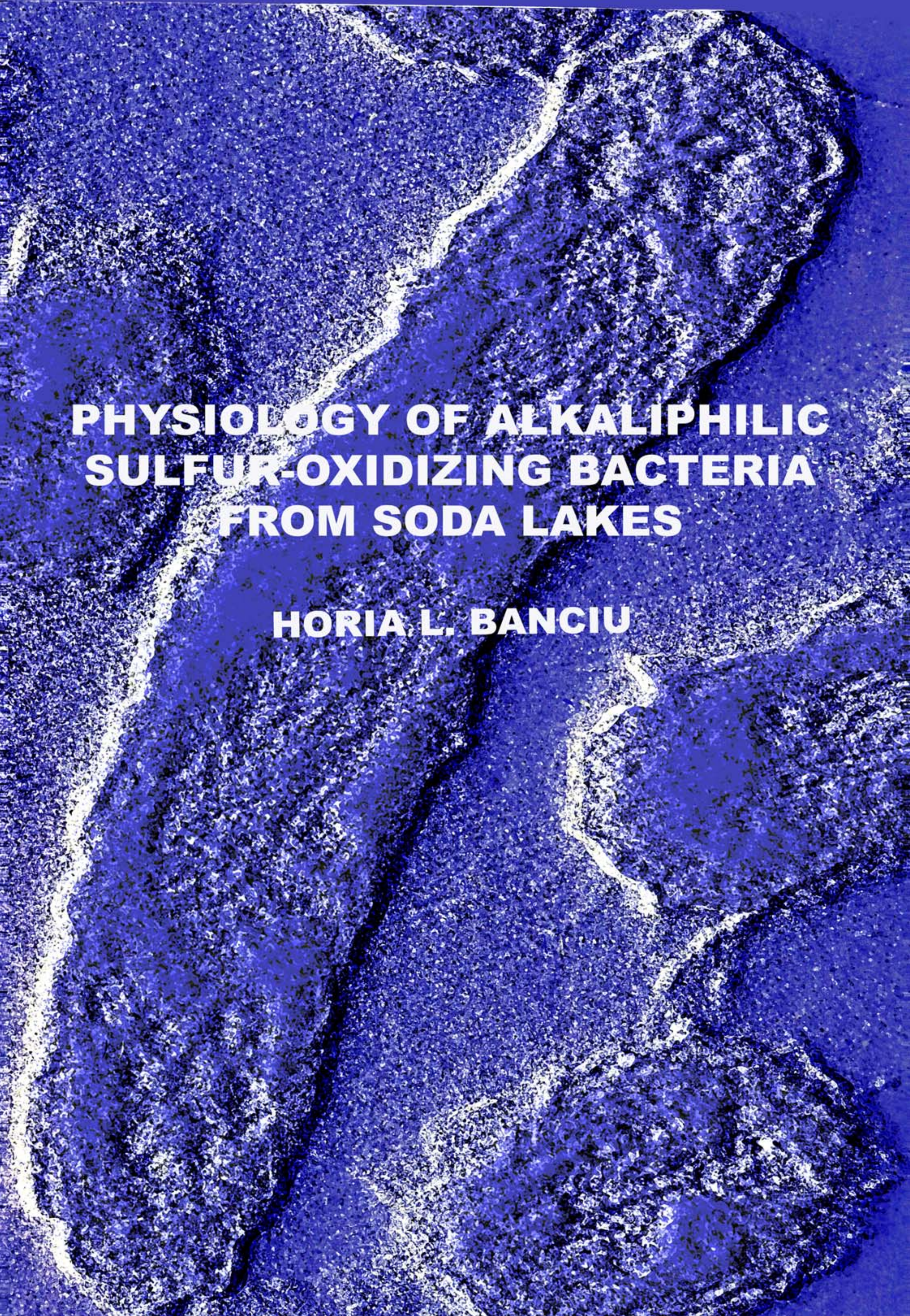

PHYSIOLOGY OF ALKALIPHILIC SULFUR-OXIDIZING BACTERIA FROM SODA LAKES

HORIA L. BANCIU

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Contents

To my wife Manuela and to my parents Cornelia and Ioan-Horia

1

General introduction

1. Saline environments

Extreme environments are widely distributed on Earth and they comprise the immense desert regions, the surface and deep-sea active volcanic areas, the thermal and often acidic springs and lakes, the Arctic and Antarctic ice shields, the permafrost and glaciers, the saline and/or alkaline soils and lakes etc. Despite the apparently adverse physico-chemical conditions, these extreme environments are densely populated by microorganisms. In the past decades a growing number of new genera and species of extremophilic microorganisms have been discovered and described. However, in spite of these recent advances, the cultivated bacteria only make up a small percentage of the microorganisms known to be present in these environments.

The present thesis concerns one particular segment of these extreme milieus, namely saline and alkaline environments. Most saline and/or alkaline lakes are located in the arid areas in Middle East and Central Asia, Eastern Africa, the western and northern part of the U.S.A and Central Australia. Remote saline lakes have been also found in other part of the world, such as the hypersaline Antarctic lakes (Lawson et al., 2000). The saline lakes may have originated from a complex interaction of biogeochemical, geographical and climatic conditions. Based on these particularities acidic, neutral or alkaline saline lakes can occur. Saline lakes may contain water permanently, intermittently or transiently. They range from deep to shallow, from small to extremely large (Great Salt Lake, Dead Sea).

Based on their marine or continental origin, the saline lakes are divided into thalassohaline (with the same salt composition as seawater) and athalassohaline lakes (with a salt composition different from that of the seawater) (**Table 1**) (Oren, 2002).

Table 1. Concentration of ions in thalassohaline and athalassohaline brines (modified after Grant et al., 1998; Zavarzin et al., 1999; Imhoff et al., 1979)

Ion	Concentration (g/l)						
	Seawater	Great Salt Lake, U.S.A.	Dead Sea	Big Soda Lake, U.S.A.	Lake Hadyn, Siberia	Lake Magadi, Kenya	Lake Zugm-Wadi Natrun, Egypt
Na^+	10.8	105.0	39.7	8.1	3.9	161.0	142.0
Mg^{2+}	1.3	11.1	42.4	0.32	0.28	0	0
Ca^{2+}	0.4	0.3	17.2	0.15	0	0	0
K^+	0.4	6.7	7.6	0.005	0	2.3	2.3
Cl^-	19.4	181.0	219.0	7.1	2.6	111.8	154.6
SO_4^{2-}	2.7	27.0	0.4	5.8	3.8	16.8	22.6
CO_3^{2-}/HCO_3^-	0.34	0.72	0.2	4.1	2.4	23.4	67.2
pH	8.2	7.7	6.3	9.7	8.4	11.0	11.0

The neutral saline lakes (pH 6-8) contain NaCl as the major salt and their buffering capacity is low. On the other hand, the alkaline saline (soda) lakes (pH 9-11) are characterized by the presence of large amounts of sodium carbonates (Na_2CO_3 + $NaHCO_3$) that confer the water a high buffering capacity (**Fig. 1**). Naturally occurring alkalinity is usually associated with salinity (Grant and Tindall, 1986). Other major ions found in salt lakes are Mg^{2+}, Ca^{2+} and K^+ as cations, and SO_4^{2-} and Br^- as anions. In the soda lakes, one of the major chemical characteristics is the lack of solubilized divalent cations (Mg^{2+}, Ca^{2+}) due to their strong tendency of precipitation as carbonates under alkaline conditions. The removal of divalent cation carbonates drives the solubilization of sodium or potassium carbonates, thus increasing the monovalent cation concentration. In this way brines are formed. In some parts of the world, the shallow lakes may end as a layer of solid rock (trona – crystalline sodium sesquihydrate, Na_2CO_3•$NaHCO_3$•$2H_2O$) by evaporation during dry seasons. A broad range of intermediate saline and/or alkaline lakes occur by the mixing of the minerals in various ratios. Several examples of saline lakes an their chemical composition are presented in **Table 1**.

