Two strains of haloalkaliphilic, obligately autotrophic, sulfur-oxidizing bacteria were isolated from the oxygen-sulfide interface water layer of stratified alkaline and saline Mono Lake, California, USA. Strain ALM 1\(^T\) was a dominant species in enrichment on moderate-saline, carbonate-buffered medium (0 < $6\text{M} \text{Na}_2\text{CO}_3$, pH 10) with thiosulfate as an energy source and nitrate as a nitrogen source. Cells of ALM 1\(^T\) are open ring-shaped and are non-motile. It has a high growth rate and activity of thiosulfate and sulfide oxidation and very low sulfur-oxidizing activity. Genetic comparison and phylogenetic analysis suggested that ALM 1\(^T\) (DSM 14477\(^T\) = JCM 11371\(^T\)) represents a new species of the genus *Thioalkalimicrobium* in the $\gamma$-Proteobacteria, for which the name *Thioalkalimicrobium cyclicum* sp. nov. is proposed. Another Mono Lake isolate, strain ALM 2\(^T\), dominated in enrichment on a medium containing 2 \(\text{M} \text{Na}_2\text{CO}_3\) (pH 10). It is a motile vibrio which tolerates up to 4 \(\text{M} \text{Na}_2\text{CO}_3\) and produces a membrane-bound yellow pigment. Phylogenetic analysis placed ALM 2\(^T\) as a member of genus *Thioalkalivibrio* in the $\gamma$-Proteobacteria, although its DNA hybridization with the representative strains of this genus was only about 30%. On the basis of genetic and phenotypic properties, strain ALM 2\(^T\) (DSM 14478\(^T\) = JCM 11372\(^T\)) is proposed as *Thioalkalivibrio jannaschii* sp. nov.

**Keywords:** haloalkaliphilic sulfur-oxidizers, soda lakes, Mono Lake, *Thioalkalimicrobium*, *Thioalkalivibrio*
Siberia and Mongolia. In general, these bacteria differ from the well-known neutrophilic species by their ability to grow optimally at pH > 9 and up to 10-5-10-6 in media strongly buffered by a sodium bicarbonate/carbonate mixture. All these bacteria have been assigned into two new genera *Thioalkalimicrobium* and *Thioalkalivibrio* in the γ-Proteobacteria (Sorokin et al., 2001a). The two genera differ from each other in many aspects of the growth kinetics, metabolic activity and genetics. In general, the genus *Thioalkalimicrobium* includes fast-growing species with high activity of thiosulfate and sulfide oxidation but relatively low salt tolerance. Most of the strains were obtained from the low-mineralized steppe soda lakes. The genus is a member of the *Thiomicrospira* cluster. In contrast, the genus *Thioalkalivibrio* is represented by the slow-growing but more salt-tolerant organisms isolated mostly from the highly concentrated Kenyan soda lakes. Some of the isolates were even capable of growth in saturated soda brines. The genus *Thioalkalivibrio* is related to sulfur purple bacteria of the genus *Ectothiorhodospira* (Trüper & Schlegel, 1964). This genus also includes several strains capable of growth with thiocyanate as electron donor and nitrogen source (Sorokin et al., 2002a) which form two new *Thioalkalivibrio* species (Sorokin et al., 2002b). In this paper we describe two new species of haloalkaliphilic, obligately chemolithoautotrophic, sulfur-oxidizing bacteria isolated from the stratified, alkaline and saline Mono Lake in California, USA.

**METHODS**

**Sampling.** Water samples were collected in July 1999 from the sulfide-oxygen interface layer (depth 19–25 m) at the deepest point (48 m) of Mono Lake, California, using 51 sampling bottles. The mean pH and total salinity values of the Mono Lake water were 9.8 and 80 g l–1, respectively. The HS\(^-\) concentration decreased upwards from several hundred micromolar in the anaerobic layer to several micromolar in the upper part of the interface layer. The samples were brought into a laboratory within 5 h of collection and kept at 4 °C until use.

