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Thioalkalimicrobium aerophilum gen. nov., sp. nov. and *Thioalkalimicrobium sibericum* sp. nov., and *Thioalkalivibrio versutus* gen. nov., sp. nov., *Thioalkalivibrio nitratis* sp. nov. and *Thioalkalivibrio denitrificans* sp. nov., novel obligately alkaliphilic and obligately chemolithoautotrophic sulfur-oxidizing bacteria from soda lakes

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Forty-three strains of obligately chemolithoautotrophic sulfur-oxidizing bacteria were isolated from highly alkaline soda lakes in south-east Siberia (Russia) and in Kenya using a specific enrichment procedure at pH 10. The main difference between the novel isolates and known sulfur bacteria was their potential to grow and oxidize sulfur compounds at pH 10 and higher. The isolates fell into two groups that were substantially different from each other physiologically and genetically. Most of the Siberian isolates belonged to the group with a low DNA G+C content (48·0–51·2 mol%). They were characterized by a high growth rate, a low growth yield, a high cytochrome content, and high rates of oxidation of sulfide and thiosulfate. This group included 18 isolates with a DNA homology of more than 40%, and it is described here as a new genus, *Thioalkalimicrobium*, with two species *Thioalkalimicrobium aerophilum* (type species) and *Thioalkalimicrobium sibericum*. The other isolates, mainly from Kenyan soda lakes, fell into a group with a high DNA G+C content (61·0–65·6 mol%). In general, this group was characterized by a low growth rate, a high molar growth yield and low, but relatively equal, rates of oxidation of thiosulfate, sulfide, elemental sulfur and polythionates. The group included 25 isolates with a DNA homology of more than 30%. It was less compact than *Thioalkalimicrobium*, containing haloalkalophilic, carotenoid-producing, nitrate-reducing and facultatively anaerobic denitrifying strains. These bacteria are proposed to be assigned to a new genus, *Thioalkalivibrio*, with three species *Thioalkalivibrio versutus* (type species), *Thioalkalivibrio denitrificans* and *Thioalkalivibrio nitratis*. Phylogenetic analysis revealed that both groups belong to the γ-*Proteobacteria*. The *Thioalkalimicrobium* species were closely affiliated with the neutrophilic chemolithoautotrophic sulfur bacteria of the genus *Thiomicrospira*, forming a new alkaliphilic lineage in this cluster. In contrast, *Thioalkalivibrio* was not related to any known chemolithoautotrophic taxa, but was distantly associated with anaerobic purple sulfur bacteria of the genus *Ectothiorhodospira*.

Keywords: *Thioalkalimicrobium*, *Thioalkalivibrio*, sulfur-oxidizing bacteria, alkaliphilic bacteria, soda lakes

The GenBank/EMBL accession numbers for the 16S rDNA sequences of strains ALJD^T, AL 2^T, ALJ 12^T, AL 3^T and AL 7^T are AF126545–AF126549, respectively.

INTRODUCTION

Microbial communities in natural alkaline environments such as soda lakes have recently attracted attention as potential sources of industrially potent enzymes (Horikoshi, 1996) and as a new perspective on microbial diversity (Zavarzin, 1993; Duckworth *et al*., 1996; Jones *et al*., 1998). Generally, shallow and eutrophic soda lakes are characterized by a high level of primary production (Melack, 1981; Cloern *et al*., 1983) which, in turn, gives rise to active sulfidogenesis (Isachenko, 1951; Abdel Malek & Rizk, 1963; Vamos & Tasnadi, 1975; Imhoff *et al*., 1979). Early studies have shown that a dense community of anaerobic phototrophic bacteria is responsible for the oxidation of sulfide produced in soda lake sediments (Isachenko, 1951; Imhoff *et al*., 1979; Tindall, 1980). However, our recent search revealed the presence of a diverse population of aerobic sulfur-oxidizing bacteria in these alkaline environments. First, obligately heterotrophic alkaliphiles capable of oxidation of sulfur compounds to tetrathionate aerobically and under denitrifying conditions were isolated and characterized (Sorokin *et al*., 1996a; Sorokin & Mityushina, 1998). Next, two strains of obligately chemolithoautotrophic sulfur-oxidizing alkaliphilic bacteria from soda lake Hadyn in Tuva (Siberia) were described (Sorokin *et al*., 2000). These autotrophs represented two metabolic types that differ in many aspects of their physiology and genomic characteristics. Further investigation of various samples from soda lakes in different geographic areas (Sorokin *et al*., 1996b) resulted in the isolation of another 41 strains of obligately autotrophic alkaliphilic sulfur bacteria, all of which fitted into one of the two metabolic groups mentioned above. This paper describes the taxonomic properties of the two groups and proposes the creation of two new genera, *Thioalkalimicrobium* and *Thioalkalivibrio*, to accommodate these sulfur-oxidizing bacteria.

METHODS

Samples. The samples of surface sediments and underlying water were obtained from a number of Siberian soda lakes during summer expeditions in 1989, 1995, 1996 and 1997. These lakes are situated in dry steppe regions; they are mostly small, shallow, highly productive and of low to moderate salinity. The samples from Kenyan soda lakes (East African Rift Valley) were collected during two expeditions in 1992 and 1996. These lakes are bigger and more saline than the steppe lakes. The general information on the samples is presented in Tables 1 and 2.

Bacterial strains. Forty-three strains of obligately chemolithoautotrophic sulfur-oxidizing bacteria isolated from various soda lakes were investigated. A list of the strains is presented in Tables 1 and 2. The type strain of *Thiomicrospira pelophila* was obtained from the culture collection of Delft University (LMD) and the type strain of *Thiomicrospira crunogena* was kindly provided by C. Wirsen (Woods Hole Oceanographic Institute).

Enrichment, isolation and culture conditions. The alkaliphilic sulfur bacteria were enriched and isolated using mineral medium, buffered at pH 10 with sodium carbonate/bicarbonate and with thiosulfate as electron donor, containing Na_2CO_3, 21 g l^{-1}; $NaHCO_3$, 9 g l^{-1}; NaCl, 5 g l^{-1}; K_2HPO_4, 0·5 g l^{-1}; KNO_3, 5 mM; $MgCl_2.6H_2O$, 0·5–1·0 mM; trace elements (Pfennig & Lippert, 1966), 2 ml l^{-1}; and a final pH of 10·05–10·10 after sterilization. Thiosulfate was sterilized separately and added at final concentrations of 40–80 mM. Solid media were prepared by mixing 4 % (w/v) agar and double-strength mineral base at 55–60 °C to prevent agar caramelization at high pH. During work with extremely saline samples from Kenyan soda lakes, the medium was supplied with an additional 0·5 M NaCl. The haloalkaliphilic strain ALJ 15 was enriched and isolated on a medium containing 2 M NaCl in addition to the soda base. Another haloalkalophilic strain, ALJ 22, was enriched and isolated on a medium containing 4 M total Na^+ as sodium carbonates.

Nitrate-to-nitrite reducing strains ALJ 12^T and ALD 2 were enriched anaerobically on medium containing 20 mM nitrate in addition to the usual soda base. Despite the successful enrichment, the resulting pure cultures were unable to grow anaerobically by reducing nitrate to nitrite. This ability seems to help these bacteria, however, to grow under microaerobic conditions because of their very weak oxidase activity. The only true denitrifier, strain $ALJD^T$, was enriched and then isolated in pure culture when an anaerobic enrichment was performed with N_2O (0·5 atm. overpressure) as electron acceptor and thiosulfate as electron donor at pH 10.

Pure cultures of the alkaliphilic sulfur bacteria were maintained on liquid mineral medium with thiosulfate at pH 10 with a 2 month storage interval between transfers. $MgCl_2$ (5 mM) was added as a cell-wall stabilizing agent before conservation at 4 °C. Prolonged storage on solid medium was not efficient. For long-term conservation, concentrated biomass samples were mixed with glycerol (10 %, v/v, final concentration) and kept at −70 °C.

The *Thiomicrospira* species were grown on thiosulfate mineral base, pH 7·5, according to original formulations (Kuenen & Veldkamp, 1972; Jannasch *et al*., 1985), except that 50 mM HEPES was added as a buffer.