In general, at a salt concentration higher than that found in seawater (35 g/l, w/v), the lake is considered highly saline or hypersaline (Grant et al., 1998). The saline lakes have attracted the attention of investigators due to their unique chemical and biological features. Soda lakes are considered as models of ancient Martian or Archaean terrestrial aquatic biotopes (Kempe and Degens, 1985; Kempe and Kazmierczak, 1997). The (hyper)saline lakes are populated mostly with halophilic neutrophilic organisms while the alkaline saline lakes are the habitats of haloalkaliphilic species. The organisms living in such environments possess special adaptation mechanisms that make them interesting for industrial application (Margesin and Schinner, 2001).

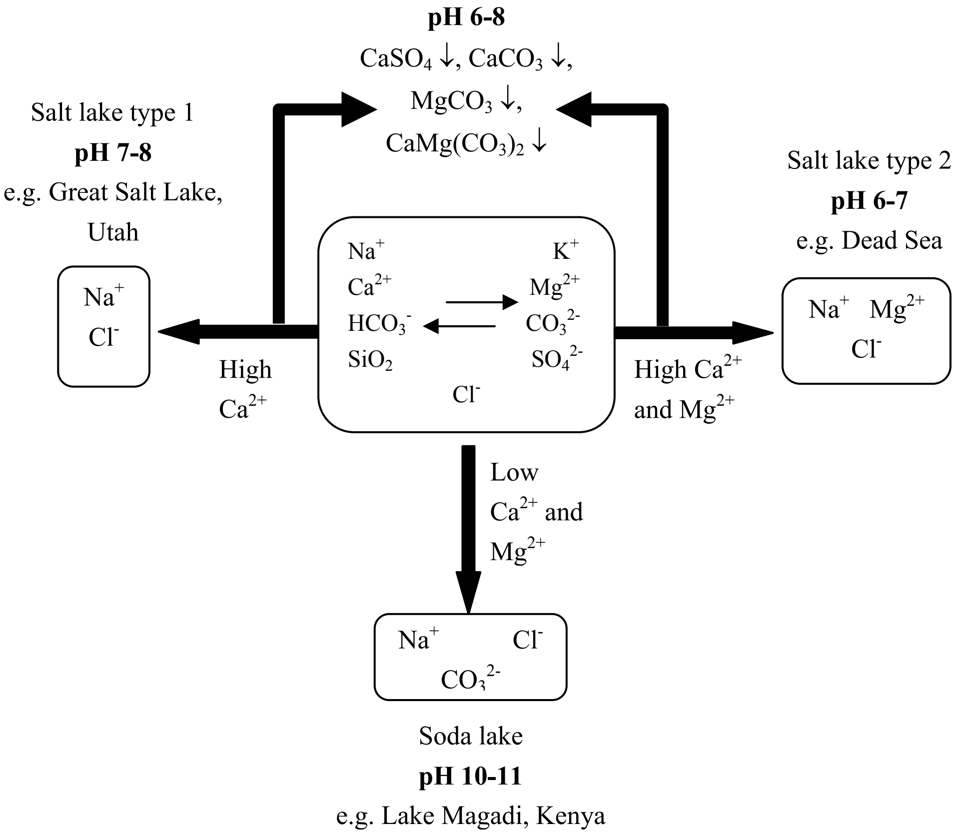

Figure 1. Schematic representation of the genesis of acidic, neutral and alkaline saline lakes (modified after Grant et al., 1998; Jones et al., 1994)

2. Diversity of halophilic microorganisms

In the past decades the studies revealed a large diversity of organisms that thrive in highly saline and alkaline lakes (Duckworth et al., 1996; Humayoun et al., 2003; Jones et al., 1998; Oren, 1994, 2002). Based on their salt tolerance and optimal salt concentration, the halophilic organisms can be grouped into several categories: halotolerants, low-salt halophiles, moderate halophiles, borderline extreme halophiles, extreme halophiles and haloversatiles (**Table 2**) (Grant et al., 1998; Ventosa, 1989). The halophiles that have their pH optimum at alkaline values are called haloalkaliphiles. On the reverse, many alkaliphiles are low salt or nonhalophilic alkaliphiles (Hamamoto and Horikoshi, 1992). The term "natronophilic" (or "trona–loving" organisms) has been suggested for the organisms living at high soda (carbonate/bicarbonate) concentrations rather than at high NaCl concentrations. This quality may apply to those haloalkaliphilic organisms isolated from soda lakes and growing optimally at high pH and high soda concentrations. In the following text we will use the terms "halophilic" or "halophiles" for those organisms that are at least low-salt halophiles (require a minimum of 0.2 M or 15 g/l salts).

Table 2. Categories of halophilic microorganisms (modified after Grant et al., 1998; Ventosa, 1989)

Category	Salt concentration (M)		
	Range	Optimum	Example
Nonhalophiles	0-1.0	<0.2	*Escherichia coli*
Low-salt halophiles	0.2-2.0	0.2-0.5	*Thioalkalimicrobium aerophilum*
Moderate halophiles	0.4-3.5	0.5-2.0	*Desulfovibrio halophilus*
Borderline extreme halophiles	1.4-4.0	2.0-3.0	*Halorhodospira abdelmalekii*
Extreme halophiles	2.0-5.2	>3.0	halophilic *Archaea* (e.g. *Halococcus* sp.)
(Extreme) Halotolerants	0->1.0 (5.0)	<0.2	*Dunaliella*
Haloversatiles	0->3.0	0.2-0.5	*Halothiobacillus kellyi*

Halophilic organisms are found in all three domains of life: *Archaea*, *Bacteria* and *Eucarya*. A large nutritional and ecological spectrum of halophiles assures the natural cycling of the elements in the saline environments. The taxonomy, physiology and molecular aspects of halophiles and halophilic adaptations have been extensively reviewed in the past (Grant et al., 1998; Kushner, 1989; Oren, 1986; 1999; 2002; Reed, 1986; Ventosa, 1989; Ventosa et al., 1998; Baumgarte, 2003).

