Thialkalivibrio nitratireducens sp. nov., a nitrate-reducing member of an autotrophic denitrifying consortium from a soda lake

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Strain ALEN 2T was isolated from a mixed culture capable of complete autotrophic denitrification with thiosulfate as electron donor at pH 10; the mixed culture was enriched from sediment from Lake Fazda (Wadi Natrun, Egypt), a hypersaline alkaline lake. The isolate had large, non-motile, coccoid or barrel-shaped cells with intracellular sulfur globules. The bacterium was obligately chemolithoautotrophic. It grew with reduced sulfur compounds aerobically and anaerobically with nitrate as electron acceptor, nitrate being reduced to nitrite. It was moderately halophilic and obligately alkaliphilic. On the basis of genetic analysis and its unique phenotype, strain ALEN 2T (= DSM 14787T = UNIQEM 213T) is proposed as the type strain of a novel species of the genus Thialkalivibrio, Thialkalivibrio nitratireducens.

Only a few species of lithotrophic sulfur-oxidizing bacteria (SOB) are capable of anaerobic growth with sulfur compounds and nitrogen oxides as electron acceptors. In particular, obligately autotrophic Thiobacillus denitrificans, Thiomicrospira denitrificans and facultatively autotrophic Paracoccus species perform the complete denitrification of nitrate to nitrogen gas, whereas filamentous, vacuolated, SOB Beggiatoa and Thioploca species appear to conduct dissimilatory nitrate reduction to ammonia with sulfide as electron donor (Kuenen et al., 1992; McHatton et al., 1996; Otte et al., 1999). Such SOB play an important role in mineral cycling and waste removal by linking the sulfur and nitrogen cycles (Kuenen & Robertson, 1987; Robertson & Kuenen, 1992). Of the haloalkaliphilic SOB isolated from soda lakes, currently represented by nearly 100 isolates (Sorokin et al., 2000, 2001a, 2002a, 2002b), only one, described as Thialkalivibrio denitrificans ALJD7T, has the potential for anaerobic growth. This strain differs from its neutrophilic analogues Thiobacillus denitrificans and Thiomicrospira denitrificans in that it does not possess a dissimilatory nitrate reductase. It grows with nitrite or N2O as electron acceptor and thiosulfate or polysulfide as electron donor (Sorokin et al., 2001b). Recently, an enrichment culture has been obtained from a sediment sample from an alkaline hypersaline lake in Wadi Natrun (Egypt) that completely reduced nitrate to nitrogen gas, with thiosulfate as electron donor, at pH 10. In this report, the nitrate-reducing member of this haloalkaliphilic autotrophic denitrifying association is described.

Sediment from Lake Fazda, a hypersaline soda lake (250 g total salts l−1; pH 10) in the Wadi Natrun (Egypt), was used as inoculum to enrich for denitrifying SOB. The basic hydrochemical and microbiological characteristics of the Wadi Natrun lakes have been described by Taher (1999) and Imhoff et al. (1979), respectively. The enrichment was performed in 100 ml serum bottles with butyl-rubber stoppers filled with 50 ml alkaline base containing 0.6 M total Na+, pH 10 (Sorokin et al., 2001a), with 20 mM thiosulfate and 30 mM nitrate. Anoxic conditions were achieved by five cycles of evacuation–flushing with argon with active degassing of the liquid. Solid medium was prepared by 1:1 mixing of the above-mentioned alkaline base containing a double concentration of substrates and 4% (w/v) agar at 50 °C. Anaerobic plate incubation was performed using closed jars filled with pure argon in the presence of anaerobic catalyst (Oxoid). Growth with H2 as electron donor was tested in 100 ml bottles closed with butyl-rubber stoppers containing 10 ml medium under an
atmosphere of 98% (v/v) H2 and 2% (v/v) O2. Methyl-

otrophy was tested with methanol and formate (5 mM)