Respiratory experiments. Cells for the respiratory test were grown in liquid medium with thiosulfate at pH 10 until all thiosulfate was consumed. Haloalkalophilic strains ALJ 15 and ALJ 22 were grown in a medium containing 2 M total Na^+. After centrifugation, the pellets were washed and resuspended in concentrated form (about 20 mg protein ml^{-1}) in soda buffer at pH 10. The activity was tested at pH values between 6 and 11·0–11·5 using buffers containing 0·6 M total Na^+ and 50 mM KCl: for pH 6·0–7, 0·1 M HEPES/NaOH plus NaCl was used; for pH 8, freshly prepared $NaHCO_3$; for pH 9·0–11·5, a combination of $Na_2CO_3/NaHCO_3$ was used. The carbonate dependence of the respiration was examined using 0·1 M Tris/HCl plus NaCl for pH 9–10. For haloalkaliphilic strains, the buffers were supplemented with an additional 1 M NaCl. The respiration rates were measured in a 5 ml thermostatted chamber mounted on a magnetic stirrer fitted with a Clark-type oxygen electrode (Yellow Spring Instruments). The final biomass concentration in the respiration chamber was in the range 0·02–0·20 mg protein ml^{-1}. Stock solutions of sulfide, polysulfide (S_6^{2-}) and sulfite were prepared anaerobically in 0·1 M Tris/HCl, pH 10, with 5 mM EDTA to prevent auto-oxidation, and introduced into the chamber at final concentrations of 25–50 mM. Elemental sulfur was

Table 1 Isolation of obligately autotrophic, alkaliphilic, sulfur-oxidizing bacteria of the '*Thioalkalimicrobium*' group (low G+C group)

Lake	Sample			Isolate		
	Type*	Water pH	Water salinity (g l^{-1})	Strain	G+C (mol%)	Morphology
Soda lakes in south-east Siberia, Russia						
Tuva Republic,1995						
Hadyn	2	10·0	30	AL 3^T	49·5	Rod
Kunkur steppe (Chita region),1996						
Hilgatyn	1	9·8	40	AL 6	48·5	Curved rod
	3			AL 7^T	48·9	Vibroid rod
Unnamed-2	2	10·0	5	AL 8	48·7	Vibroid rod
Low Mukei	3			AL 9	49·5	Rod
	1	9·5	14	AL 14	49·9	Rod
	2			AL 15	51·2	Rod
Ulan-Nor	2	10·5	22	AL 10	48·3	Vibroid rod
				AL 17	48·5	Vibroid rod
Gorbunka	3	9·5	6·5	AL 11	48·7	Vibroid rod
				AL 16	49·3	Vibroid rod
Unnamed-3	2	10·0	5	AL 12	49·4	Vibroid rod
Heito-Holvo-Torum	1	9·8	15	AL 13†	48·9	Rod
	3			AL 18†	48·5	Rod
Barguzin river valley,1997						
Nuhe-Nur	2	9·9	10	AL 20	48·0	Vibroid rod
Kenya, 1996						
Elmenteita	2	10·0	26‡	ALJ 13†	49·5	Spirillum
				ALJ 14†	49·6	Spirillum
Bogoria	2	10·2–10·5	65·6‡	AL 19	48·0	Vibroid rod

* Sample types: 1, water above the sediments or mats; 2, sediments; 3, microbial mat surface.

† Strains required 0·5 M NaCl in addition to normal soda-based medium.

‡ Water conductivity values given (mS cm^{-1}; 60 mS cm^{-1} conductivity corresponds to approximately 1 M total Na^+).

introduced as a saturated solution in acetone at a final concentration of 70 mM and the results were compared with tests using acetone alone. Thiosulfate and polythionates were used at final concentrations of 50–200 mM from freshly prepared concentrated stock solutions in distilled water.

Enzyme activities. Cell-free extracts were prepared by ultrasonic disruption of the same cell suspensions used in respiratory experiments. Unbroken cells and cell debris were removed by centrifugation at 50000 ***g*** for 10 min. All operations were carried out at 0–4 °C. The activity of sulfite dehydrogenase was measured colorimetrically using ferricyanide as an artificial electron acceptor (Kelly & Wood, 1994). Cytochrome *c* oxidase activity was measured spectrophotometrically using reduced TMPD as a substrate in 0·1 M Tris/HCl, pH 8, plus 0·3 M NaCl, which was found to be optimal. Cytochrome spectra were recorded on a diode-array UV-visible spectrophotometer 8453 (Hewlett Packard) in total extracts or its fractions obtained after centrifuging the crude extracts at 144000 ***g*** for 4 h.

SDS-PAGE. Gel electrophoresis of total proteins was performed under denaturing conditions using gel concentrations of 8–12%. Cells grown with thiosulfate at pH 10 were resuspended in distilled water and sonicated. The resulting extracts were adjusted to a protein concentration of 1 mg ml^{-1} and boiled in sample buffer with 2-mercaptoethanol and dithiothreitol (15 µl sample plus 5 µl sample buffer); the final preparations were applied to a gel after cooling and centrifuging for 2 min.

Total DNA analysis. The isolation of the DNA and subsequent determination of the DNA G+C content and 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 rRNA genes, the DNA was obtained by standard phenol/chloroform extraction. The 16S rRNA genes were selectively amplified using primers 5′-AGAGTTTGATCCTGGCTCAG-3′ (forward) and 5′-TACGGTTACCTTGTTACGACTT-3′ (reverse). PCR products were purified from low-melting agarose using the Wizard PCR Prep kit (Promega) according to the manufacturer's instruction. Almost complete sequencing (1400–1450 nt) was performed using the Promega Silver Sequencing kit (Promega) according to the manufacturer's instructions with minor modifications.

16S rDNA sequence analysis. The sequences were aligned manually with sequences obtained from the database of small subunit rRNA collected from the the EMBL database.

Table 2 Isolation of obligately autotrophic, alkaliphilic, sulfur-oxidizing bacteria of the '*Thioalkalivibrio*' group (high G+C group)

Lake	Sample				Isolate		
	Period	Type*	Water pH	Water conductivity (mS cm^{-1})	Strain	G+C (mol %)	Morphology
Kenya							
Magadi	1992	1	10·0–10·5	109	ALJ 1	62·9	Vibrio
	1996	2	—	—	ALJ 18	62·1	Rod
					ALJ 22†‡	65·6	Vibrio
Bogoria	1992	1	11·0	60	ALJ 3	64·9	Vibrio
					ALJ 6	63·9	Spirillum
					ALJ 7	64·2	Spirillum
					ALJ 10	65·0	Vibrio
	1996	1	10·2–10·5	66	ALJ 15†‡	64·9	Vibrio
					ALJ 16	65·3	Vibrio
					ALJ 20	63·7	Vibrio
					$ALJD^T$§	63·7	Curved rod
Elmenteita	1992	1	10·5	28	ALJ 2	63·2	Vibrio
	1996	1	10·0	26	ALJ 17	63·8	Thin curved rod
Crater lake (Sonachi)	1992	1	10·5	15	ALJ 4	64·9	Vibrio
					ALJ 5	64·7	Vibrio
	1996	1	10·0	23	ALJ 19	64·5	Vibrio
					ALJ 21†	61·3	Curved rod
Nakuru	1992	1	10·5	51	ALJ 8	62·9	Vibrio
					ALJ 9	64·2	Vibrio
					ALJ 11	63·7	Vibrio
					$ALJ\ 12^T$	62·1	Rod
South-east Siberia							
Hadyn	1989	1	10·0	30‖	$AL\ 2^T$	63·7	Curved rod
Tsaidam	1995	1	9·8	15‖	AL 5	63·3	Curved rod
Low Mukei	1996	1	9·5	14‖	ALD 1	64·7	Vibrio
					ALD 2	61·7	Rod

* Samples: 1, surface sediments plus water; 2, salt crust from the salt pan.

† Strains ALJ 15, ALJ 21 and ALJ 22 form yellow carotenoids.

‡ Strains ALJ 15 and ALJ 22 are halophilic (1·5–2·0 M Na^+ optimum).

§ Strain $ALJD^T$ is a facultatively anaerobic denitrifying bacterium.

‖ Salinity (g l^{-1}).

The sequences were compared with those of members of the *Proteobacteria*. Regions that were not sequenced in one or more reference organisms were omitted from the analyses. Pairwise evolutionary distances (expressed as estimated changes per 100 nt) were computed by using the Jukes & Cantor method. The resulting phylogenetic tree was constructed by the neighbour-joining method. Bootstrap analysis (100 replications) was used to validate the reproducibility of the branching pattern of the trees.

Electron microscopy. Whole cells were fixed with glutaraldehyde (2·5%, w/v, final concentration) directly in their culture medium and then positively stained with 1% (w/v) phosphotungstic acid. Samples for ultra-thin sectioning were first pre-fixed by adding 0·2% (w/v) OsO_4 (final concentration) to cell suspension in hydrocarbonate buffer (pH 8) for 1 h. Then, the cells were centrifuged, resuspended in 2% (w/v) NaCl solution (pH 8), fixed with 1% (w/v) final concentration OsO_4 for 12 h at 4 °C, dehydrated and embedded into the resin. Thin sections were stained with uranyl acetate and lead citrate. To identify the intracellular accumulation of elemental sulfur, cells were sedimented, stained with a solution containing 2% (w/v) $AgNO_3$ and 2% (w/v) glutaraldehyde for 10 h and then fixed with OsO_4. Post-sectional staining was omitted in this case.

Chemical analysis. Sulfur compounds were analysed by cyanolytic procedures after Kelly *et al.* (1969) and Sörbo (1957). Nitrite and nitrate were analysed spectrophotometrically according to Gries-Romijn-van Eck (1966) and Bhandari & Simlat (1986), respectively. Biomass protein was measured with Folin reagent (Lowry *et al.*, 1951) after removal of interfering sulfur compounds either by repeating washing with soda buffer (water-soluble compounds) or by an overnight acetone extraction of the pellets (elemental sulfur).

RESULTS

1. '*Thioalkalimicrobium*' group

Strains and morphology. The alkaliphilic sulfur-oxidizing bacteria with a low DNA G+C content (48–51 mol %) were isolated mostly from fresh samples of the low-saline Siberian soda lakes (about 80 % positive enrichments). Only three strains of a total of 18 originated from the Kenyan soda lakes (Table 1). The group was tentatively named '*Thioalkalimicrobium*'.