Most of the halophilic organisms are prokaryotes and only a few are eukaryotes. The unicellular eukaryotes, such as green algae of genus *Dunaliella* can live up to saturation values of NaCl. The green algae are the trophic base for higher eukaryotes in the (hyper)saline lakes like the brine shrimps (*Artemia franciscana*, *A. monica*) and the brine flies (*Ephydra gracilis*, *E. hians*). Species of protozoa, fungi, invertebrates and plants tolerating high concentrations of NaCl have also been described (DasSarma and Arora, 2002).

The primary biomass producers in the soda lakes are mostly the haloalkalpihilic cyanobacteria that can produce up to 10 g C/ m^2 day (Grant et al., 1990). The low saline soda lakes turn often into a red color because of seasonal blooming of halophilic cyanobacteria while the primary biomass producers in hypersaline soda lakes are both haloalkaliphilic cyanobacteria and anoxygenic phototrophic bacteria. The prolific development of haloalkaliphilic cyanobacteria (*Cyanospira*, *Arthrospira*, *Spirulina, Synechococcus*) provide the trophic base for large populations of aquatic birds (Krienitz et al., 2003; Zavarzin et al., 1999). Cyanobacteria are essential both for N_2 fixation and for O_2 production in the saline lakes.

Haloalkaliphilic phototrophic anoxygenic bacteria are represented mainly by purple sulfur bacteria of the genera *Ectothiorhodospira* and *Halorhodospira*. They use inorganic sulfur compounds like sulfide and elemental sulfur as electron donor for C fixation and for growth. The organic compounds are further mineralized through biological processes catalyzed aerobically by halophilic heterotrophic bacteria of the genus *Halomonas* (Duckworth et al., 2000). The final step in the organic matter degradation occurs primarily under anaerobic conditions. In principle all major metabolic group are represented among the halophiles. Aerobic phototrophic (e.g. *Roseinatronobacter thioxidans*) (Sorokin et al., 2000), phototrophic nonsulfur bacteria (e.g. *Rhodobaca bogoriensis*) (Milford et al., 2000), photoheterotrophic heliobacteria (e.g. *Heliorestis daurensis*, *H. baculata*) (Bryantseva et al., 1999, 2000) have been found in low-saline East African or Siberian lakes. At extreme salt concentrations (>150 g/l) several physiological groups of organisms could not be found: autotrophic methanogens, acetoclastic methanogens, dissimilatory sulfate-reducers that perform the complete oxidation of their substrate and autotrophic ammonia and nitrite oxidizers. According to Oren (1999) the reason for the absence of these nutritional groups at hypersaline conditions might be the bioenergetic constraints. It is speculated that since these organisms perform a less energetically efficient metabolism, they could not sustain an energetically expensive osmoregulation.

3. Sulfur cycle and trophic relationships among haloalkaliphilic sulfur bacteria

Sulfur is one of the most important elements for sustaining life on Earth. The sulfur chemistry is complicated by the many oxidation states sulfur can assume (**Table 3**).

Table 3. Oxidation states of sulfur in common compounds (after Steudel, 2000; Brüser et al., 2000)

Oxidation state	Compounds
-2	Dihydrogen sulfide H_2S, hydrogen sulfide ion HS^-, sulfide ion S^{2-} as in FeS; thiocyanate SCN^-
-1	Disulfane H_2S_2; disulfide S_2^{2-} as in pyrite FeS_2; thiosulfate sulfane S^{1-}; polysulfides $^-S(S)_nS^-$
0	Elemental sulfur S_n; organic polysulfanes $R\text{-}S_n\text{-}R$; polythionates $^-O_3S(S)_nSO_3^-$
+1	Dichlorodisulfane Cl-S-S-Cl
+2	Sulfur dichloride SCl_2; sulfoxylate SO_2^{2-}
+3	Dithionite $S_2O_4^{2-}$
+4	Sulfur dioxide SO_2; sulfite SO_3^{2-}; bisulfite HSO_3^-
+5	Dithionate $S_2O_6^{2-}$; sulfonate RSO_3^-; thiosulfate sulfone SO_3^-
+6	Sulfur trioxide SO_3; sulfate SO_4^{2-}; peroxosulfate SO_5^{2-}

Geochemically, sulfur is very abundant and several sources of production, emission or storage can be identified (**Table 4**). The biochemical significance of sulfur is tremendous. The origin of life has been linked with iron sulfide (pyrite) that becomes catalytically active at elevated temperature and at high pressures (Wächtershäuser, 1988; Cody et al., 2000; Martin and Russell, 2003). Sulfur plays a catalytical role in the iron-sulfur clusters within respiratory enzymes. Sulfur containing aminoacids (cysteine, cystine and methionine), sulfolipids, and many co-enzymes (glutathione, coenzyme A, biotin, lipoic acid) are essential for cell metabolism.

Table 4. Main sources of sulfur

Source of sulfur	Dominant sulfur compound
Volcanic activity	SO_2
Biogenic emissions (from vegetation, wetlands, lands)	H_2S, dimethyl sulfide, carbonyl sulfide
Biogenic emissions from oceanic environments	SO_4^{2-}, dim ethylsulfide
Anthropogenic activities	SO_2 , SO_3
Sulfur storage products	Gypsum ($CaSO_4 \bullet 2H_2O$), metal sulfides, elemental sulfur (S^0)

In the natural environment the element sulfur is part of a closed cycle with alternating oxidized and reduced sulfur species, in the organic and inorganic forms. The chemical sulfur cycle strongly interacts with biological activity resulting in utilization, transformation and storage of sulfur compounds (**Figure 2**). Nevertheless, the biological importance of sulfur compounds resides in their capacity to serve as electron donor and acceptor for anaerobic respiration or aerobic light-dependent CO_2 reduction and moreover, as an aerobic energy source for ATP production (Lens and Kuenen, 2001).

The high salinity and hence high density of the hypersaline and many soda lakes explain why they are often hydrologically stratified. This often results in a layer of less saline water permanently covering the concentrated salt layer (known as meromixis). (Hollibaugh et al., 2001). Only the upper layers of the water contain oxygen and can support eukaryotic and aerophilic prokaryotic life. Due to the lack of mixing, the bottom waters are anoxic and at alkaline pH values, toxic inorganic compounds as sulfide or ammonia accumulate. The stratification of physical properties (e.g. temperature, light) or chemical parameters (dissolved oxygen, pH, salinity) is reflected in a stratification of microbial community.

Microorganisms involved in the sulfur cycle from saline and alkaline environments like soda lakes have been well studied. The haloalkaliphilic sulfur-oxidizing phototrophic and anoxygenic bacteria are members of the genus *Ectothiorhodospira* (*E. haloalkaliphila, E. vacuolata*) (Imhoff et al., 1979; Tindall, 1980) and of the genus *Halorhodospira* sp. (*H. halophila*, *H. halochloris*, *H. abdelmalekii*) (Imhoff and Süling, 1996; Oren 2002).