under aerobic and denitrifying conditions. The pH range for
growth was tested on media prepared using 0.1 M HEPES/
Na2CO3 (pH 7–8) supplemented with 0.6 M NaCl or
sodium carbonate/sodium bicarbonate (pH 8–11) with
0.6 M total Na+. Na+ tolerance was tested on sodium
bicarbonate/bicarbonate-based mineral medium containing
0.1–2.0 M total Na+ at pH 10. The same pH and salinity
buffers were used in experiments with washed cells. Res-
piration activity, reduction of nitrogen oxides by washed
cells, sulfur-metabolizing activity, denitrifying enzymes and
cytochrome spectra were measured as described previously
(Sorokin et al., 2002c). Inorganic sulfur compounds, nitrate,
nitrite, N2O and protein concentrations were determined by
spectrophotometric and GC methods as described pre-
viously (Sorokin et al., 2001a, b). DNA extraction, DNA
G+C content determination and DNA–DNA hybridization
were performed according to standard protocols (Marmur,
1961; De Ley et al., 1970). For amplification and sequencing
of the 16S rRNA gene, DNA was obtained by standard
phenol/chloroform extraction. The 16S rRNA gene was
selectively amplified using primers 5’-AGAGTTTGATCC-
TGCTACAG-3’ (forward) and 5’-TACGTTACCTGTATT-
ACGACTT-3’ (reverse). PCR products were purified from
low-melting-point agarose using the Wizard PCR-Prep kit
(Promega) according to the manufacturer’s instructions.
Almost-complete sequencing (1420 nt) was performed
using the Promega Silver Sequencing kit according to the
manufacturer’s instructions, but with minor modifications.
Primary comparative analysis of the 16S rRNA gene
sequence of strain ALEN 2T with database sequences was
done using BLAST. On the basis of results of the BLAST search,
the 16S rRNA gene sequences of strain ALEN 2T and its
closest relatives were aligned using CLUSTAL_X (Thompson
et al., 1997). Regions that were not sequenced in one or more
reference organisms were omitted from subsequent ana-
lyses. An unrooted phylogenetic tree based on 16S rRNA
gene sequences of the studied bacteria was constructed by
the neighbour-joining method available in the TREECON
package (Van de Peer & De Wachter, 1994). Bootstrap
analysis (100 replications) was used to validate reproduc-
ibility of the branching pattern of the tree.

The anaerobic enrichment resulted in a stable binary culture
consisting of large irregular coccoid cells with intracellular
sulfur globules and thin straight rods that were occasionally
motile. The latter were responsible for nitrite reduction. On
the basis of their physiology and genetic properties, the thin
straight rods resembled the previously described halo-
alkaliphilic SOB Thialkalivibrio denitrificans (Sorokin et al.,
2001b). Further work focused on isolation and character-
ization of the unusual coccoid morphotype that was
responsible for the reduction of nitrate to nitrite.

The cocoid organism was present in relatively low numbers
in the mixed denitrifying culture, which made it difficult to
isolate. When plated onto nitrate/thiosulfate alkaline agar
under an argon atmosphere, a surprisingly low proportion
of the total cells present in liquid culture produced colonies.
It appeared that the numerically dominant small rods
were dependent on the cocoid phenotype for growth, resulting in
formation of mixed colonies. By picking up the colony
material containing mostly the cocoid morphotype and
replacing it, the colony number of the desired organism
gradually increased. Eventually, pure colonies of the cocoid
phenotype were obtained, and the isolated bacterium was
designated strain ALEN 2T. It formed colonies of variable
size and shape, 1–3 mm, some flat, some dome-like. The
young colonies were shining white and full of sulfur, turning
reddish and transparent with time. Cells in the colonies were
extremely pleomorphic, mostly cocoid, with multiple
intracellular sulfur globules. Cells grown aerobically on
liquid medium with thiosulfate at pH 10 were cocoid,
8–20 μm in diameter and aggregated in chains of
different lengths (Fig. 1a, c). The cells grown anaerobically
in liquid culture were barrel-shaped and less aggregated
(Fig. 1b, d).