The group includes strains with rod-shaped, vibroid and spirilloid cells. Vibriods and spirilla were motile by means of one polar flagellum. Rod-shaped strains could have up to three polar and subpolar flagella from the one end (Fig. 1a, c). Isolates with rod- and spirillum-shaped cells retained their morphology under different growth conditions, whereas strains with vibroid cells had a tendency to grow as short rods in shaken cultures and as vibrios or bent rods under static microaerobic conditions. All strains had a similar ultrastructural organization, with an undulating cell wall of the Gram-negative type and multiple carboxysome-like structures localized in the central region of the cell (Fig. 1b, d). The cell wall in these bacteria was very unstable under low-osmotic conditions and during storage. Cells were lysed immediately after suspension in distilled water or after thawing from the frozen stage. The presence of Mg^{2+} in millimolar concentrations had a stabilizing effect on the cell wall and prolonged the successful storage at 4 °C for up to 2 months. Four strains (AL 6, AL 8, AL 16 and AL 18) survived storage for 12 months. On alkaline thiosulfate agar, colonies were reddish, without sulfur deposition, spreading or compact.

Growth characteristics. *Thioalkalimicrobium* strains were obligate chemolithoautotrophs. They were able to grow only in the presence of thiosulfate or sulfide. Although some strains tested assimilated a limited amount of organic compounds (acetate, yeast extract), the bacteria were not able to grow heterotrophically. They grew relatively fast (for autotrophic sulfur bacteria) on thiosulfate mineral medium at pH 10. In batch cultures, bacteria grew optimally at pH values higher than 9. In pH-controlled thiosulfate-limited continuous culture, growth occurred within a pH range of 7·5–10·6, with an optimum around 10. At pH values lower than 8, most of the cells showed signs of autolysis. The maximum specific growth rate in chemo-

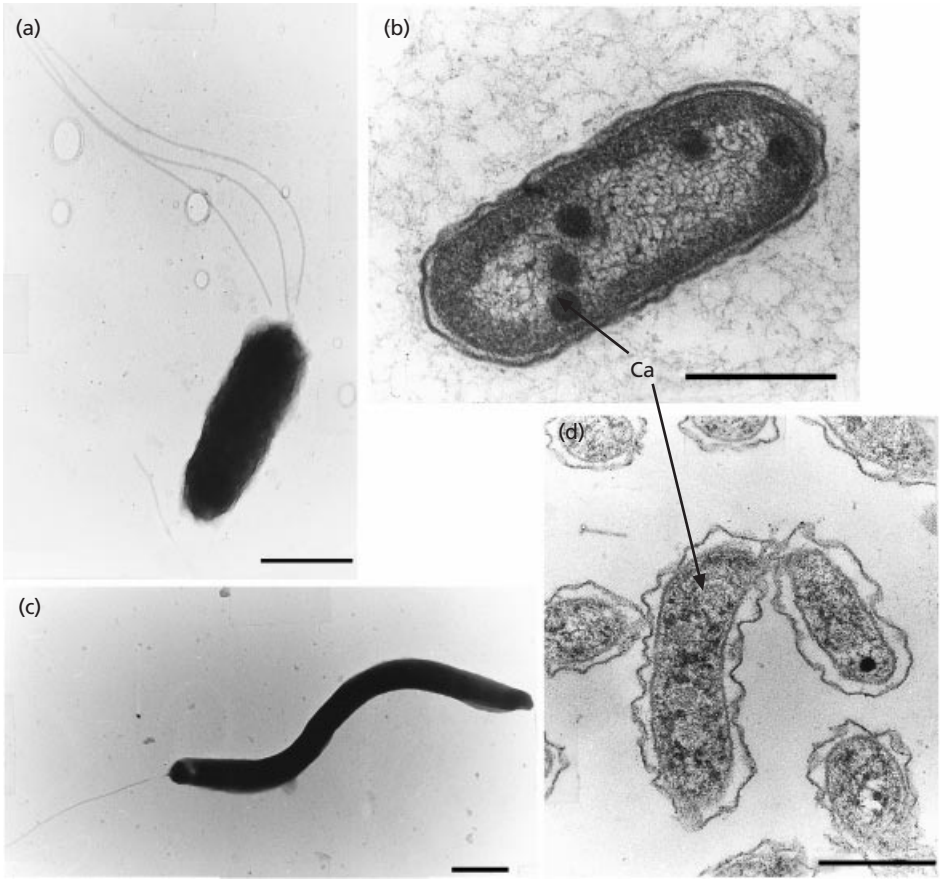

Fig. 1. Morphology of alkaliphilic sulfur bacteria from the *Thioalkalimicrobium* group grown with thiosulfate at pH 10. (a, c) Total preparations; (b, d) ultrathin sections; (a, b) strain AL 3^T; (c, d) strain ALJ 14. Ca, Carboxysome-like structures. Bars, 0·5 μm.

Table 3 Similarity of groups within the alkaliphilic genus *Thioalkalimicrobium*

Strain	Total protein similarity*	Oxygen demand†	Colony‡	Tetrathionate oxidation§
AL 7^T	1	1	1	1
AL 11	1	1	1	1
AL 17	1	1	1	1
AL 10	1	1	1	1
AL 12	1	1	1	1
AL 8	1	1	1	2
AL 9	1	2	1	2
AL 16	1	2	2	2
AL 19	1	2	1	2
AL 13	2	2	2	2
ALJ 13	2	2	1–2	1
ALJ 14	2	2	1–2	1
AL 3^T	3	2	1	2
AL 14	3	2	2	2
AL 18	3	2	2	2
AL 6	2	2	1–2	1
AL 15	2	2	2	2

* Groups of total protein similarity were estimated according to a position of three major haem *c*-containing bands at approximately 50, 40 and 30 kDa.

† Groups of oxygen demands: 1, growth rate in static cultures more than in shaken cultures; 2, growth rate in static cultures equal or less than in shaken cultures.

‡ Colony groups: 1, compact colonies; 2, flat, spreading colonies; 1–2, intermediate type.

§ Tetrathionate-oxidation groups: 1, activity range 0·0–0·1 nmol O_2 (mg protein)$^{-1}$ min^{-1}; 2, activity range 0·3–1·1 nmol O_2 (mg protein)$^{-1}$ min^{-1}.

stat culture (four strains tested) was 0·33 h^{-1}; in batch culture, it varied from 0·08 to 0·22 h^{-1}. The molar growth yield on thiosulfate was relatively low; in batch culture, it varied from 1·8 to 2·7 g protein (mol thiosulfate)$^{-1}$ and, in the chemostat, it reached a maximum value of 3·5 g protein (mol thiosulfate)$^{-1}$. No intermediate elemental sulfur formation was observed during batch cultivation. Instead, a small amount of sulfite was detectable in some cultures under unfavourable pH conditions. Elemental sulfur production was only observed during polysulfide oxidation by cell suspensions and in cultures grown with thiosulfate and sulfide under strong oxygen limitation.

On the basis of their oxygen requirement, *Thioalkalimicrobium* strains could be divided into two categories: preferentially aerobic and preferentially microaerobic. Aerobic strains grew better under conditions of non-limiting oxygen supply. This category included mostly rod-shaped bacteria isolated from the water or surface sediments of the Siberian soda lakes (Table 1). They formed large, flat, spreading colonies. The exceptions were the Kenyan strains ALJ 13, ALJ 14 and AL 19 which, being aerobic, formed spirilloid cells and compact colonies. The microaerobic strains mostly originated from the anaerobic sediments. They grew faster under conditions of limited oxygen supply. Some of them, however, were only slightly inhibited by forced aeration as well. The latter strains were represented by vibroid cells and formed compact colonies (Table 3).

All strains were sodium-dependent, with a minimal sodium ion requirement of about 0·2–0·3 M. The upper limit of sodium ion concentration was 1·2–1·5 M.

Sulfur-oxidizing potential. The bacteria from the *Thioalkalimicrobium* group had, in general, high sulfide- and thiosulfate-dependent respiration activity, which contrasted with their low cytochrome *c* oxidase activity [0·2–0·3 mmol TMPD (mg protein)$^{-1}$ min^{-1}]. They had a very high concentration of cytochromes of the *c* type [up to 3 nmol haem *c* (mg membrane protein)$^{-1}$]. On the other hand, cytochromes of the *b* type became spectroscopically detectable only after their chromatographic separation from the bulk of cytochrome *c*. A special study undertaken with strains AL 3^T and AL 7^T (unpublished results) revealed the presence of cytochrome *c* oxidase of the cbb_3 type in their membranes – a recently discovered cytochrome *c* oxidase with high affinity to oxygen (Preisig *et al.*, 1993; García-Horsman *et al.*, 1994). This species was the only cytochrome *c* oxidase found in the neutrophilic sulfur bacterium *Thiobacillus neapolitanus* W5 (Visser *et al.*, 1997).

All members of this group oxidized thiosulfate, sulfide and polysulfide with maximum rates at pH 9–10 and

Table 4 Respiratory activity of washed cells of the two genera of alkaliphilic autotrophic sulfur bacteria grown with thiosulfate at pH 10

V_{max}, Rate of oxygen consumption at optimum pH (minus endogenous rate) mmol O_2 (mg protein)$^{-1}$ min^{-1}; *n*, number of strains tested.