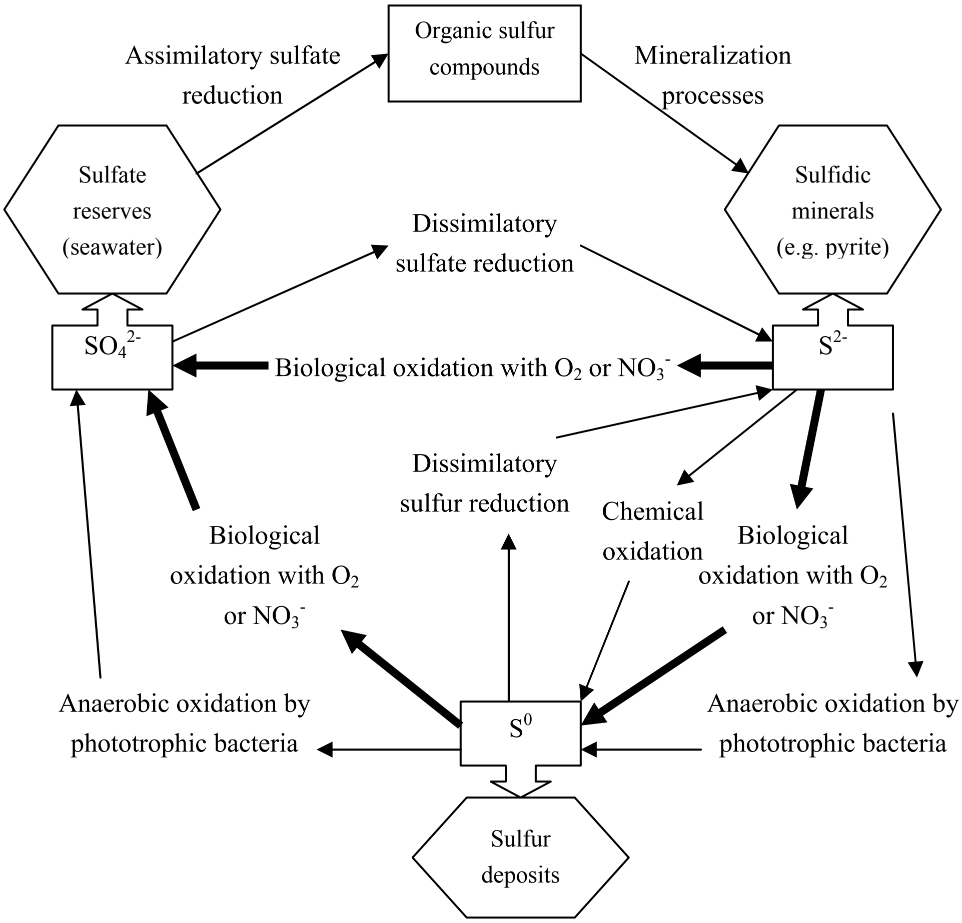

Figure 2. The biological sulfur cycle (Robertson and Kuenen, 1992). The thickened arrows indicate the oxidation processes that occur in the alkaliphilic SOB from soda lakes.

The halophilic sulfate-reducing bacteria are found within *Desulfonatronovibrio* sp. (*D. hydrogenovorans*) (Zhilina et al., 1997) and *Desulfonatronum* (*D. lacustre*, *D. thiodismutans*) (Pikuta et al., 1998, 2003). Together with recently discovered obligately chemolithoautotrophic sulfur-oxidizing bacteria of the genera *Thioalkalimicrobium* and *Thioalkalivibrio* (Sorokin et al., 2001b, c, 2002), the heterotrophic sulfur-oxidizers and sulfate-reducers constitute an ecologically balanced microbial community that ensures the recycling of sulfur in the saline and alkaline lakes (**Fig. 3**).

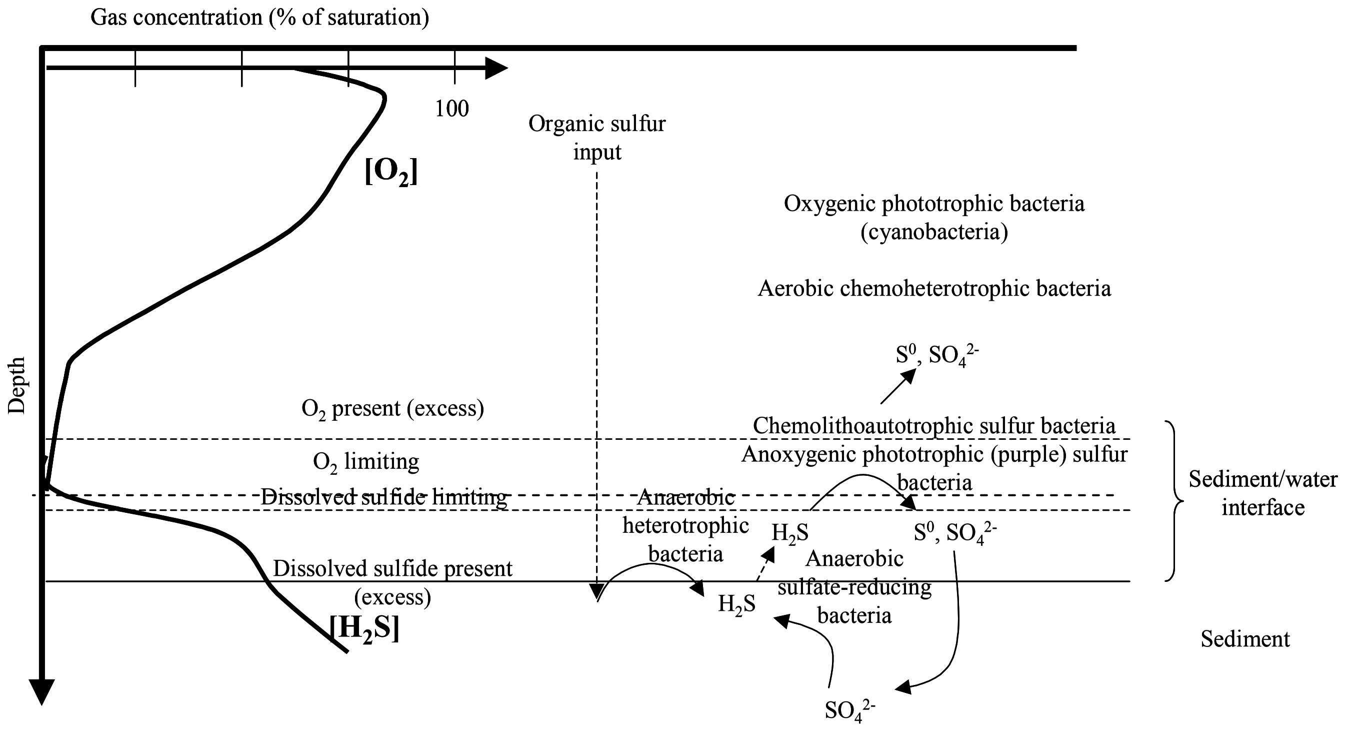

Figure 3. The diagram of the biological sulfur cycling in a saline alkaline lake. The alkaliphilic, chemolithoautotrophic SOB bacteria inhabit the O_2/HS^- interface where two situations are possible: a coexistence of O_2 with HS^- and the presence of a shuttle system between O_2, Fe^{2+}, Mn^{2+} and HS^-