The bacterium was obligately autotrophic. It grew well
under fully aerobic conditions, oxidizing 40 mM thiosulfate
at pH 10 within 3 days with prominent formation of
intracellular sulfur (8–10 mM), which was finally oxidized
to sulfate within another 3–5 day period. Maximum specific
growth rate with thiosulfate under aerobic conditions was
0.08 h⁻¹, with a yield of 5.5–6.0 mg protein mmol⁻¹. The
bacterium belonged to the obligate alkaliphiles (pH range for
growth of 8.0–10.5, with optimum at 9.5–10.0) and
moderate halophiles (salt range for growth of 0.2–1.5 M
total Na+, with an optimum at 0.4–0.5 M). Respiration
activity of the washed cells with sulfate and thiosulfate was
maximal at pH 10 and still substantial at pH 11 (20 %
from maximum; data not shown). Strain ALEN 2T actively
oxidized sulfide and, at much lower rates, polysulfide and
thiosulfate. The oxidation activity of elemental sulfur was an
order of magnitude lower than that of thiosulfate (Table 1),
which accounts for a heavy sulfur accumulation in cultures.

In static aerobic cultures with thiosulfate, strain ALEN 2T
reduced up to 15 mM nitrate to nitrite. Full aeration inhi-
bited nitrite production. Under anaerobic conditions, the
bacterium was able to grow with nitrate as electron acceptor
and thiosulfate, sulfide and polysulfide (2 mmol l⁻¹
portions, fed-batch supply for the latter two) as substrates.
With 20 mM thiosulfate/40 mM nitrate, rapid nitrite pro-
duction and transient sulfur accumulation were observed.
When all the nitrate was consumed, growth was arrested.
Finally, 38 mM nitrite was produced per 11 mM thiosulfate
oxidized to sulfate, which corresponds to a two-electron
nitrate reduction to nitrite (assuming that approximately
10% electrons are used for CO₂ reduction). No anaerobic
growth was observed with either nitrite (10 mM) or N₂O as
electron acceptors. The fed-batch growth with sulfide/

take proceeded in two phases. At first, when nitrate was
still present, each 2 mM addition of sulfide was followed by
cell growth, transient sulfur formation with its further
oxidation to sulfate and a build-up in nitrite concentration, similar to growth with thiosulfate. When nitrate was consumed, biomass increase stopped, but sulfide was still oxidized to elemental sulfur, accompanied by slow nitrite consumption with a molar H$S^2^-$/NO$\_2^-$ ratio of 2.0–2.5:1. The latter implies a one-electron reduction of nitrite to NO. Although NO was not measured, at this stage some N$_2$O in the headspace was registered. However, no anaerobic growth was observed with sulfide and nitrite or N$_2$O as electron acceptors. From these experiments, it was concluded that strain ALEN 2$^T$ is a high-capacity nitrate-to-nitrite reducer and, therefore, may serve as a nitrite provider in a denitrifying association with a nitrite reducer.

Further experiments with washed cells of strain ALEN 2$^T$ confirmed its inability to reduce nitrite and N$_2$O with thiosulfate as electron donor. However, low nitrite-reducing activity was observed with polysulfide and H$_2$ as electron donors (Table 2). In both cases, N$_2$O was produced in the gas phase. Moreover, H$_2$ also stimulated the reduction of elemental sulfur by washed cells of strain ALEN 2$^T$. Despite these facts, the bacterium was incapable of anaerobic growth with H$_2$ as electron donor and nitrite or elemental sulfur as electron acceptors, which implies that some important links between the dissimilatory enzymes and the energy-generating system are missing in this unusual alkaliphilic SOB species.

In the soluble fraction of cell-free extract of strain ALEN 2$^T$, rapid nitrate-dependent oxidation of reduced methyl viologen was observed, but nitrite, the usual product of nitrate reductase activity, was not detected. Replacement of nitrate by nitrite in the reaction mixture resulted in extremely high rates of methyl viologen oxidation and nitrite consumption [8.0–10.5 μmol NO$\_2^-$ (mg protein)$^{-1}$ min$^{-1}$].