Substrate	*Thioalkalimicrobium*			*Thioalkalivibrio*		
	V_{max}	pH_{opt}	*n*	V_{max}	pH_{opt}	*n*
$S_2O_3^{2-}$	2·0–4·3	9–10	17	0·2–1·0	9–10	25
HS^-	2·6–5·2	9–10	17	0·3–0·8*	9–10	25
S_6^{2-} (polysulfide)	1·1–3·0*	10	15	0·2–0·85*	10	20
S_8	0·0–0·2	10	17	0·2–0·6	10–11	25
SO_3^{2-}	0·0	—	15	0·0–0·2	10	20
$S_3O_6^{2-}$	0·0	—	6	0·0–0·2	9	10
$S_4O_6^{2-}$	0·0–1·1	9	17	0·05–0·5	9	25
$S_5O_6^{2-}$	0·0	9	6	0·1–0·8	9	10

* Biphasic kinetics.

up to pH 11 (Table 4). The specific rates are comparable with the highest values observed in neutrophilic sulfur bacteria (Stefess, 1993; Visser, 1997). On the other hand, only a few of the *Thioalkalimicrobium* strains were able to oxidize tetrathionate and most could not oxidize elemental sulfur or did it with a very low specific activity. As the affinity constant for tetrathionate was at least one order of magnitude higher than that for thiosulfate and sulfide (about 80–100 mM) and because of the instability of tetrathionate under alkaline conditions at high concentrations, it is very unlikely that tetrathionate can serve as a natural substrate for the *Thioalkalimicrobium* group. In contrast, polysulfides can be regarded as specific substrates for alkaliphilic sulfur bacteria because they become chemically stable at alkaline pH. The stoichiometry of oxygen consumption and accumulation of colloidal sulfur during polysulfide oxidation indicated that only the terminal sulfur atoms of S_6^{2-} were oxidized completely to sulfate by the *Thioalkalimicrobium* strains.

These results demonstrated that inorganic polysulfur compounds like polysulfide are unlikely to be involved as intermediates of sulfide oxidation in these bacteria. Together with the complete inability to oxidize sulfite, the absence of sulfite dehydrogenase activity and the very low tetrathionate synthase activity in cell-free extracts, results suggest that the *Thioalkalimicrobium* representatives oxidize sulfur compounds directly to sulfate by a mechanism similar to that found in several neutrophilic sulfur bacteria like *Thiobacillus versutus* (Kelly, 1989, 1999). Purified sulfide dehydrogenase from strain AL 3^T oxidized sulfide via a one-electron mechanism implying the formation of a sulfide radical as immediate product (Sorokin *et al.*, 1998). Further oxidation may proceed via some kind of enzyme-bound [S–S] intermediate with a step-by-step oxidation of sulfur atoms to sulfate.

DNA–DNA homology. All strains with a low DNA G+C content were members of one genetic group at the genus level with a DNA similarity higher than 40% (Fig. 2). Inside the group, three major genetic clusters and two individual strains, which did not cluster with

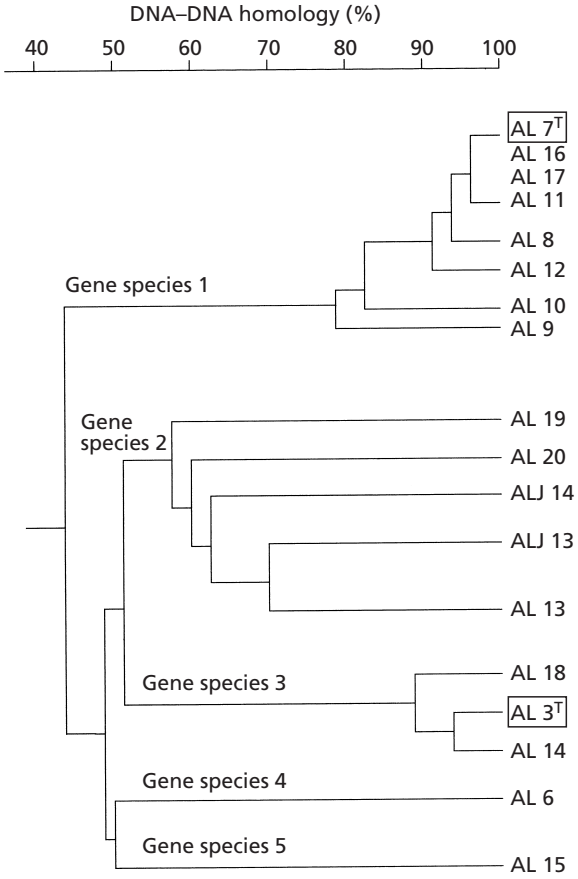

Fig. 2. Dendrogram of DNA–DNA homology among strains of the *Thioalkalimicrobium* group.

any other strains, could be distinguished. The microaerobic vibroid Siberian isolates formed one separate gene cluster with a DNA homology of more than 70%. These strains had 43–45% homology with the rod-shaped aerobic Siberian strains and about 50% with the spirilloid aerobic Kenyan isolates. Because the separation of the two gene clusters mentioned above correlated with morphological and physiological properties (Table 3), this was taken as a basis for the description of the two species inside the group. Two tight subclusters were found inside the second gene cluster, which corresponded to gene species 2 and 3 (Fig. 2). Single-strain gene species AL 6 and AL 15 could be distinct species on the basis of DNA–DNA homology data, but it was not possible to separate them clearly from the other strains on the basis of phenotypic characteristics. In particular, these two strains were phenotypically similar to the subcluster AL 3^T/AL 14/AL 18 (gene species 3 in Fig. 2), which included aerobic rod-shaped Siberian strains. On the other hand, genetic subcluster AL 13/ALJ 13/ALJ 14/AL 19/AL 20 included members that differed in their morphology and respiratory and protein profiles (Table 3).

Phylogenetic analysis. 16S rRNA gene sequence analysis of the rod-shaped strain AL 3^T and the vibroid strain AL 7^T revealed that the *Thioalkalimicrobium* group belongs to the *γ-Proteobacteria*, with a relatively close affiliation to the neutrophilic sulfur-oxidizing bacteria of the genus *Thiomicrospira* (see Fig. 5). The genus *Thiomicrospira* includes two major lineages, which are substantially different in many characteristics. The *Thioalkalimicrobium* group clustered with the *Thiomicrospira pelophila* lineage. It had about 4 and 10% total sequence difference with *Thiomicrospira pelophila* and *Thiomicrospira crunogena* type strains, respectively. Despite the relatively high sequence dissimilarity level, the two *Thiomicrospira* lineages and the alkaliphilic *Thioalkalimicrobium* representatives possessed similar signature regions in their 16S rRNA gene. It has been previously demonstrated (Brinkhoff & Muyzer, 1997) that *Thiomicrospira* 16S rDNA contains two specific motifs at positions (*Escherichia coli*) 128–145 and 830–849. These were also present in the genes of alkaliphilic strains AL 3^T and AL 7^T.

2. '*Thioalkalivibrio*' group

Strains and morphology. This group includes 25 strains with a DNA G+C content of 61·0–65·6 mol%. The strains were isolated mostly from the Kenyan soda lakes (Table 2) which are, in general, more alkaline and saline than the Siberian steppe lakes. The group is represented typically by vibrio-shaped bacteria with one polar flagellum. In cultures of strains ALJ 6 and ALJ 7, spirilla-like cells dominated. The cells of the nitrate-reducing strains ALJ 12^T, ALD 2 and ALJ 18 were predominantly rod-shaped, although some bent cells could be seen in old cultures. Strains ALJD^T and ALJ 17 differed from the others by formation of curved filamentous rod-shaped cells. The ultrastructural organization of the *Thioalkalivibrio* cells was generally similar to that of the *Thioalkalimicrobium* representatives; in most strains, the Gram-negative cell wall was undulating. Also, most of the strains, except the nitrate-reducing and denitrifying strain ALJD^T, formed multiple carboxysome-like structures in the centre of the cells (Fig. 3e, f). In contrast to the *Thioalkalimicrobium* strains, cells of the *Thioalkalivibrio* strains were more resistant to osmotic shock and survived much longer during storage in liquid cultures at 4 °C; only 5 out of 25 strains did not retain viability after 12 months. A substantial difference in cell fine structure was observed only in the haloalkalophilic strains ALJ 15 and ALJ 22. The cell wall of ALJ 15 cells had multiple tubular extensions filled with electron-dense material (Fig. 3b, c). The cells of another haloalkalophilic strain, ALJ 22, were surrounded by a large capsule, sometimes shared by several cells which tended to aggregate (Fig. 3a, d). The *Thioalkalivibrio* strains formed relatively large colonies (up to 5 mm) on alkaline thiosulfate agar, developing through several stages. Young colonies were usually brightly white and filled with sulfur. With time, the sulfur gradually disappeared and the colonies turned yellowish, sometimes even orange. The colour change was a result of the transformation of insoluble elemental sulfur into a cell-bound soluble polysulfur compound like polysulfide, as judged from the spectra of cell extracts prepared from the colonies and from a qualitative cyanolytic test.

Growth characteristics. Similarly to representatives of *Thioalkalimicrobium*, none of the *Thioalkalivibrio* strains was able to grow on purely organic media without reduced sulfur compounds, thus being obligately chemolithoautotrophic. Optimal growth on thiosulfate mineral medium in batch culture occurred at a pH around 9–10. The actual pH range for growth was detected in pH-controlled continuous culture (four strains); it was within the pH range 7·50–10·65, with an optimum at 10·0–10·2. Some of the Kenyan isolates failed to grow at a pH lower than 10. During growth in batch culture with thiosulfate, elemental sulfur was always produced in the early growth phase and then disappeared in the late exponential phase. In thiosulfate-limited chemostat cultures, elemental sulfur was formed only when the dilution rate approached the maximum growth rate and at a pH lower than 8. The bacteria of this group grew more slowly than the *Thioalkalimicrobium* strains; their maximum specific growth rate in chemostat cultures was 0·18 h^{-1} and that in batch cultures was 0·1 h^{-1} (strains AL 2^T and ALJ 2). On the other hand, the molar growth yield on thiosulfate was about two times higher than in *Thioalkalimicrobium* strains [5–6 g protein (mol thiosulfate)$^{-1}$]. Most strains decreased their growth rate, and especially the molar growth yield, after 2–4 years maintenance in liquid batch cultures.

