus included nine recognized species (Sorokin et al., 2006) and more than 100 undescribed isolates from hypersaline soda lakes in south-eastern Siberia, north-eastern Mongolia, Kenya, Egypt and California (Foti et al., 2006; Sorokin et al., 2011). The genus is a member of the family Ectothiorhodospiraceae, which is known to accommodate halophilic and haloalkaliphilic species (Imhoff,
According to 16S rRNA gene sequence-based phylogeny, the genus *Thioalkalivibrio* is monophyletic despite the relatively large phylogenetic divergence of its core subgroup, clustering around the type species, *Thioalkalivibrio versutus*, and the other two subgroups. This heterogeneity is also evident from the substantial phenotypic differences within the genus and from the phylogeny of important functional genes, such as *cbbL* (Tourova *et al.*, 2005, 2006, 2007). However, the integrity of the genus is justified by the common core metabolism (sulfur-based obligate chemolithoautotrophy) and the 16S rRNA gene sequence-based phylogeny.

Our recent analysis of a full-scale bioreactor removing H$_2$S from biogas under moderately haloalkaline conditions resulted in the isolation of a sulfide-specialized microaerophilic SOB related to one of the *Thioalkalivibrio* strains isolated earlier from a Kenyan soda lake. These two strains are described here as members of a novel species of the genus *Thioalkalivibrio*.

For the isolation of strain HL-EbGr7$^T$, biomass was used from the full-scale sulfide-removing reactor of the WWTP in Eerbeek, The Netherlands. The SOB within the reactor oxidize HS$^-$ to elemental sulfur under oxygen-limited and moderately haloalkaline conditions (Janssen *et al.*, 2009). Strain ALJ17 was isolated in 1999 from surface sediments of the soda lake Elmenteita in Kenya, the brines of which had a pH of 9.5–10.0 and a salt content of approx. 50 g l$^{-1}$.

The mineral base medium used for enrichment of SOB from the bioreactor was based on sodium bicarbonate (0.6 M) and contained 0.2 M NaCl and 1 g K$_2$HPO$_4$ l$^{-1}$. The pH after sterilization was 8.8. The mineral base medium for enrichment of soda-lake SOB included sodium carbonate/bicarbonate buffer at pH 10 containing 0.5 M total Na$^+$, 0.1 M NaCl, 0.5 g K$_2$HPO$_4$ l$^{-1}$ and 1 g KNO$_3$ l$^{-1}$. After sterilization, the media were supplemented with 1 mM MgCl$_2$, 6H$_2$O, 4 mM NH$_4$Cl and 0.1 or 1.0 ml trace metal solution l$^{-1}$ (Pfennig & Lippert, 1966) (for sulfide- and thiosulfate-containing media, respectively).

To enrich and isolate microaerophilic, sulfide-utilizing SOB from the bioreactor, dilution series were made in 15 ml sulfide–oxygen gradient tubes. These were prepared by overlaying 4 ml 2 % (w/v) agar containing 10 mM Na$_2$S with 10 ml 0.2 % (w/v) thoroughly washed agar in anoxic medium at pH 8.8. The tubes were closed with rubber stoppers and incubated for 2 days to establish a gradient before inoculation. The soda-lake strain was enriched in liquid medium at pH 10 containing 20 mM thiosulfate under microaerobic static conditions (2 % O$_2$ in the gas phase) and further purified from colonies grown on solid medium. Culture purity was checked by microscopy, by sequencing of the 16S rRNA gene and by the absence of growth on medium in which the sulfur substrate was replaced by 1 g yeast extract l$^{-1}$ to detect the potential

**Fig. 1.** Cell morphology of strains HL-EbGr7$^T$ (a, c) and ALJ17 (b) grown at pH 10 with thiosulfate. (a, b) Phase-contrast photomicrographs; (c) TEM photomicrograph. Bars, 10 $\mu$m (a, b) and 1 $\mu$m (c).
presence of heterotrophic bacteria. Phase-contrast photomicrographs were obtained using a Zeiss Axioskop Imaging 2 microscope. For electron microscopy, cells were fixed in glutaraldehyde (final concentration 3 % v/v) in 0.5 M NaCl and, after removal of the fixative, stained with 1 % (w/v) neutralized phosphotungstate. The pH and salinity profiles for growth and activity were evaluated with thiosulfate as a substrate using a range of buffered mineral media containing 0.6 M total Na\(^+\) for pH tests and a range of carbonate concentrations from 0.1 to 2.0 M total Na\(^+\) at pH 10 (Sorokin, 2005).