**Media and growth conditions.** Enrichment for and cultivation of aerobic alkaliphilic sulfur bacteria was performed using a mineral medium strongly buffered by NaHCO\(_3\)/Na\(_2\)CO\(_3\) (0.6–4 M total Na\(^+\)) at pH 10–10.1, as described previously (Sorokin et al., 2001a). Thiosulfate (40–80 mM) served as the energy source and nitrate (5 mM as KNO\(_3\)) as the nitrogen source. Solid alkaline media with final salt concentrations 0.6 and 2 M total Na\(^+\) were prepared by 1:1 mixing of 4% agar and double-strength mineral base at 50 °C. Enrichments for denitrifying sulfur bacteria were performed in 100 ml serum bottles with butyl rubber stoppers filled with 50 ml of alkaline base with 20 mM thiosulfate and 20 mM nitrate. Anaerobiosis was achieved by five cycles of evacuation-flushing with argon.

**Activity tests.** The activity tests were performed with washed cells obtained from batch cultures grown at pH 10 with thiosulfate. Cells were harvested by centrifugation, washed twice with sodium buffer and resuspended in the same buffer at 20 mg protein ml\(^{-1}\) and kept on ice. This concentrated suspension was diluted 100–1000 times immediately before experiments. The pH influence on the activity of thiosulfate oxidation was tested with an oxygen electrode as described previously (Sorokin et al., 2001a) using 0.1 M HEPES + 0.6 M NaCl for the pH range 6–8 and NaHCO\(_3\)/Na\(_2\)CO\(_3\) buffer for higher pH values. All buffers contained 50 mM KCl. The influence of salt concentration on the activity of thiosulfate oxidation was investigated using sodium buffer, pH 10, with total Na\(^+\) concentration from 0.3 to 4 M.

**Total DNA analysis.** The isolation of the DNA and subsequent determination of the G+C content of the DNA and the DNA–DNA hybridization were performed according to standard procedures (Marmur, 1961; De Ley et al., 1970).

**Amplification and sequencing of 16S rRNA genes.** For amplification and sequencing of 16S rDNA genes, the DNA was obtained by standard phenol/chloroform extraction. The 16S rDNA genes were selectively amplified using primers 5'-AGAGTTTGTACCTGGCTCAG-3' (forward) and 5'-TACGGTTACCTTGTTACGACTT-3' (reverse). PCR products were cloned, and transformed using Invitrogen kit. Sequencing had been done by MWG Biotech, Inc (High Point North Carolina) with Lico machine using custom-designed primers to sequence samples to an accuracy of > 99%. A combination of both available sequencing reactions which include dye primer on the Lico Long Read IR 4200 sequencers and dye terminator on the ABI3700 capillary sequencers were used. Dye primer chemistry provided read lengths from 750 to over 1100 bases, while the dye-terminator reaction was used to confirm base calls and provide gap closure with read lengths ranging from 500 to 750 bases. Nearly complete 16S rDNA gene sequences were obtained for the Mono Lake isolates ALM 1<sup>T</sup> and ALM 2<sup>T</sup> (1450–1470 nucleotides).

**16S rDNA sequence analysis.** The sequences were aligned manually with sequences obtained from the database of small-subunit rRNAs in EMBL. The sequences were compared with those of the members of the Proteobacteria. Regions that were not sequenced in one or more reference organism were omitted from the analyses. Pairwise evolutionary distances (expressed as estimated changes per 100 nucleotides) were computed by using the Jukes & Cantor method. A resulting phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) with bootstrap analysis of 100 trees using programs of the TRECON package (Van de Peer & De Wachter, 1994). Bootstrap analysis (100 replications) was used to validate the reproducibility of the branching pattern of the trees.