4. Biological oxidation of inorganic sulfur

The most abundant form of sulfur available in nature for use by living organisms is in the oxidized state (SO_4^{2-}). Sulfate is biologically reduced under anaerobic conditions by sulfate-reducing bacteria (SRB) using different substrates as electron donors (organic compounds or H_2). Bacterial sulfate reduction in the presence of low concentration of oxygen has also been observed (Canfield and Des Marais, 1991). The process of sulfate reduction is synonym to sulfide (H_2S) production or sulfidogenesis. In a next step of biological sulfur cycle, H_2S, the most reduced sulfur compound, serves as electron donor and energy source for chemolithotrophic microorganisms. The anaerobic phototrophic sulfur-oxidizing bacteria (SOB) such as *Allochromatium*, *Chlorobium*, some *Rhodospirillaceae*, *Ectothiorhodospiraceae* and some cyanobacteria when grown anaerobically use H_2S as the electron donor for CO_2-fixation. In the chemolithoautotrophic nutrition, the reduced sulfur compound has a dual role, i.e. as electron donor as is the case in the phototrophs, and as energy source. The oxidation of sulfur compounds leads to the build-up of a proton motive force, which may generate ATP for CO_2-fixation. The proton motive force is also used to drive reversed electron transport to provide the reducing power (as NADH) for CO_2-fixation. The CO_2 fixation pathway in most known phototrophic and chemotrophic bacteria is the Calvin cycle, but also the reversed tricarboxylic acid cycle has been detected in a variety of (non)phototrophic bacteria.

Two groups of lithotrophic SOB have been distinguished previously; members of one group are able to utilize polythionates, and members of the other group are not able to do this (Kelly et al., 1997). On the basis of physiological and biochemical data, at least two major pathways have been proposed for different SOB: (i) the sulfur oxidation pathway and (ii) the S_4 intermediate pathway involving polythionates (Kelly et al., 1997; Friedrich et al., 2001).

The product of biological sulfide oxidation is SO_4^{2-} (complete oxidation), S^0 (elemental sulfur) or both SO_4^{2-} and S^0 (incomplete oxidation). Occasionally also thiosulfate was detected as end product both under aerobic and anaerobic conditions (Jørgensen, 1990; de Zwart et al., 1996). Elemental sulfur can be excreted in the environment or it can be stored extra- or intracellularly. The forms of stored elemental sulfur were investigated recently and it was shown that sulfur atoms are associated in chains or rings to which organic radical groups are attached. In this way, the biologically stored elemental sulfur differs in structure and composition from that of sulfur deposits resulting from chemical reactions (Prange et al., 1999, 2002). The excreted and the stored elemental sulfur are further oxidized to SO_4^{2-} by the same organism or by other SOB to yield supplementary energy. Several phototrophic bacteria, when grown in the dark, can also use elemental sulfur as acceptor for electrons derived from the storage compounds. Another category of SOB is able to use H_2S under anaerobic condition with NO_3^- as electron acceptor (denitrifying colorless sulfur bacteria). Interestingly, a number of strictly anaerobic bacteria are capable of “fermenting” partially reduced sulfur compounds, such as sulfur, thiosulfate and sulfite into a mixture of H_2S and sulfate. In this way a complete turnover of inorganic sulfur compounds is possible through biological processes. When seasonal or accidental changes occur in the physico-chemical or geological parameters of the natural environments, perturbation of the sulfur cycle can follow. A predominance of sulfide production may lead to accumulation of this toxic compound, which will diffuse toward the aerobic zones. A gradient of sulfide is thus established. In a very narrow layer, at the aerobic-anaerobic interface, H_2S meets O_2. H_2S can be oxidized either chemically or biologically under aerobic conditions. The factors that influence the rate of chemical oxidation are the concentration of the reaction components, the pH and the presence of metal ions (Kuenen, 1975). Several groups of microorganisms are able to oxidize reduced sulfur compounds such as H_2S under aerobic or microaerobic conditions. Three main groups of SOB can be distinguished: the anoxygenic phototrophs (e.g. green and purple sulfur bacteria), the obligate and the facultatively autotrophic colorless sulfur bacteria among which one can find the morphologically conspicuous bacteria (**Table 5**). There also exist sulfur-dependent Archaea (e.g. *Thermococcus*, *Sulfolobus*, *Acidianus*). A group called purple nonsulfur bacteria was originally thought to be unable to use sulfide as an electron donor for the reduction of CO_2 to cell material. However, under certain conditions, sulfide at low concentrations can be used by most purple nonsulfur species (Hansen and van Gemerden, 1972). Representatives of the SOB can be isolated from acidic, neutral or alkaline environments, from cold, moderate or hot habitats, as well as from low to highly saline waters and soils.

Table 5. Categories of SOB

Category	Metabolic type	Location	S compound used as electron donor	Representatives
Green sulfur bacteria	Anaerobic photolithoautotrophs	Mud and anoxic water	H_2S, S^0	*Chlorobium*
Purple sulfur bacteria	Anaerobic or microaerophilic (photo)lithoautotrophs	Oxic and anoxic water, above green sulfur bacteria layer	H_2S, $S_2O_3^{2-}$ S^0	*Chromatium , Rhodospirillum, Rhodobacter, Thiospirillum, Thiocapsa, Ectothiorhodospira, Halorhodospira*
Obligate autotrophic colorless sulfur bacteria	Aerobic and anaerobic obligate chemolithoautotrophs	Soil, sediments, oxic/ anoxic interfaces of water, sulfur springs and other volcanic sources	H_2S, metal sulfides, $S_2O_3^{2-}$, S^0, $S_3O_6^{2-}$ $S_4O_6^{2-}$	*Thiobacillus thioparus, Thermithiobacillus tepidarius, Acidithiobacillus thiooxidans, Acidithiobacillus ferrooxidans*, *Halothiobacillus neapolitanus, Halothiobacillus halophilus, Thiomicrospira pelophila*
Facultatively autotrophic colorless sulfur bacteria	Aerobic and anaerobic facultative chemoautorophs		H_2S, metal sulfides, $S_2O_3^{2-}$, S^0, $S_4O_6^{2-}$	*Starkeya novella, Thiobacillus aquaesulis, Thiomicrospira. thyasirae, Paracoccus denitrificans, Paracoccus versutus;* Morphologically conspicuous bacteria as *Beggiatoa, Thiothrix, Thioploca, Achromatium, Macromonas, Thiobacterium, Thiospira, Thiomargaritha*

Examples of the energy-yielding reactions used by colorless sulfur bacteria are presented in **Table 6**. From this table it can be noticed that, in general, the oxidation of inorganic sulfur compounds releases high amounts of energy, which is trapped as proton-motive force or sometimes, directly as ATP (by substrate-level phosphorylation). The complete oxidation of inorganic sulfur leads to production of sulfuric acid and therefore there is a strong tendency of acidification of the surrounding environment. The microbial oxidation of sulfides is important for the formation of sulfuric acid in coal mines and in sulfur deposits. The acidification resulting from the biological activity has a strong impact on large natural areas (Gonzalez-Toril et al., 2003; Lopez-Achilla et al., 2001). The sulfur-oxidizing (leading to H_2SO_4) and sulfate-reducing (H_2S) activities of microorganisms are often related to strong corrosion in sewer systems, concrete structures and in the equipment used to mine, store or transport coal (Little et al., 2000).