All members of the group, except strain ALJ 17 and the denitrifying ALJD^T, grew faster under unlimited aeration, although in static cultures less sulfur was

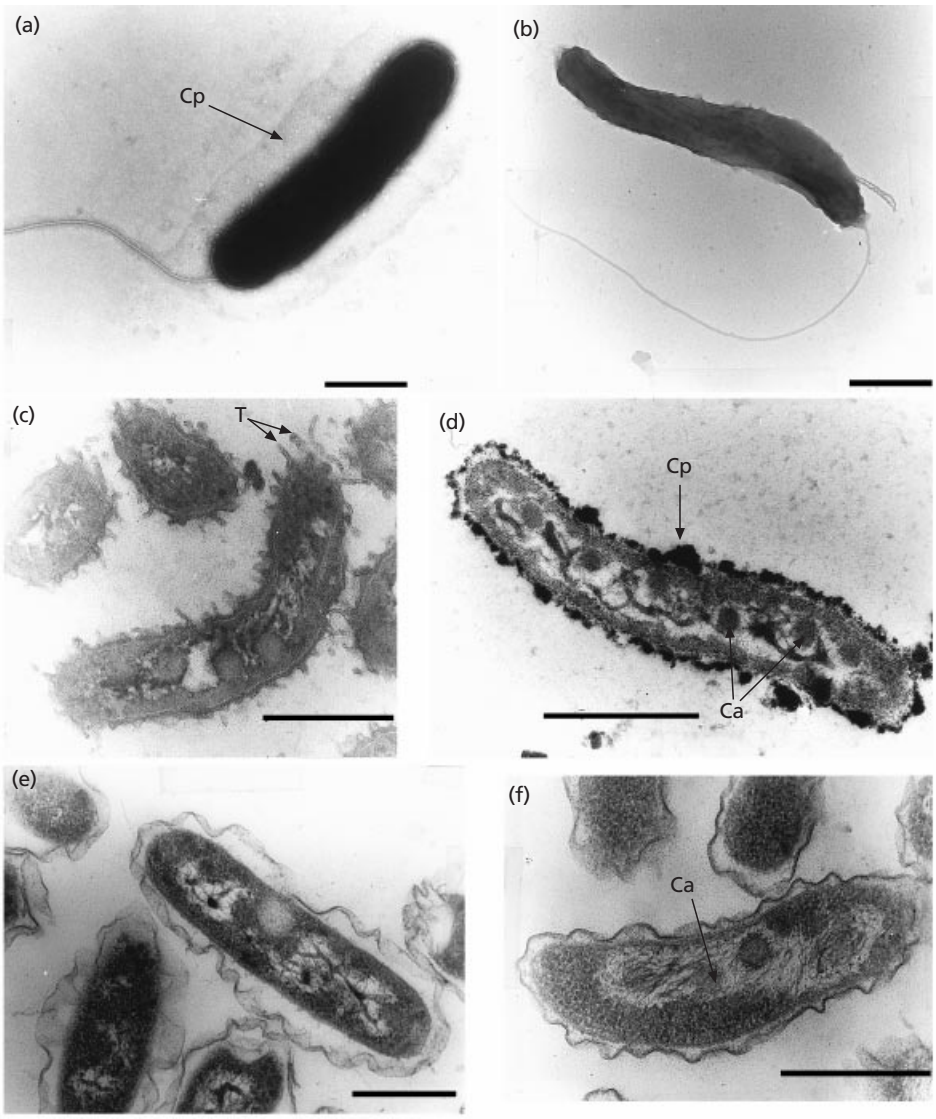

Fig. 3. Morphology of the alkaliphilic sulfur bacteria from the *Thioalkalivibrio* group grown with thiosulfate at pH 10. (a, b) Total preparations; (c–f) thin sections; (a, d) strain ALJ 22 (grown with 3 M total Na^+); (b, c) strain ALJ 15 (grown with 2 M total Na^+); (e) strain AL 2^T; (f) strain ALJ 5. Ca, Carboxysome-like structures; Cp, capsule material; T, tubular structures. Bars, 0·5 μm.

produced during the initial growth phase. The bacteria also grew faster with 0·2–0·5 mM Mg^{2+} in the medium. Higher concentrations of Mg^{2+} inhibited growth, resulting in the incomplete oxidation of thiosulfate to elemental sulfur. All strains were obligately Na^+-dependent. The minimal sodium ion concentration for growth was about 0·3 M and growth was possible generally up to 1·2–1·5 M total Na^+. The halo-alkaliphilic strains ALJ 15 and ALJ 22 had an absolute requirement for at least 1 M Na^+. They were able to grow in the presence of up to 4 M total Na^+, with an optimum at 2 M. In media (or in buffers for resting cells) containing less than 1 M Na^+, immediate and heavy sulfur formation from thiosulfate and sulfide was observed.

Cytochrome composition. Cells of the *Thioalkalivibrio* members, in general, contained much lower concentrations of cytochromes than the *Thioalkalimicrobium* strains. However, the relative abundance of *b* type cytochromes was higher, so that the latter could be easily identified spectroscopically in total extracts. In addition to normally presented *b* type cytochromes with an α-maximum at 556–560 nm, in nitrate-reducing strains a cytochrome with an α-maximum at 595–598 nm was observed. Because a maximum in the γ-region, typical for cytochrome *a*, in reduced extracts was absent and because the CO spectrum was of the cytochrome *b* type, the 595 nm maximum could be attributed to a special form of a high-spin haem *b* (Lowrence *et al.*, 1986). The cytochrome oxidase

activity of nitrate-reducing strains was much lower than in other isolates, which may be the reason for the positive influence of nitrate on their growth. Cells of the denitrifying strain ALJD^T grown with nitrite or N_2O as electron acceptors had a high concentration of a cytochrome component with spectroscopic characteristics of nitrite reductase type cd_1. Also, ALJD^T was the only strain among the isolated alkaliphiles that contained cytochrome aa_3 in the membranes (absorption maxima at 452 and 601 nm in the α- and γ-regions, respectively). The CO-difference spectra of the cell-free extract of seven strains unable to reduce nitrate had a pattern typical of cytochrome *o*. The isolation of pure protein will be necessary to make a final conclusion on the dominant cytochrome oxidase type. The activity of the cytochrome *c* oxidase in cell-free extracts of these *Thioalkalivibrio* strains was 5–50 times higher than in the members of *Thioalkalimicrobium* and it was at least five times less sensitive to CN^-. The latter confirmed the spectroscopic evidence on the different nature of cytochrome *c* oxidases in the two groups of these sulfur-oxidizing alkaliphiles.

Sulfur-oxidizing potential. Despite their much higher oxidase activity, the *Thioalkalivibrio* strains had much lower actual rates of substrate-dependent respiration than the *Thioalkalimicrobium* members (Table 4). There was also a substantial difference in the substrate specificity. Most of the *Thioalkalivibrio* strains were able to oxidize elemental sulfur with the same order of activity as for sulfide and thiosulfate. Polythionates were also actively oxidized. Experiments with a cell suspension of strain AL 2^T demonstrated that this bacterium was capable of anaerobic hydrolysis of tetrathionate to thiosulfate, sulfur and sulfate, which is similar to results observed in acidophilic thiobacilli (Pronk *et al.*, 1990), except that the pH optimum for the reaction was 9. Some of the strains were able to consume oxygen in the presence of sulfite. Polysulfide (S_6^{2-}) was oxidized completely to sulfate at pH 10 without sulfur formation, in contrast to the *Thioalkalimicrobium* strains. The bacteria were active within a pH range 7–11 (some up to 11·5), with an optimum of 9–10. The activity below pH 8 was usually very low, especially with sulfide as substrate. The Kenyan isolates were more active than the Siberian ones at extremely alkaline pH values ($> 10{\cdot}5$). At pH values lower than 9, the usual biphasic kinetics of oxygen consumption with sulfide, sulfur and polysulfide were observed.

All strains of *Thioalkalivibrio*, in contrast to the *Thioalkalimicrobium* representatives, exhibited an AMP-independent sulfite dehydrogenase activity. It varied from 0·2 to 5 mmol (mg protein)$^{-1}$ min^{-1} in different strains. The activity was obligately dependent on the presence of $NaHCO_3$ (optimum at 0·4 M and pH 8·5) and was insensitive to the usual inhibitors of sulfite dehydrogenases (*p*-chloromercuribenzoate, NEM, atebrin, CN^-) in concentrations up to 10^{-3} M. Despite the very high sulfite dehydrogenase activity in cell-free extracts of some of the strains, the actual sulfite-oxidizing activity by whole cells was always low, probably because of sulfite toxicity even at the micromolar concentrations used in the respiration experiments.