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Table 1. Respiratory activity of washed cells of strain ALEN 2$^T$, grown aerobically at pH 10 and 0.6 M Na$^+$ with thiosulfate

Results are the means from two experiments.

<table>
<thead>
<tr>
<th>Substrate (50 μM)</th>
<th>Respiration rate [nmol O$_2$ (mg protein)$^{-1}$ min$^{-1}$]</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS$^-^$</td>
<td>2810</td>
<td>Sulfur</td>
</tr>
<tr>
<td>S$_2^-^$</td>
<td>430</td>
<td>Sulfur</td>
</tr>
<tr>
<td>S$_8^-^$</td>
<td>32</td>
<td>ND</td>
</tr>
<tr>
<td>S$_2$O$_3^-^$</td>
<td>175</td>
<td>Sulfur + sulfate</td>
</tr>
<tr>
<td>S$_4$O$_4^-^$</td>
<td>38</td>
<td>ND</td>
</tr>
<tr>
<td>SO$_3^-^$</td>
<td>8</td>
<td>Sulfate</td>
</tr>
</tbody>
</table>

ND, Not determined.
This implies that strain ALEN 2\textsuperscript{T} possesses both nitrate and nitrite reductase activities despite the fact that it is unable to use nitrite as an electron acceptor \textit{in vivo}. Antipov \textit{et al.} (2003) have demonstrated the presence of a nitrate/nitrite reductase in strain ALEN 2\textsuperscript{T} with unusual properties. Activities of the sulfur-metabolizing enzymes thiosulfate reductase, sulfite dehydrogenase and sulfide dehydrogenase were found in the soluble fraction of cell-free extract of strain ALEN 2\textsuperscript{T} at pH 10 \cite{690, 260 and 60 nmol (mg protein)\textsuperscript{2} min\textsuperscript{-1}, respectively}. The cell membranes of strain ALEN 2\textsuperscript{T} contained high amounts of cytochrome types \textit{c} and \textit{b} (absorption maxima in the alpha region at 554 and 558 nm, respectively). CO difference spectra of the membranes indicated the presence of a cytochrome oxidase of type \textit{bb} (troughs in alpha region at 558 and 563 nm). The \textit{G} + \textit{C} content of the DNA of strain ALEN 2\textsuperscript{T} was 64·8 ± 0·5 mol\%. Phylogenetic analysis placed this bacterium in the genus \textit{Thialkalivibrio} (Fig. 2), which accommodates the high-\textit{G} + \textit{C}-containing species of haloalkaliphilic SOB \cite{Sorokin \textit{et al.}, 2001a}. The isolate had highest similarity (98·2 \%) to the thiocyanate-utilizing species \textit{Thialkalivibrio paradoxus} \cite{Sorokin \textit{et al.}, 2002b}. Indeed, strain ALEN 2\textsuperscript{T} resembled this species in its specific cell morphology and accumulation of intracellular sulfur due to a low elemental sulfur oxidizing capacity. These two strains also had the highest level of DNA–DNA relatedness (54 \%) compared to values obtained with other species of \textit{Thialkalivibrio} (<30 \%). However, strain ALEN 2\textsuperscript{T} was substantially different physiologically from \textit{Thialkalivibrio paradoxus}: strain ALEN 2\textsuperscript{T} was incapable of thiocyanate and carbon disulfide oxidation, whereas \textit{Thialkalivibrio paradoxus} cannot utilize nitrate either as electron acceptor nor as nitrogen source. Overall, these differences suggest that strain ALEN 2\textsuperscript{T} should be regarded as a novel species of the genus \textit{Thialkalivibrio}, for which the name \textit{Thialkalivibrio nitratireducens} is proposed.