**Electron microscopy.** For total preparations, cells were washed and resuspended in 0.5–1 M neutral NaCl solution, pre-fixed (2 h) and then fixed (10 h) at 4 °C in 0.1% and 2.5% (v/v, final) glutaraldehyde solutions, respectively, containing the same amount NaCl, and then positively stained with 1% (w/v) uranyl acetate. For ultrathin sectioning cells after fixation were postfixed in 1% (w/v) OsO\(_4\) solution containing 0.6–2 M NaCl for 10 h at 4 °C. Then the cells were washed, dehydrated and embedded into the resin. Thin sections were stained with 1% solutions (w/v) of uranyl acetate and lead citrate.

**Chemical analysis.** Thiosulfate in batch cultures and in experiments with washed cells was determined by the iodimetric titration after acidification of the samples by acetic acid to pH 5. Sulfide was measured colorimetrically (Trüper & Schlegel, 1964) after precipitation with zinc acetate (1%, w/v, final). Elemental sulfur was extracted from the cell pellets with acetone overnight and was assayed by a cyanolytic procedure (Sörbo, 1957). The biomass
protein was determined by the Lowry method, interfering sulfur compounds being removed either by several washings of the cell pellet with 0–6 M NaCl (thiosulfate) or by a double acetone extraction (elemental sulfur). The pigment from the cells of strain ALM 2 was extracted with a methanol/acetone (1:1) mixture. Its absorption spectrum was recorded on the UV-Visible diode-array Hewlett Packard HP 8453 spectrophotometer.

RESULTS AND DISCUSSION

Enrichment and isolation of pure cultures

Two different positive enrichment cultures from the interface water layer of Mono Lake were obtained using alkaline mineral medium with thiosulfate and different salt content. In low-salt enrichment (0–6 M Na\(^+\)) a phenotype with coccoid, non-motile cells was seen. Tentative enumeration showed 10\(^{-6}\)–10\(^{-7}\) cells ml\(^{-1}\). In high-salt enrichment (2–5 M Na\(^+\)) slightly curved motile rods dominated, and the cultures turned yellowish after complete consumption of thiosulfate and intermediately produced elemental sulfur. Plating of the cultures onto solid media with corresponding salt concentrations resulted in isolation of two different pure cultures of aerobic sulfur-oxidizing bacteria, strains ALM 1\(^T\) (0–6 M Na\(^+\)) and ALM 2\(^T\) (2 M Na\(^+\)).

Morphology

The colonies of strain ALM 1\(^T\) were up to 3 mm in diameter, reddish, transparent, without sulfur deposition. Under the light microscope the cells looked like tiny, non-motile, irregular spheres, often aggregated. The electron microscopy demonstrated that the cells of ALM 1\(^T\) were shaped as the open rings (Fig. 1a, b) with a diameter 0.5–0.8 \(\mu\)m and cell width 0.3–0.4 \(\mu\)m. Many cells contained multiple carboxysome-like structures (Fig. 1a). The colonies of strain ALM 2\(^T\) were up to 4 mm in diameter, yellowish, with sulfur deposition. ALM 2\(^T\) cells are curved rods, 0.3–0.4 \(\times\) 1–2 \(\mu\)m, motile by a single polar flagellum (Fig. 2a). Cells often contained intracellular sulfur globules (Fig. 2b, c) apparently encapsulated via invagination of the cell membrane (Fig. 2d). The biomass of ALM 2\(^T\) was yellow-coloured due to membrane-associated pigment. Methanol/acetone extract of the cell membranes had absorption maxima at 405, 430 (main) and 450 nm. The specific pigment content in the biomass increased about twofold when salt content of the growth medium increased from 0.6 to 2–2.5 M total Na\(^+\), remaining at constant level upon further increase of salinity to 3–4 M Na\(^+\).