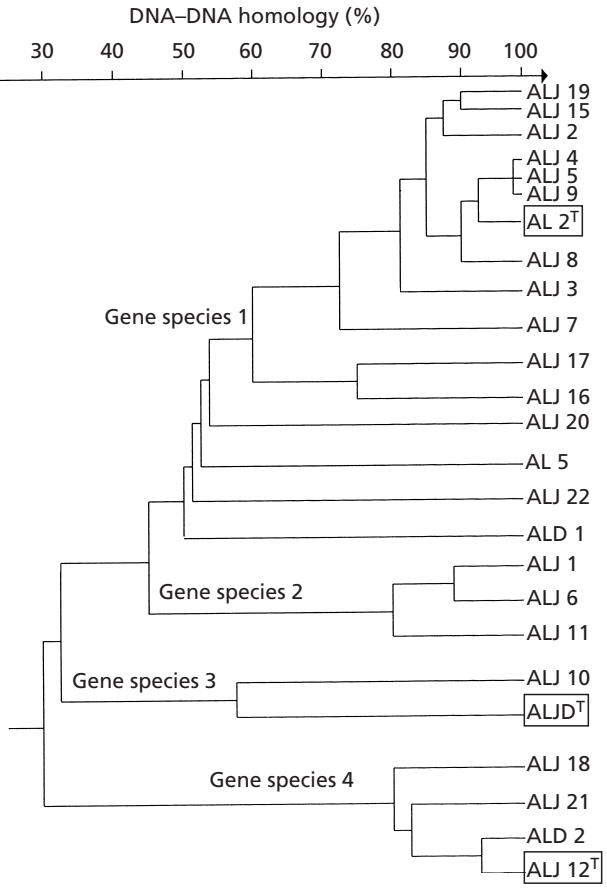

Fig. 4. Dendrogram of DNA–DNA homology among strains of the *Thioalkalivibrio* group.

Overall, the results demonstrated that the bacteria of the *Thioalkalivibrio* group appear to employ a pathway of sulfide oxidation via polysulfur (sulfur or possibly polysulfide) compounds and sulfite, similar to many acidophilic and some neutrophilic sulfur-oxidizing bacteria (Kelly, 1989, 1999; Pronk *et al.*, 1990).

DNA–DNA hybridization and clustering. Genetically, as well as phenotypically, the *Thioalkalivibrio* group is less compact than the *Thioalkalimicrobium* group. DNA–DNA hybridization demonstrated that it includes both highly related strains with more than 90% DNA homology and only weakly related representatives with a DNA similarity of about 30% with the other strains (Fig. 4). On the basis of DNA homology, four gene clusters could be distinguished. However, only one of them strongly correlated with phenotypic differences. This cluster included four nitrate-reducing strains (cluster 4), which were most distant not only genetically but also different from the other isolates with respect to their cytochrome com-

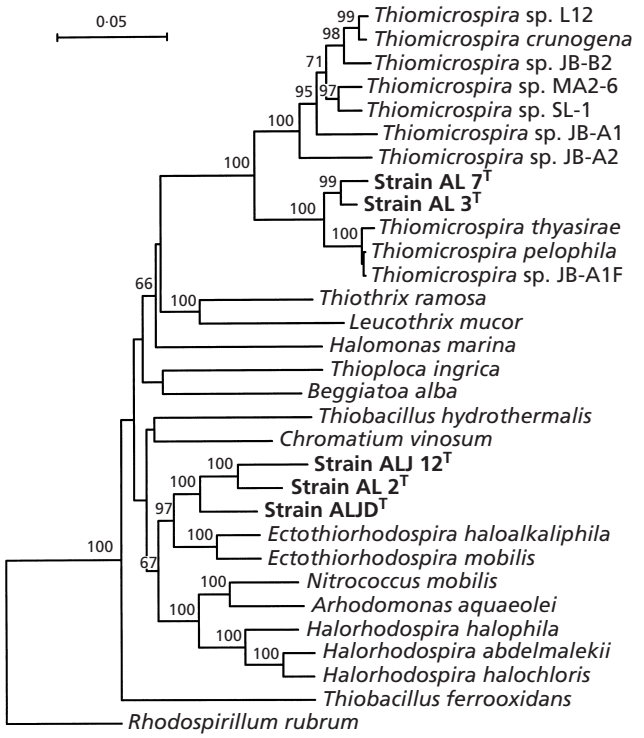

Fig. 5. Phylogenetic tree showing the relationships of the alkaliphilic sulfur-oxidizing bacteria (marked in bold) in the *γ-Proteobacteria*. The sequence of *Rhodospirillum rubrum* was used as an outgroup. The numbers on the branches refer to bootstrap values; only values above 50% are shown.

position and morphology. The other gene clusters were less phenotypically determined, although there was some correlation with the protein profiles and the level of sulfite dehydrogenase activity. Cluster 1 (Fig. 4) included a major proportion of the isolates with a DNA homology more than 40%; in general, its members had a high level of sulfite dehydrogenase in contrast to the small cluster 3. Although there were at least two tight subclusters inside cluster 1, substantial phenotypic descriptors to isolate these subclusters into separate species were not found.

Controversially, three strains which possessed important phenotypic differences were not separated genetically from the others. Two haloalkalophilic strains, ALJ 15 and ALJ 22, and one nitrate-reducing strain, ALJ 21, were able to produce membrane-bound yellow carotenoids but genetically they could not be separated from the colourless strains. The yellow pigment could be extracted from the wet biomass with acetone. In strain ALJ 15, it was represented by a group of highly hydrophilic homologous carotenoid species which are currently under investigation (S. Takaichi, personal communication). Actually, this is a first known example of pigment formation among aerobic 'colourless' sulfur bacteria. In the haloalkalophilic strains, the amount of carotenoid produced positively correlated with salt concentration in the growth medium. Assuming a phylogenetic relation with the purple sulfur bacteria (see below), the coloured strains were also analysed for the possible presence of the bacteriochlorophylls, but the results were always negative.

Strains ALJ 15 and ALJ 22 not only possessed carotenoids, but were also the most halotolerant and thermotolerant among the alkaliphilic isolates. All other strains were moderately halotolerant. The haloalkalophilic strains had been isolated from the highly saline samples by a special enrichment on a medium with 2–4 M total Na^+. They grew actively in the presence of more than 0·5 M and up to 4 M total Na^+ with an optimum at 1–2 M. While growing on 2 M Na^+-containing medium, strain ALJ 15 tolerated temperatures up to 45 °C and ALJ 22 up to 47 °C. Both strains grew optimally at 40 °C. Together with the carotenoid formation and unusual cell ultrastructure, these strains showed enough phenotypic differences from the other *Thioalkalivibrio* isolates to form a separate species, but this was not confirmed by the DNA analysis. Strains ALJ 15 and ALJ 22 were not specifically related to each other and both belonged to genetic cluster 1 together with the colourless non-halophilic strains (Fig. 4).

Another interesting example is strain ALJD^T. It was the only facultatively anaerobic denitrifying strain among the isolated alkaliphilic sulfur bacteria. This bacterium was isolated with thiosulfate as electron donor and N_2O as electron acceptor at pH 10. It grew very slowly under microaerobic conditions and anaerobically with nitrite. Anaerobic growth with nitrate was not observed at all, presumably because of the lack of dissimilatory nitrate reductase. Best growth occurred anaerobically with thiosulfate as a substrate and N_2O as an electron acceptor. The ability to denitrify is very rare among obligately autotrophic sulfur bacteria and only two species have thus far been described (Kuenen *et al.*, 1992). Therefore, the value of such a phenotypic property should correspond to at least a separate species level. Indeed, strain ALJD^T had a relatively low DNA similarity with the non-denitrifying *Thioalkalivibrio* strains except strain ALJ 10. With the latter, it showed 55–58% similarity, which is above gene species level within the *Thioalkalivibrio* group (Fig. 4). ALJD^T also had a protein profile very similar to that of the non-denitrifying strain ALJ 10. All attempts to grow ALJ 10 anaerobically with different nitrogen oxides as electron acceptors were not successful. The reason for such an obvious discrepancy between the genetic similarity and physiological difference is not clear. It might be speculated that this could be the result of the complete deletion of the DNA region responsible for denitrification in strain ALJ 10.

Phylogenetic analysis. A preliminary sequence analysis of the first 500 nt from the 16S rDNA gene of ten Kenyan strains (ALJ 1–10) demonstrated that they form a monophyletic group in the *γ-Proteobacteria*

with only a distant clustering to some of the methanotrophic bacteria (data not shown). Analysis of the complete 16S rDNA gene sequence of the representative strains AL 2^T, ALJ 12^T and ALJD^T confirmed that the *Thioalkalivibrio* group belongs to the *γ-Proteobacteria* and that its position is quite different from that of the *Thioalkalimicrobium* group (Fig. 5). The sequence difference between the type strains of these three species was also higher (5–6%). The *Thioalkalivibrio* group had no immediate relatives among the other chemolithotrophic members of the *γ-Proteobacteria*. On the other hand, their monophyletic branch clustered well with the anaerobic purple sulfur bacteria of the genus *Ectothiorhodospira* (up to 91% sequence similarity). Comparative analysis of the secondary structure of 16S rRNA of strains AL 2^T, ALJ 12^T and ALJD^T revealed some helical regions (60–90 and 1000–1040; *E. coli* numbering) that differed from the helical regions of *Ectothiorhodospira* species. These regions could be used to construct specific molecular probes for the detection of the *Thioalkalivibrio* group in the natural environment.

DISCUSSION

The results of these morphological, physiological and genetic studies of a range of new sulfur-oxidizing chemolithotrophic bacteria from a number of natural alkaline habitats demonstrated that all of them belong to two distinct groups, different both from each other and from known neutrophilic species with similar metabolism. The most striking difference is the potential of the new bacteria to grow and oxidize sulfur compounds under highly alkaline conditions. The main properties of the two different groups are summarized in Table 5.