**Description of \textit{Thialkalivibrio nitratireducens} sp. nov.**

\textit{Thialkalivibrio nitratireducens} (ni.tra.ti.re.du’cens. N.L. n. \textit{nitras} nitrate; L. part. adj. \textit{reducens} converting to a different state; N.L. adj. \textit{nitratireducens} reducing nitrate).

Cells are mostly coccoid or barrel-shaped, often in chains and aggregates, 0·8–2·0 μm in diameter, often with long

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**Table 2. Anaerobic activity of washed cells of strain ALEN 2\textsuperscript{T}**

Cells were suspended in sodium carbonate/bicarbonate buffer containing 0·6 M Na\textsuperscript{+}, pH 10, at a protein concentration of 0·3–0·6 mg ml\textsuperscript{-1}.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>Products of:</th>
<th>Reduction rate [nmol (mg protein)\textsuperscript{-1} min\textsuperscript{-1}]*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S\textsubscript{2}O\textsubscript{3}\textsuperscript{-}</td>
<td>NO\textsubscript{3}\textsuperscript{-}</td>
<td>S\textsubscript{8} + SO\textsubscript{4}\textsuperscript{2-}</td>
<td>NO\textsubscript{2}\textsuperscript{-}</td>
</tr>
<tr>
<td>NO\textsubscript{2}\textsuperscript{-}</td>
<td>NO\textsubscript{3}\textsuperscript{-}</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>N\textsubscript{2}O</td>
<td>N\textsubscript{2}O</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S\textsubscript{2} (polysulfide)</td>
<td>NO\textsubscript{3}\textsuperscript{-}</td>
<td>S\textsubscript{8} + SO\textsubscript{4}\textsuperscript{2-}</td>
<td>NO\textsubscript{2}\textsuperscript{-}</td>
</tr>
<tr>
<td>NO\textsubscript{2}\textsuperscript{-}</td>
<td>NO\textsubscript{2}\textsuperscript{-}</td>
<td>S\textsubscript{8}</td>
<td>N\textsubscript{2}O</td>
</tr>
<tr>
<td>N\textsubscript{2}O</td>
<td>N\textsubscript{2}O</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NO\textsubscript{2}\textsuperscript{-}</td>
<td>NO\textsubscript{2}\textsuperscript{-}</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>N\textsubscript{2}O</td>
<td>N\textsubscript{2}O</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S\textsubscript{8}</td>
<td>S\textsubscript{8}</td>
<td>–</td>
<td>S\textsubscript{2}\textsuperscript{-}</td>
</tr>
</tbody>
</table>

*Rates of nitrite production or consumption during nitrate or nitrite reduction and sulfane atom production during sulfur reduction; endogenous rates without electron donors are subtracted.

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**Fig. 2.** Phylogenetic tree showing the position of the novel haloalkaliphilic strain ALEN 2\textsuperscript{T} in the γ-Proteobacteria. Numbers on the branches refer to bootstrap values; only values above 90 \% are included. Bar, 5 nt substitutions per 100 nt. Accession numbers are shown in parentheses.
sulfur globules inside. Non-motile. Colonies are up to 3 mm in size, full of sulfur, turning reddish with age. Obligately chemolithoautotrophic. Oxidizes thiosulfate, sulfide, polysulfide and, much less actively, elemental sulfur and tetrasulfide to sulfate. Facultatively anaerobic. Grows anaerobically with nitrate as electron acceptor and thiosulfate, sulfide or polysulfide as electron donor. The sole product of nitrate reduction is nitrite. Obligately alkaliphilic and moderately halophilic. Genetically most closely related to a thiocyanate-oxidizing species, *Thialkalivibrio paradoxus*.

The type strain is ALEN 2T (= DSM 14787T = UNIQUEST 213T), isolated from sediments of Lake Fazda (Wadi Natrun, Egypt), a hypersaline soda lake. Its DNA G+C content is 64.8 ± 0.5 mol% (T_m method). Other properties are as for the genus.

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**References**