Phylogeny

Phylogenetic analysis of nearly complete 16S rDNA gene sequences of strains ALM 1\(^T\) and ALM 2\(^T\) demonstrated their close relationship to two recently described genera of alkaliphilic sulfur bacteria in the \(\gamma\)-Proteobacteria. Strain ALM 1\(^T\) is a member of the genus Thioalkalimicrobium with a high level of sequence similarity to type strains AL 3\(^T\) and AL 7\(^T\) (96.8–98.5\%). Strain ALM 2\(^T\) is more distantly related but definitely belongs to a phylogenetic cluster of the genus Thioalkalivibrio (92.7–96.6% sequence similarity) (Fig. 3).

Genetic analysis

The results of total DNA analysis of the Mono Lake isolates are given in Table 1. The G+C content in DNA of both strains fitted well into the range typical
for the corresponding genera of previously described haloalkaliphilic sulfur bacteria. Comparison of total DNA of strain ALM 1<sup>T</sup> and the type strains of the genus *Thioalkalimicrobium* confirmed the results of phylogenetic analysis, in that this strain, despite its peculiar morphology, is a member of the genus *Thioalkalimicrobium*. It had relatively high hybridization to rod-shaped type strain *Thioalkalimicrobium aerophilum* AL 3<sup>T</sup> but it was still less than is usual between strains of the same species (<70%; Stackebrandt & Goebel, 1994). In contrast, strain ALM 2<sup>T</sup> showed a close phenotypic similarity to the members of genus *Thioalkalivibrio*, in particular with the yellow coloured, extremely salt-tolerant subgroup, but displayed a very low level of DNA hybridization (<32%) with the representative strains of *Thioalkalivibrio*. Nevertheless, with clear phenotypic and phylogenetic indications, strain ALM 2<sup>T</sup> should be considered as a member of the genus *Thioalkalivibrio*.

**Physiological properties**

Both Mono Lake isolates belong to obligately chemolithoautotrophic sulfur-oxidizing bacteria. Growth with hydrogen as electron donor and denitrification (thiosulfate + nitrate, nitrite or N<sub>2</sub>O) were not observed. Like other sulfur-oxidizing strains isolated previously from various soda lakes, the Mono Lake isolates were obligately alkaliphilic. Growth in batch culture with thiosulfate at 0.6 M Na<sup>+</sup> salt content was possible within the pH range 7.5–10.5. The thiosulfate-dependent O<sub>2</sub> consumption by washed cells of strain ALM 1<sup>T</sup> and ALM 2<sup>T</sup> was observed within the pH range 6.5–11 and 7.0–11.2, respectively, with an optimum at pH 9.5 for both organisms (Fig. 4). The salt tolerance of strain ALM 1<sup>T</sup> was in the moderate range while ALM 2<sup>T</sup> belongs to an extremely tolerant type, being able to function within a very broad salinity range from 0.4 to 4 M total Na<sup>+</sup> (Fig. 5). The difference between the strains was more evident with growing cultures (measured by the rate of thiosulfate consumption, Fig. 5a) as compared to activity of the resting cells (Fig. 5b). Strain ALM 1<sup>T</sup> never formed sulfur as an intermediate during growth with thiosulfate, while prolific sulfur production was observed in ALM 2<sup>T</sup> cultures at salt concentration less than 2 M total Na<sup>+</sup>. The respiratory profiles of both strains (Table 2) are consistent with the patterns of representatives of the genera *Thioalkalimicrobium* and *Thioalkalivibrio*. As is typical for members of the genus *Thioalkalimicrobium*
Haloalkaliphilic sulfur-oxidizing bacteria

Figure 3. Phylogenetic tree showing the relationships of the Mono Lake strains of alkaliphilic sulfur-oxidizing bacteria in the γ-Proteobacteria. The numbers on the branches refer to bootstrap values; only values above 90% are shown. The bar represents 5% sequence divergence.