The *Thioalkalivibrio* group is clearly different from all known sulfur autotrophs and therefore can be described as a new genus. Some *Ectothiorhodospira* species were found to dominate in the anaerobic phototrophic populations in alkaline soda lakes (Isachenko, 1951; Imhoff *et al.*, 1979; Tindall, 1980; V. Gorlenko, personal communication). Moreover, a potential for microaerobic growth was discovered at least for some species (Kondrat'eva *et al.*, 1976), although they were not entirely lithoautotrophic. In addition, there are some phenotypic similarities between some of the *Ectothiorhodospira* species (in particular with *Ectothiorhodospira haloalkalophila*) and *Thioalkalivibrio*, including cell morphology, salt tolerance, pH optimum and elemental sulfur formation outside of the cells. It might be speculated that the described alkaliphilic aerobic sulfur-oxidizing chemolithotrophic bacteria represent the direct aerobic descendants of the phototrophic sulfide-oxidizing anaerobes. In this respect, it will be interesting to undertake comparative studies of these two kinds of sulfur-oxidizing bacteria to understand more clearly the mechanisms of metabolic evolution after the appearance of oxygen.

The taxonomic status of the *Thioalkalimicrobium* group is less clear. The phylogenetic closeness of the alkaliphilic strains to the *Thiomicrospira pelophila* cluster indicated that the latter may be related to the alkaliphilic group. Therefore, a physiological and genetic comparison between *Thiomicrospira pelophila* and *Thioalkalimicrobium* was performed. The former was unable to grow on soda base medium at pH 10, which was routinely used for the cultivation of soda-lake isolates. However, in contrast to *Thiomicrospira crunogena*, *Thiomicrospira pelophila* appeared to belong to the alkalitolerant organisms, being able to initiate its growth at a pH as high as 9·5 in a medium containing up to 0·3 M sodium bicarbonate. In this case, the pH went down from 9·5 to 8·1 at the end of the growth period. The major difference in the respiratory profile between *Thiomicrospira pelophila* and the *Thioalkalimicrobium* strains was found around pH 10·0 in media with high sodium carbonate concentrations. *Thiomicrospira pelophila* actively respired thiosulfate up to pH 9·5 in NaCl and sodium bicarbonate-containing buffers (data not shown) with an optimum at pH 7. In sodium carbonate-based buffers at pH 10 this bacterium was inactivated, while these conditions were optimal for the activity of soda-lake isolates. The genetic variability inside the alkaliphilic *Thioalkalimicrobium* group was itself sufficient for a genus range (Fig. 2) but obviously higher than is usual for the species level. Direct genetic comparison between two representative strains of *Thioalkalimicrobium* (AL 3^T and AL 7^T) and *Thiomicrospira pelophila* and *Thiomicrospira crunogena* demonstrated a very low DNA homology level (about 15% with *Thiomicrospira pelophila* and 6% with *Thiomicrospira crunogena*). Therefore, assuming substantial phylogenetic divergence, physiological differences and low genetic similarity, the alkaliphilic sulfur bacteria of *Thioalkalimicrobium* group should be described as a new genus. It should, however, be pointed out that *Thioalkalimicrobium* formed a coherent phylogenetic cluster with the *Thiomicrospira pelophila* lineage and therefore these alkaliphilic and neutrophilic sulfur bacteria may be members of the same family. Another conclusion from the genetic comparison is that *Thiomicrospira crunogena* should be reclassified because of its extremely low levels of genetic (6% DNA homology) and phylogenetic (about 10% sequence dissimilarity) similarity with *Thiomicrospira pelophila*.

Description of *Thioalkalimicrobium* gen. nov.

Thioalkalimicrobium (Thi.o.al.kal.i.mi.cro′bi.um. Gr. n. *thios* sulfur; N.L. n. *alcali* Arabic *al* the; *qaliy* soda ash; Gr. adj. *micros* small; Gr. masc. n. *bios* life; M.L. n. *Thioalkalimicrobium* sulfur alkaline microbe).

Cells vary from straight rods with sharp edges to spirilla, 0·4–0·5 × 0·8–1·5 μm, motile by means of 1–3 polar flagella at one end. Gram-negative. Cell wall is undulating and unstable in low osmotic environment. Mg^{2+} stabilizes the cell wall. Cells contain multiple

Table 5 Characteristics of the two genera of alkaliphilic sulfur-oxidizing bacteria

Property	*Thioalkalivibrio* (25 strains)	*Thioalkalimicrobium* (18 strains)
Source of isolate	Kenyan (21) and Siberian (4) soda lakes	Siberian (15) and Kenyan (3) soda lakes
DNA G+C (mol%)	61·0–65·6	48·0–51·2
Cell morphology	From rods to spirilla	From rods to spirilla
Flagellation	Single polar	1–3 polar from one side
Periplasmic sulfur globules	+/−	−
Carboxysomes	+/−	+
Colony morphology	Compact, often with sulfur (white) and polysulfide (yellow)	Compact or spreading, without sulfur, pink
Growth		
pH range	7·50–10·65	7·5–10·6
pH optimum (chemostat)	10·0–10·2	9·8–10·0
Upper temperature limit (°C)	47	41
Upper salt limit [NaCl or sodium carbonates; total Na^+ (M)]	4·0–4·3	1·2–1·5
Growth yield on thiosulfate	Up to 6 g protein mol^{-1}	Up to 3·5 g protein mol^{-1}
Growth rate	Up to 0·18 h^{-1}	Up to 0·33 h^{-1}
Survival after 12 months storage	20 strains	4 strains
Mg^{2+} influence	Growth stimulation	Stabilization of the cell wall, growth stimulation
Sulfur-oxidizing potential		
Sulfide and thiosulfate	Moderate	High
Sulfur	Moderate	Very low
Tetrathionate	+	+/−
Trithionate and pentathionate	+/−	−
Sulfite	+/−	−
Sulfite dehydrogenase	+	−
RuBPcase	+	+
Denitrification		
$NO_2^- \rightarrow N_2$	1 strain	−
$NO_3^- \rightarrow NO_2^-$	4 strains	−
Extreme halotolerance	2 strains	−
Yellow pigment	3 strains	−
Major ubiquinone*	Q-8	Q-8
N sources for growth (pH 10)		
NH_3 (low concn)	+	+
NO_2^-, NO_3^-	+	+
Urea and organic N	−	−
Thiocyanate	11 strains from 15 tested	7 strains from 12 tested
Dominating cytochrome	*c* and *b*	*c*
Cytochrome *c* oxidase	?	cbb_3
K_i50 of oxidase to CN^- (mM)	3–5	< 1
Sensitivity to CCCP and DCCD	Low	High

* Detected by B. Tindall (DSMZ, Braunschweig) in strains AL 3^T and AL 2^T.

carboxysomes. Colonies are reddish, without sulfur, convex or spreading. Obligately aerobic and chemolithoautotrophic. Grows best in soda-buffered media at pH 10 with thiosulfate or sulfide as electron donor. Under pH-controlled conditions, growth is possible within the pH range 7·5–10·6. Oxidizes sulfide and thiosulfate to sulfate at high rates within the pH range 7–11 (optimum at pH 9–10). Some strains oxidize tetrathionate. Only a few strains oxidize elemental sulfur with low activity. Polysulfide is oxidized to a mixture of sulfate and elemental sulfur. Unable to oxidize sulfite. Maximum specific growth rate and molar yield on thiosulfate at pH 10 is about 0·3 h^{-1} and 3·5 g protein mol^{-1}, respectively. The level of cytochrome *c* oxidase (cytochrome cbb_3) activity is relatively low. The level of cytochrome *b* is extremely low compared to the cytochrome *c* content. Carbon is assimilated via Calvin cycle. All strains are halotolerant, being able to grow in media containing up to 1·2–1·5 M total Na^+. NH_3 (at low concentrations),

NO_2^- and NO_3^- are utilized as nitrogen sources. Many strains are able to assimilate nitrogen from thiocyanate. Major ubiquinone is Q-8. DNA G+C content is between 48 and 51·2 mol% (T_m method). Isolated from soda lakes. Member of the *γ-Proteobacteria* and closely related to the *Thiomicrospira pelophila* group. Genus includes strains with a DNA homology level more than 40% that are assigned to two species– *Thioalkalimicrobium sibericum* and *Thioalkalimicrobium aerophilum*. The type species is *Thioalkalimicrobium aerophilum*.

Description of *Thioalkalimicrobium aerophilum* sp. nov.

Thioalkalimicrobium aerophilum (ae.ro.phi′lum. Gr. masc. n. *aër* gas; Gr. adj. *philus* loving; M.L. n. *aerophilus* air-loving).

The species includes seven rod- and vibrio-shaped strains from the water and surface sediments of Siberian soda lakes (AL 3^T, AL 6, AL 13, AL 14, AL 15, AL 18, AL 20) and three spirilloid strains from Kenyan soda lake sediments (ALJ 13, ALJ 14, AL 19) with DNA–DNA homology levels more than 40%. All strains grow faster under fully aerobic conditions. Most form flat, spreading colonies and oxidize tetrathionate. The Kenyan spirilloid strains form a tight gene cluster with the Siberian rod-shaped isolate AL 13. They have a similar protein profile and demand a higher sodium ion concentration for optimal growth than other strains of the genus. Among the rod-shaped strains, AL 3^T forms a tight gene cluster with AL 14 and AL 18. The other strains are less related. The DNA G+C content is between 48 and 51·2 mol% (T_m method). Other properties are as for the genus. Type strain is AL 3^T (= CBS 100465^T = DSM 13739^T). The type strain AL 3^T is a rod with up to three polar flagella, forms compact colonies and oxidizes tetrathionate. Its maximum specific growth rate and molar growth yield with thiosulfate at pH 10 are 0·33 h^{-1} and 3·5 g protein mol^{-1}, respectively. DNA G+C content is 49·5 mol%. Isolated from the surface sediments of Siberian soda lake (Tuva Republic).