Table 6. Examples of the reactions used by colorless sulfur bacteria to gain energy for growth (Robertson and Kuenen, 1992)

$$\begin{aligned}
H_2S + 2O_2 &\rightarrow H_2SO_4 \\
2H_2S + O_2 &\rightarrow S^0 + 2H_2O \\
2S^0 + 3\,O_2 + 2H_2O &\rightarrow 2H_2SO_4 \\
Na_2S_2O_3 + 2O_2 + H_2O &\rightarrow Na_2SO_4 + H_2SO_4 \\
4Na_2S_2O_3 + O_2 + 2H_2O &\rightarrow 2Na_2S_4O_6 + 4NaOH \\
2Na_2S_4O_6 + 7O_2 + 6H_2O &\rightarrow 2Na_2SO_4 + 6H_2SO_4 \\
2KSCN + 4O_2 + 4H_2O &\rightarrow (NH_4)_2SO_4 + K_2SO_4 + 2CO_2 \\
5H_2S + 8KNO_3 &\rightarrow 4K_2SO_4 + H_2SO_4 + 4N_2 + 4H_2O \\
5S^0 + 6KNO_3 + 2H_2O &\rightarrow 3K_2SO_4 + 2H_2SO_4 + 3N_2
\end{aligned}$$

5. Taxonomy and morphology of the obligately chemolithoautotrophic, alkaliphilic SOB from soda lakes

The biology of inorganic sulfur oxidation was well documented in neutral (Kuenen and Beudeker, 1982; Kuenen et al., 1992) and acidic conditions (Harrison, 1984). In the neutral hypersaline environments purple sulfur bacteria use light as energy source and inorganic sulfur compounds (H_2S, S^0, $S_2O_3^{2-}$) as electron donors. The inorganic sulfur oxidation by obligate haloalkaliphilic chemolithoautotrophs was only recently discovered and investigated. The autotrophic SOB bacteria capable of oxidation of inorganic sulfur compounds at moderate to high salt concentration and at high pH can be divided into three genera: *Thioalkalimicrobium* (low-salt tolerant alkaliphiles), *Thioalkalivibrio* (extremely salt tolerant and extremely halophilic alkaliphiles) (Sorokin et al., 2001a) and *Thiomicrospira* (Sorokin et al., 2002b). These genera belong to the γ subdivision of the Proteobacteria (**Fig. 4**). The haloalkaliphilic SOB play a crucial role in the natural sulfur-cycle in the saline, alkaline environments. A large number of alkaliphilic SOB strains have been isolated and characterized in our laboratory (Sorokin et al. 1996, 2000, 2002 a, b, 2003).

5.1. Genus *Thioalkalimicrobium*

The genus *Thioalkalimicrobium* comprises species with a low DNA G-C content (48-51 mol%) isolated from the low saline Siberian soda lakes, from Kenyan soda lakes and from the saline and alkaline Mono Lake in U.S.A. The cells are rod-shaped, vibroid, spirilloid and coccoid. Some strains include motile cells with one to three polar flagella while other strains are non-motile. The cells of *Thioalkalimicrobium* sp. survive 4-12 months at 4^oC. They can be cultivated on alkaline thiosulfate agar medium and the colonies are reddish, without sulfur deposition. The ultrastructural study of *Thioalkalimicrobium* cells showed a similar organization in all strains, with an undulating cell wall of the Gram-negative type and multiple carboxysome-like structures localized in the central region of the cell. The cell wall in these bacteria was very unstable under low-osmotic conditions and during storage.

The 16S rRNA gene sequence analysis of the type strains revealed that the *Thioalkalimicrobium* group has a relatively close affiliation to the neutrophilic sulfur-oxidizing bacteria of the genus *Thiomicrospira* (4 and 10% total sequence difference with *Thiomicrospira pelophila* and *Thiomicrospira crunogena* type strains, respectively).

Based on phylogenetic analysis including 16S rDNA sequence analysis and DNA-DNA hybridization supplemented by phenotypic characterization, in the *Thioalkalimicrobium* group three species have been described to date: *Thioalkalimicrobium aerophilum*, *Thioalkalimicrobium sibiricum* and *Thioalkalimicrobium cyclicum*.

The strains of ***Thioalkalimicrobium aerophilum*** were isolated from the water and surface sediments of Siberian soda lakes (e.g. the type strain AL 3^T, DSM 13739^T) and from Kenyan soda lake sediments.

Thioalkalimicrobium sibiricum type strain, AL 7^T (DSM 13740^T) was isolated from the sediments of Siberian soda lake in Buriatia (Russia) (Sorokin et al., 2001a).