Table 1. DNA–DNA hybridization between Mono Lake isolates and members of genera Thioalkalimicrobium and Thioalkalivibrio

<table>
<thead>
<tr>
<th>Strain</th>
<th>DNA G+C content (mol%)</th>
<th>DNA–DNA hybridization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thioalkalimicrobium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group 1</td>
</tr>
<tr>
<td>ALM 1T</td>
<td>49.6</td>
<td>60</td>
</tr>
<tr>
<td>ALM 2T</td>
<td>63.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, No data.

(Sorokin et al., 2001a), strain ALM 1T oxidized thiosulfate and sulfide, less actively polysulfide and tetrathionate and had no activity with elemental sulfur. Strain ALM 2T oxidized all five sulfur compounds with more or less equal but relatively low activity, which is a characteristic of the members of genus Thioalkalivibrio (Sorokin et al., 2001a). Addition of sulfite did not stimulate oxygen consumption in the cell suspensions of the new isolates.

Mono Lake belongs to a comparatively rare type of stratified alkaline and highly saline lakes. In this lake, a hypersaline, anaerobic, sulfide-containing water body is overlaid by less saline, aerobic waters. The result is a rather stable oxic/anoxic interface that has been shown to act as an active aerobic chemolithotrophic ‘filter’ for reduced inorganic compounds, such as ammonia, methane and sulfide (Joye et al., 1999; Ward et al., 2000). Given the high sulfate...
investigation demonstrated that at least two different interface of Mono Lake was suspected as well. Our presence of sulfur-oxidizing bacteria in the redox concentration (130 mM) (Oremland & Miller, 1993), the presence of sulfur-oxidizing bacteria in the redox interface of Mono Lake was suspected as well. Our investigation demonstrated that at least two different cultivable types of obligately chemolithoautotrophic, aerobic, haloalkaliphilic sulfur bacteria inhabit this lake. One type (represented by strain ALM 1T), enriched at moderate salt concentration, probably corresponds to a population adapted to the surface aerobic waters of the Mono Lake mixolimnion. The other type (represented by strain ALM 2T), enriched at extremely high salt concentrations, functions opti-

Table 2. Respiration rate of washed cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ALM 1T</th>
<th>ALM 2T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiosulfate ($S_2O_3^{−}$)</td>
<td>2.50</td>
<td>0.60</td>
</tr>
<tr>
<td>Sulfide (HS)$^+$</td>
<td>3.04</td>
<td>0.70</td>
</tr>
<tr>
<td>Polysulfide ($S_n^{−}$)</td>
<td>1.80</td>
<td>0.66</td>
</tr>
<tr>
<td>Sulfur (S$^+$)</td>
<td>0.00</td>
<td>0.52</td>
</tr>
<tr>
<td>Tetrathionate ($S_4O_6^{−}$)</td>
<td>0.42</td>
<td>0.33</td>
</tr>
</tbody>
</table>

*ALM 1T and ALM 2T were grown with thiosulfate at pH 10 and 0.6 M Na+. Values are given as µmol O$_2$ (mg protein)$^−$ 1 min$^−$ 1 (means of 2–3 independent measurements); endogenous rates were subtracted.

Fig. 4. Influence of pH on the activity of thiosulfate-dependent oxygen consumption by washed cells of the Mono Lake isolates ALM 1T ( ● ) and ALM 2T ( ○ ). All buffers contained 0.6 M total Na$. pH 6.5–8, HEPES + NaCl; pH 8–11.2, NaHCO$_3$/Na$_2$CO$_3$. Means from the two independent experiments.

Fig. 5. Influence of salt concentration (NaHCO$_3$/Na$_2$CO$_3$/NaCl) on growth and activity of thiosulfate oxidation by washed cells of the Mono Lake isolates at pH 10. (a) Amount of thiosulfate consumed during 24 and 47 h growth in batch cultures of the strains ALM 1T and ALM 2T, respectively; (b) activity of thiosulfate-dependent respiration. ●, ALM 1T; ○, ATM 2T. Means from the two independent experiments.