Aerobic enrichments with thiosulfate from the bioreactor biomass gave positive results up to 10\(^{-8}\) dilution, but the SOB isolated (Thiomicrospira and Thioalkalimicrobium strains) either were not visible or represented a minor component of the reactor community according to DGGE profiles. On the other hand, dilution series in sulfide–oxygen gradient tubes were positive up to 10\(^{-10}\) and the dominant SOB strain (designated HL-EbGr7\(^T\)) obtained by this approach represented a dominant DGGE band in the reactor biomass (Fig. S1, available in IJSEM Online). The reason for this result became clear during purification of the strain. It took a long time to adapt the strain to growth with thiosulfate as substrate. When tested directly for substrate-dependent oxygen consumption, the reactor biomass showed a clear preference for sulfide over thiosulfate, probably as a result of the reactor conditions. The soda-lake enrichment under micro-oxic conditions and with thiosulfate as substrate resulted in the isolation of a haloalkaliphilic SOB, strain ALJ17, which differed from other isolates obtained from the same habitats under fully aerobic conditions by its inability to start growing when cultivated in shaken flasks.

These two SOB isolates, one from a bioreactor and the other from a soda lake, were closely related phylogenetically (99 % 16S rRNA gene sequence similarity). Cells of both strains were relatively long, slender and slightly curved rods, motile with a polar flagellum (Fig. 1). Both required low concentrations of oxygen (2–5 %) or the addition of 1 mM sulfide at the beginning of growth when the cell density was low but, as soon as the cell numbers increased, the cultures could be cultivated under fully aerobic conditions.

Genomic DNA was extracted by the phenol/chloroform method (Marmur, 1961) and the G+C content was analysed by the thermal denaturation/reassociation technique (Marmur & Doty, 1962) using DNA from Escherichia coli K-12 as a standard. The values determined were 65 mol% for HL-EbGr7\(^T\) and 63.5 mol% for ALJ17. DNA–DNA hybridization was conducted according to De Ley et al. (1970). The DNA relatedness between the two newly described strains was 83 %, indicating that they are members of the same genetic species, whereas the relatedness with Thioalkalivibrio denitrificans ALJD\(^T\) and Thioalkalivibrio thiocyanodentificans ARhD1\(^T\) (closest phylogenetic relatives) was significantly below the species level (41 and 35 %, respectively).

For molecular analysis, DNA was extracted from cells using the UltraClean Microbial DNA isolation kit (MoBio Laboratories). The nearly complete 16S rRNA gene was obtained from pure cultures using primers GM3f and GM4r and a partial (550 bp) fragment for DGGE analysis with primers 341f and GC/907r (Schafer & Muyzer, 2001). DGGE analysis was performed according to Schäfer & Muyzer (2001) using a 30–70 % denaturing gradient. PCR

![Fig. 2. Phylogenetic position of the novel SOB strains within the genus Thioalkalivibrio based on 16S rRNA gene sequence analysis. The tree was reconstructed from evolutionary distances by using the neighbour-joining method. Percentages of bootstraps were derived from 1000 resamplings. Values greater than 70 % were considered as significant and are indicated by filled circles at nodes. Bar, 2 % sequence divergence.](image-url)
products were purified using the Qiagen gel extraction kit. The sequences were first compared to sequences stored in GenBank using the BLAST algorithm. Subsequently, the sequences were imported into the ARB software program (Ludwig et al., 2004), automatically aligned and added to a phylogenetic tree using the Quick-add tool. A definitive tree was built using the neighbour-joining algorithm with automatically selected correction settings.

16S rRNA gene sequence analysis demonstrated that the two SOB isolates were closely related to each other, forming a new lineage inside the cluster of *Thioalkalivibrio* denitrificans, which is the most distant group of the genus *Thioalkalivibrio* (Figs 2 and S2). Only two environmental sequences that were related to this group were found in GenBank. One of the sequences, from a deep subsurface alkaline brine inclusion in China (Zhang et al., 2005), is particularly interesting because another alkaliophilic isolate from the same bioreactor, the sulfur-respiring anaerobe *Desulfurispirillum* alkaliphilum, also had a closely related sequence (Sorokin et al., 2007). This seems unlikely to be a coincidence, and indicates that alkaliophiles might disseminate from deep subterrestrial alkaline strata to the ocean and could be selectively enriched subsequently under specific conditions.

Despite the close phylogenetic relationship to the two denitrifying *Thioalkalivibrio* species in the *T. denitrificans* cluster, the novel SOB isolates were obligately aerobic, and strain HL-EBGr7T was not even able to utilize nitrate or nitrite as a nitrogen source. Both strains grew chemolithoautotrophically with thiosulfate and sulfide, but the bioreactor isolate had difficulties in adapting to thiosulfate when freshly isolated, preferring sulfide as a substrate. Washed cells of both strains, pre-grown with thiosulfate, also actively oxidized polysulfide, elemental sulfur and tetrathionate. Genome sequencing of strain HL-EBGr7T showed the presence of a hybrid sulfur-oxidizing system including reverse sulfate reduction elements (*dsr* and *aps*) and a truncated Sox system, probably responsible for thiosulfate-sulfane atom oxidation to sulfur (Muyzer et al., 2011).