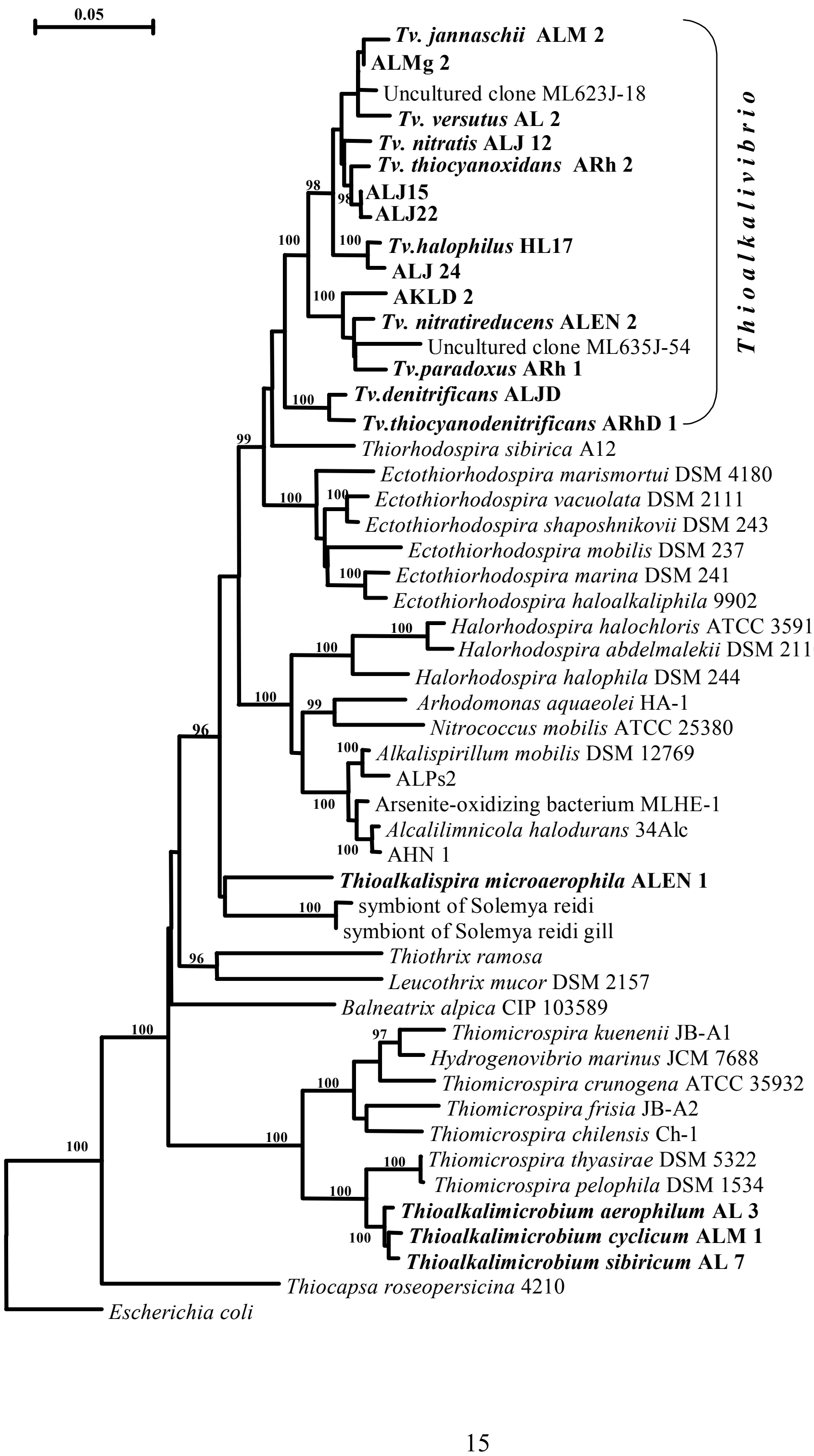

Figure 4. Phylogenetic tree demonstrating position of the three new genera of haloalkaliphilic SOB isolated from the soda lakes. Numbers on the branches indicates bootstrap values (only the highest values are included). Unaffiliated strains among the genus *Thioalkalivibrio*: extremely salt tolerant strains from Mongolia (AL Mg 2) and Kenya (ALJ 15, ALJ 22, ALJ 24); AKLD 2 – facultatively anaerobic nitrate-reducing strain from Kulunda. Bar, 5% sequence divergence.

Thioalkalimicrobium cyclicum (type strain ALM 1^T, DSM 14477^T) was isolated on solid agar medium from the oxygen-sulfide interface water layer of Mono Lake (California, U.S.A) (Sorokin et al., 2002a). Unlike strains of *Thioalkalimicrobium sibiricum* that are rather microaerophilic, the strains of *Thioalkalimicrobium aerophilum* grow faster under fully aerobic conditions. Another phenotypic difference is the tetrathionate-oxidizing capacity, which is present in *Thioalkalimicrobium aerophilum* and *Thioalkalimicrobium cyclicum* and very low or absent in *Thioalkalimicrobium sibiricum*. Moreover, *Thioalkalimicrobium aerophilum* strains demand a higher sodium ion concentration for optimal growth than *Thioalkalimicrobium sibiricum* strains (Sorokin et al., 2001a). The growth characteristics of *Thioalkalimicrobium* species are detailed later in this chapter.

5.2. Genus *Thioalkalivibrio*

The genus *Thioalkalivibrio* includes obligately autotrophic sulfur-oxidizing species with a high DNA G-C content (61.0-65.6 mol%). The *Thioalkalivibrio* group belongs to the *γ-Proteobacteria* and has no immediate relatives among the other chemolithotrophic members of the *γ-Proteobacteria*. The group bears a distant relationship to the anaerobic purple sulfur bacteriaof the genus *Ectothiorhodospira*.

The strains were isolated mostly from the Kenyan soda lakes, which are, in general, more alkaline and saline than the Siberian steppe lakes. To date, one species of *Thioalkalivibrio* was isolated from Mono Lake (U.S.A.) (Sorokin et al., 2002a). The group is represented mainly by vibrio-shaped bacteria with one polar fagellum. Some strains show spirilla-, rod-, filamentous rod-shaped cells or curved barrel-like-cells with thick capsules. It also may include strains with non-motile cells. The Gram-negative cell wall of *Thioalkalivibrio* cells is undulating and the multiple carboxysome-like structures are present in the center of the cells with the exception of the denitrifying species. 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 4^0C. A substantial difference in cell fine structure was observed only in the haloalkaliphilic strains. Strain ALJ 15 presents a cell wall with multiple tubular extensions filled with electron-dense material. The cells of another haloalkaliphilic strain, ALJ 22, are surrounded by a large capsule, sometimes shared by several cells that tend to aggregate.

Genetically, as well as phenotypically, the *Thioalkalivibrio* group is more heterogenous 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. The group includes strains that possessed important phenotypic differences and could not be separated from the others based on its 16S RNA or DNA-DNA homology. There are moderate halotolerant and extremely halophilic and halotolerant strains. The latter are able to produce membrane-bound yellow carotenoids but genetically they could not be separated from the colorless strains. This is a first known example of pigment formation among the aerobic 'colorless' sulfur bacteria. In the haloalkaliphilic strains, the amount of carotenoid produced positively correlated with the salt concentration in the growth medium. The colored strains do not contain bacteriochlorophylls.

Several strains of *Thioalkalivibrio* are able to grow with thiocyanate (SCN^-) as the sole energy and nitrogen source. They were isolated from soda lakes in South-East Siberia, Kenya and Egypt and classified into two separate species.

Thioalkalivibrio versutus (type strain AL 2^T, DSM 13738^T) includes strains of vibrio- to spirilla-shaped bacteria isolated from Kenyan and Siberian soda lakes. Two strains (ALJ 15 and ALJ 22) are halophilic, thermotolerant and produce a membrane-bound yellow pigment. The type strain was isolated from the surface sediments of a Siberian soda lake (Tuva Republic) (Sorokin et al., 2001a).