Description of *Thioalkalimicrobium sibericum* sp. nov.

Thioalkalimicrobium sibericum (si.be′ri.cum. M.L. masc. adj. *sibericus* from Siberia).

This species includes eight vibrio-shaped strains isolated from Siberian soda lake sediments (AL 7^T, AL 8, AL 9, AL 10, AL 11, AL 12, AL 16, AL 17) with a DNA–DNA homology more than 75%. Most strains grow faster under microaerobic conditions, form compact colonies and have a very low tetrathionate-oxidizing activity. The DNA G+C content is between 48·3 and 49·5 mol% (T_m method). The other properties are as for the genus. Type strain is AL 7^T (= NCCB 100000^T = DSM 13740^T). The type strain AL 7^T is a vibroid rod with one polar flagellum. It grows faster under microaerobic conditions. In static batch culture with thiosulfate at pH 10, its maximum specific growth rate and molar growth yield are 0·22 h^{-1} and 2·7 g protein mol^{-1}, respectively. It forms compact colonies and does not oxidize tetrathionate. DNA G+C content is 48·9 mol%. Isolated from the sediments of Siberian soda lake (Buriatia).

Description of *Thioalkalivibrio* gen. nov.

Thioalkalivibrio (Thi.o.al.kal.i.vi′bri.o. Gr. n. *thios* sulfur; N.L. n. *alcali* Arabic *al* the; *qaliy* soda ash; L. v. *vibrio* vibrate; M.L. n. *Thioalkalivibrio* sulfur alkaline vibrio).

Cells are mostly vibroid, up to spirilla, 0·4–0·6 × 0·8–3·0 μm, motile with a single polar flagellum. Gram-negative; cell wall is usually highly rippled. Most strains contain multiple carboxysomes in the centre of the cells. Some strains are able to deposit globes of elemental sulfur in the periplasmic space. Young colonies are bright white from heavy sulfur deposition which is gradually converted to soluble polysulfide-like compounds. Matured colonies are usually yellow to orange–brown. Obligately autotrophic sulfur-oxidizing bacteria. Grows at pH 7·50–10·65 with an optimum at pH 10·0–10·2. The maximum specific growth rate with thiosulfate is 0·18 h^{-1}; the maximum molar yield on thiosulfate is 6 g protein mol^{-1}. During growth in batch cultures with thiosulfate at pH 10, elemental sulfur is always produced at the beginning of the exponential phase. Oxidizes sulfide, thiosulfate, elemental sulfur, sulfite and polythionates with relatively low activities within the pH range 7·0 to 11·0–11·5 (optimum pH 9–10). Polysulfide is oxidized completely to sulfate without intermediate sulfur formation. All strains express AMP-independent sulfite dehydrogenase. Contains cytochromes of *c* and *b* types. The cytochrome *c* oxidase activity is relatively high, with moderate sensitivity to cyanide and with spectroscopic properties of cytochrome *o*. Denitrifying strain ALJD^T contains cytochrome oxidase type aa_3. Carbon assimilation is via the Calvin cycle. Major ubiquinone is Q-8. All strains are halotolerant and able to grow in presence of up to 1·2–1·5 M total Na^+. Two strains are halophilic and thermotolerant, growing in up to 4 M total Na^+ (optimum at 1–2 M) and up to 45–47 °C (optimum at 40 °C). Three strains produce membrane-bound yellow carotenoids. Four strains reduce nitrate to nitrite under microaerobic conditions. One strain is a facultatively anaerobic denitrifier growing best with nitrous oxide as electron acceptor and thiosulfate as electron donor. NH_3 (at low concentrations), NO_2^- and NO_3^- are used as nitrogen sources. Most strains also assimilate nitrogen from thiocyanate. The DNA G+C content is between 61 and 65·6 mol% (T_m method). Isolated from soda lakes. Member of the *γ-Proteobacteria* with only a distant relationship to the anaerobic purple sulfur bacteria *Ectothiorhodospira*. Represented by strains with a DNA homology level more than 30% that are assigned to three species–

Thioalkalivibrio versutus, *Thioalkalivibrio denitrificans* and *Thioalkalivibrio nitratis*. The type species is *Thioalkalivibrio versutus*.

Description of *Thioalkalivibrio versutus* sp. nov.

Thioalkalivibrio versutus (ver.su′tus. L. adj. *versutus* versatile).

Includes 19 strains of vibrio- to spirilla-shaped bacteria with DNA homology of more than 45% and with two distinct gene clusters. Two strains (ALJ 15 and ALJ 22) are halophilic, thermotolerant and produce a membrane-bound yellow pigment with absorption maxima in acetone of 400, 425, 445 nm (ALJ 15) and 396, 415, 440 nm (ALJ 22). All strains except ALJ 17 have higher growth rates under fully aerobic conditions. Two strains oxidize trithionate in addition to tetra- and pentathionates. The DNA G+C content is between 63 and 65·6 mol% (T_m method). Other properties are as for the genus. Isolated from Kenyan (ALJ 1, ALJ 2, ALJ 3, ALJ 4, ALJ 5, ALJ 6, ALJ 7, ALJ 8, ALJ 9, ALJ 11, ALJ 15, ALJ 16, ALJ 17, ALJ 19, ALJ 20, ALJ 22) and Siberian (AL 2^T, AL 5, ALD 1) soda lakes. Type strain is AL 2^T (= CBS 100464^T = DSM 13738^T). Strain AL 2^T is a polymorphic rod with one polar flagellum. Young colonies are white with heavy sulfur deposition, old colonies becoming yellowish. Accumulates the sulfur globules in the periplasmic space and outside the cells during the initial growth phase with thiosulfate in batch cultures. The maximum specific growth rate and molar growth yield in thiosulfate-limited chemostat culture are 0·17 h^{-1} and 5 g protein mol^{-1}, respectively. Oxidizes trithionate. DNA G+C content is 63·7 mol% (T_m method). Isolated from the surface sediments of a Siberian soda lake (Tuva Republic).

Description of *Thioalkalivibrio denitrificans* sp. nov.

Thioalkalivibrio denitrificans (de.ni.tri′fi.cans. M.L. v. *denitrifico* denitrify; M.L. part. adj. *denitrificans* denitrifying).

This species includes two strains from the Kenyan soda lakes with a DNA homology of about 55%. The non-denitrifying strain ALJ 10 is a small vibrio and phenotypically similar to *Thioalkalivibrio versutus*. Strain $ALJD^T$ is a long, thin, curved rod. It is a facultatively anaerobic denitrifier. Grows best under anaerobic conditions with nitrous oxide as electron acceptor and thiosulfate as electron donor. Cannot grow with nitrate as electron acceptor. Anaerobic growth with nitrite and thiosulfate is much slower than with nitrous oxide because of the high sensitivity of strain $ALJD^T$ to nitrite. Growth is inhibited by high oxygen concentrations. DNA G+C content is between 62·3 and 65·0 mol% (T_m method). Other properties are as for the genus. Type strain is $ALJD^T$ (= NCCB 100001^T = DSM 13742^T). The type strain $ALJD^T$ is a pleomorphic, thin rod. In anaerobic cultures, long curved rods dominate. Cells possess one polar flagellum. Colonies grown anaerobically under N_2O atmosphere are brownish. Facultatively anaerobic and microaerobic. Best growth occurs with N_2O as electron acceptor and thiosulfate as electron donor. In anaerobic batch culture with thiosulfate plus N_2O, its maximum specific growth rate and molar growth yield are 0·03 h^{-1} and 4 g protein mol^{-1}, respectively. Cells grown under denitrifying conditions contain cytochrome cd_1. Contains cytochrome oxidase type aa_3. DNA G+C content is 62·9 mol%. Isolated from sediments of soda lake Bogoria in Kenya.

Description of *Thioalkalivibrio nitratis* sp. nov.

Thioalkalivibrio nitratis (ni.tra′tis. M.L. adj. *nitratis* nitrate).

Species includes four rod-shaped strains from Kenyan ($ALJ\ 12^T$, ALJ 18, ALJ 21) and Siberian (ALD 2) soda lakes with a DNA homology of more than 80%. They differ from other species of the genus by their ability to reduce nitrate to nitrite during growth with thiosulfate under oxygen-limiting conditions. Cannot grow anaerobically as denitrifiers. Strain ALJ 21 produces membrane-bound yellow pigment (absorption maxima in acetone 406, 427, 451 nm). Contains high level of a cytochrome species with reduced α-maximum at 595 nm. The DNA G+C content is between 61·3 and 62·1 mol% (T_m method). Other properties are as for the genus. Type strain is $ALJ\ 12^T$ (= NCCB 100002^T = DSM 13741^T). The type strain $ALJ\ 12^T$ is a rod, sometimes curved, with one polar flagellum. DNA G+C content is 62·1 mol%. Other properties are as for the species. Isolated from sediments of soda lake Nakuru in Kenya.

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