Aerobically grown biomass of both strains had a distinct brown colour, in contrast to the pink colour of cells grown under micro-oxic conditions with sulfide. The brown component is a soluble cytochrome with an alpha peak at 598–600 nm that is close to the peak characteristic of some types of cytochrome *a*. The unknown cytochrome remained reduced under aerobic conditions and was only oxidized by ferricyanide (Fig. S3a). However, haem analyses showed the complete absence of *a*-type haems and, instead, an increase in the content of haem *d* (Fig. S3b) haems were extracted and separated by reversed-phase chromatography on a C18 column (Microbonda Sphere S-S; Waters) at a flow rate of 0.3 ml min⁻¹ with detection at 406 nm. This is an unexpected result, since the only known soluble cytochrome containing haem *d* is the dissimilatory *cd*₆ nitrite reductase. Since the organism cannot grow anaerobically with nitrite and the nirS gene is not present in the genome of HL-EbGr7T, the soluble cytochrome *d*₉₈ is not a nitrite reductase and remains to be identified. However, involvement in protection of the microaerophilic SOB from oxygen radicals can be proposed as an obvious function.

Cellular fatty acids were extracted with acidic methanol from freeze-dried biomass of strain HL-EBGr7T grown aerobically with thiosulfate at pH 10 and analysed by GC-MS according to Zhilina et al. (1997). The analysis demonstrated a dominance of unsaturated C₁₈:₁₀₇ and saturated C₁₆:₀ constituting more than 60 % from the total, similar to other analysed species of the genus (except for the type species, which had an elevated level of C₁₉:₀ cyclo) and to other representatives of the family *Ectothiorhodospiraceae* (Table S1). In the total profile, strain HL-EbGr7T was most similar to *T. denitrificans*.

Respiratory lipoquinones were extracted with cold acetone from 100 mg freeze-dried cells. The extract was subjected to TLC in a hexane–diethyl ether (85:15) system. Absorption bands visualized under UV light were analysed further by tandem mass spectrometry (Finnigan MAT 8430) with

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**Fig. 3.** Influence of pH at 0.6 M Na⁺ (a) and salinity at pH 10.0 (b) on growth with thiosulfate (closed circles) and activity of sulfide-dependent respiration by washed cells (open circles) of strain HL-EbGr7T.
The species description is based on the properties of two closely related strains, HL-EbGr7T and ALJ17. Cells are slightly curved, long, slender rods, 0.4 × 3.0–8.0 μm, motile by a single polar flagellum, with a Gram-negative type of cell wall. The colonies are up to 2 mm in diameter, circular, appearing white at first from extracellular sulfur, which is gradually oxidized, whereby the colonies turn brownish. Obligately aerobic, but oxygen-sensitive. Chemolithoheterotrophic, utilizing sulfide (preferred substrate) and thiosulfate as electron donors for growth. Can also oxidize polysulfide, elemental sulfur and tetrathionate. Ammonia and urea (urease activity is positive) serve as nitrogen sources; strain ALJ17 can also utilize nitrate. Moderately salt-tolerant, with a salinity range for growth of 0.2–1.5 M Na⁺ at pH 10.0 (optimum at 0.4 M), and obligately alkaliphilic, with a pH range for growth at 0.6 M Na⁺ of 8.0–10.5 (optimum at pH 10.0). The dominant cellular fatty acids are C₁₈:1ω7c, C₁₆:0 and C₁₉:0 cyclo. The optimum growth temperature is 35 °C and the maximum temperature for growth is 40–41 °C. At pH 10, both known strains are sensitive to chloramphenicol, kanamycin, tetracycline and rifampicin.

The type strain is HL-EbGr7T (=NCCB 100376T =UNIQEM U246T). It has a DNA G+C content of 65 mol%. The strain was isolated from a full-scale sulfide-oxidizing bioreactor in The Netherlands. Strain ALJ17 was isolated from surface sediments of the soda lake Elmenteita, Kenya; its DNA G+C content is 63.5 mol%.

**Acknowledgements**

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**References**


### Table 1. Comparative properties of the novel SOB isolates, members of the *Thioalkalivibrio denitrificans* group and the type species of the genus

<table>
<thead>
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<th>Property</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>Anaerobic growth with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+ (preferred)</td>
</tr>
<tr>
<td>Nitrite</td>
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<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>N₂O</td>
<td>−</td>
<td>−</td>
<td>+ (preferred)</td>
<td>−</td>
<td>−</td>
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<td>Utilization of thiocyanate as electron donor</td>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Preference for sulfide over thiosulfate</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Sensitive to fully aerobic conditions</td>
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<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Nitrate/nitrite as nitrogen source</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Urea as nitrogen source</td>
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<td>+</td>
<td>−</td>
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<td>−</td>
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<tr>
<td>Presence of soluble cytochrome b₉₉₉ in aerobically grown cells</td>
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<td>+</td>
<td>−</td>
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<td>−</td>
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<td>7.5–10.6 (10)</td>
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<td>7.5–10.6 (10.0)</td>
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<td>63.5</td>
<td>63.0</td>
<td>63.1–63.7</td>
<td>63.7</td>
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tolerant, obligately chemolithoautotrophic sulfur-oxidizing Gamma-proteobacterium *Thioalkalibacter halophilus* gen. nov., sp. nov. from South-Western Siberian soda lakes. *Extremophiles* 12, 391–404.