On the basis of physiological and genetic properties, the Mono Lake isolates were clearly related to two different groups of previously described haloalkaliphilic sulfur-oxidizing bacteria isolated from the Kenyan and Siberian soda lakes (Sorokin et al., 2001a). Strain ALM 1T, being physiologically very similar to representatives of the genus *Thioalkalimicrobium*, differs from the known species of this genus by its morphology. Phylogenetically strain ALM1T is closely related to members of the genus *Thioalkalimicrobium*.
but since its DNA similarity with described species was less than 70%, this strain is proposed as a new species, *Thioalkalimicrobium cyclicum*. Strain ALM 2T, despite its very low DNA hybridization with the representative strains, phenotypically clearly resembles the extremely salt-tolerant members of the genus *Thioalkalivibrio*. In view of the results of phylogenetic analysis, this isolate should be affiliated with the genus *Thioalkalivibrio* and is proposed as a new species *Thioalkalivibrio jannaschii*. We have also found this type of sulfur-oxidizing double extremophiles dominating in highly saline alkaline lakes in Mongolia, Kenya and Wadi an Natrun in Egypt (Sorokin et al., 2001a; our unpublished results).

It is interesting to mention that these sulfur-oxidizing bacteria probably represent the only type of aerobic chemolithoautotrophs capable to grow in saturated alkaline brines. Other types of alkaliphilic chemolithoautotrophs (methane-, hydrogen-, ammonia- and nitrite-oxidizing), although being detected in and isolated from the extremely saline soda lakes, are able to grow only at salinity below 1 M Na⁺ (Ward et al., 2000; Sorokin et al., 1998, 2000a, c, 2001c).

**Description of Thioalkalimicrobium cyclicum sp. nov.**

*Thioalkalimicrobium cyclicum* (cyc.li.cum Gr. n. cyclus circle; M.L. n. cyclicum circle-like).

Cells are mostly in a form of open ring with a diameter 0.5–0.8 and cell width 0.3–0.4 µm. Many cells contain multiple carboxysome-like structures. Obligately chemolithotrophic, alkaliphilic and moderately halophilic. Grows up to 1-5 M Na⁺. Oxidizes sulfide, thiosulfate, polysulfide and tetrathionate. G+C content in DNA is 49.6 mol% (Tₘ). Isolated from the O₂-sulfide interface layer of Mono Lake (California, USA). Other properties as for the genus. The type strain is ALM 1T (= DSM 14477T = JCM 11371T).

**Emended description of genus Thioalkalimicrobium**

The description of the genus *Thioalkalimicrobium* (Sorokin et al., 2001a) should be emended by the following:

Cells vary in shape from straight rods with sharp edges to spirilla, 0.3–0.5 × 0.8–1.5 µm, motile by means of 1–3 polar flagella at one end. Also includes strains with non-motile, circular and coccolid cells.

**Description of Thioalkalivibrio jannaschii sp. nov.**

*Thioalkalivibrio jannaschii* (jann.asch.i.i. N.L. gen. n. jannaschii in honour of German microbiologist Holger Jannasch).

Cells are curved rods, 0.3–0.4 × 1–2 µm, motile by a single polar flagellum. Cells often contain intracellular sulfur globules when grown with thiosulfate at low salinity. Produces yellow, membrane-associated pigment during growth at high salinity. The methanol/acetone extract of the cell membranes has absorption maxima at 405, 430 (main) and 450 nm. Obligately chemolithoautotrophic, haloalkaliphilic bacterium. Tolerates up to 4 M Na⁺. Oxidizes sulfide, thiosulfate, polysulfide, sulfur and tetrathionate. G+C content in DNA is 63.7 mol% (Tₘ). Isolated from the O₂-sulfide interface layer of alkaline Mono Lake (California). Other properties as for the genus. The type strain is ALM 2T (= DSM 14478T = JCM 11372T).

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