Thioalkalivibrio denitrificans **(**type strain $ALJD^T$, DSM 13742^T) includes a non-denitrifying strain from the Kenyan soda lakes, phenotypically similar to *Thioalkalivibrio versutus*. The type strain $ALJD^T$, isolated from sediments of soda lake Bogoria (Kenya), is a facultatively anaerobic and microaerobic denitrifier. The strain $ALJD^T$ had a relatively low DNA similarity with the non-denitrifying *Thioalkalivibrio* strains except strain ALJ 10 (55-58% similarity). $ALJD^T$ also had a protein profile very similar to that of the non-denitrifying strain ALJ 10. Strain ALJ 10 does not grow anaerobically with different nitrogen oxides as electron acceptors. 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 (Sorokin et al., 2001a).

Thioalkalivibrio nitratis (type strain ALJ 12^T, DSM 13741^T) includes strains from Kenyan and Siberian soda lakes with a high DNA homology (80%). Unlike other species of the genus, they reduce nitrate to nitrite during growth with thiosulfate under oxygen-limiting conditions. Strain ALJ 21 produces membrane-bound yellow pigment and contains high level of cytochromes. The type strain, ALJ 12^T was isolated from sediments of soda lake Nakuru (Kenya). They store sulfur intracellularly (Sorokin et al., 2001a).

Thioalkalivibrio nitratireducens (type strain ALEN 2^T, DSM 14787^T) was isolated from the hypersaline Lake Fazda (Wadi Natrun, Egypt) and presented barrel-shaped, coccoid, non-motile cells. Strain ALEN 2^T is facultatively anaerobic, obligately alkaliphilic and moderately halophilic. The strain oxidizes thiosulfate, sulfide, polysulfide and, much less actively, elemental sulfur and tetrathionate to sulfate. They grow anerobically in the presence of nitrate as electron acceptor and thiosulfate, sulfide or polysulfide as electron donor. The sole product of nitrate reduction is nitrite. *Thioalkalivibrio nitratireducens* is genetically most closely related to the thiocyanate-oxidizing species, *Thioalkalivibrio paradoxus*. Its DNA G+C content is 64.8±0.5 mol% (*T*m method) (Sorokin et al., 2003).

Thioalkalivibrio jannaschii (type strain ALM 2^T, DSM 14478^T) was isolated from the O_2-HS^- interface layer of Mono Lake (California, USA), tolerates up to 4 M Na^+ and produces a membrane-bound yellow pigment (Sorokin et al., 2002a).

Thioalkalivibrio thiocyanoxidans **(**type strain ARh 2^T, DSM 13532^T) differs from the other *Thioalkalivibrio* species by the ability to grow with thiocyanate as the sole energy, nitrogen and sulfur source, producing cyanate as an intermediate. Cells are short vibrios and each has a single polar flagellum. The extremely salt-tolerant strains produce a membrane-bound yellow pigment. The species is obligately chemolithoautotrophic. The type strain ARh 2^T, isolated from a Kenyan soda lake, is a yellow-colored, extremely natronotolerant bacterium able to grow in soda brines (up to 4.3 M Na^+) (Sorokin et al., 2002c).

Thioalkalivibrio paradoxus **(**type strain ARh 1^T, DSM 13531^T) cells are large, non-motile, barrel-like rods with capsules. It was isolated from the sediments of Kenyan (e.g. the type strain) and Egyptian soda lakes. The strains are obligately alkaliphilic and moderately halophilic (Sorokin et al., 2002c).

5.3. Genus *Thioalkalispira*

To date the genus *Thioalkalispira* is represented by a single obligately chemolithoautotrophic sulfur-oxidizing species ***Thioalkalispira microaerophila*** (type strain ALEN 1^T, DSM 14786^T). Isolated from a soda lake in Wadi Natrun, Egypt, the *Thioalkalispira* spirillum-like bacterial cells are motile and with a single polar flagellum. They contain a membrane-associated yellow pigment. The DNA G-C content of *Thioalkalispira microaerophila* is 58.9±0.5 mol% (*T*m), which is lower than the values observed for all of the known haloalkaliphilic SOB of the genera *Thioalkalimicrobium* and *Thioalkalivibrio* isolated so far. Phylogenetic analyses of the 16S rDNA sequences of strain ALEN 1^T and its closest relatives demonstrated that this strain formed a deep branch within the *γ-Proteobacteria*, with no obvious association to any described cluster of species/genera (Sorokin et al., 2002b).

6. Growth characteristics and sulfur-oxidizing potential of alkaliphilic SOB from soda lakes

The growth physiology of the representatives of the genera *Thioalkalimicrobium* and *Thioalkalivibrio* genera have been studied under substrate excess in batch culture or substrate limitation in continuous culture, at alkaline pH and at low salt concentration (0.2-1.5 M Na^+) (Sorokin et al., 2000, 2001a, b).

6.1. Growth characteristics of *Thioalkalimicrobium* sp.

Optimal growth conditions

Species of *Thioalkalimicrobium* are obligate chemolithoautotrophic bacteria. In batch cultures, *Thioalkalimcrobium* strains grow 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 lysed. All strains are 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.

Growth kinetics

As a general property for *Thioalkalimicrobium* species a maximum specific growth rate in chemostat culture of 0.33 h^{-1} was established, while in batch culture it varied from 0.08 to 0.22 h^{-1}. The molar growth yield on thiosulfate in batch culture varied from 1.8 to 2.7 g protein (mol thiosulfate)$^{-1}$ while in chemostat culture this value did not exceed 3.5 g protein (mol thiosulfate)$^{-1}$ (Sorokin et al., 2001a).

Sulfur oxidation potential and cytochrome composition

The obligately chemolithoautotrophic *Thioalkalimicrobium* strains were able to grow only in the presence of thiosulfate or sulfide. Organic compounds (e.g. acetate, yeast extract) are assimilated without growth. Intermediate elemental sulfur formation could not be detected during batch cultivation. Under unfavorable pH conditions small amounts of sulfite were detected in some strains. During polysulfide oxidation in cell suspension or under severe oxygen limitation in thiosulfate- or sulfide-growing culture, production of elemental sulfur was observed. On the basis of their oxygen requirement, *Thioalkalimicrobium* strains have been 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. 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. The latter strains were represented by vibroid cells and formed compact colonies.

The *Thioalkalimicrobium* cells have, in general, high sulfide and thiosulfate-dependent respiration activity, and low cytochrome *c* oxidase activity [0.2-0.3 mmol tetramethyl-ethyl-phenylene-diamine (TMPD) (mg protein)$^{-1}$ min^{-1}]. They contain high concentrations of cytochromes *c* and lower concentration of cytochromes *b*. In membranes of *Thioalkalimicrobium aerophilum* and *Thioalkalimicrobium sibiricum* the cytochrome *c* oxidase is of the *cbb*3 type - a cytochrome *c* oxidase with high affnity to oxygen (Sorokin et al., 2001a). This species was the only cytochrome *c* oxidase found in the neutrophilic sulfur bacterium *Halothiobacillus neapolitanus* W5 (Visser, 1997). All members of this group oxidized thiosulfate, sulfide and polysulfide with maximum rates at pH 9-10 and up to pH 11. 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, the results suggested that the *Thioalkalimicrobium* representatives oxidize sulfur compounds directly to sulfate by a mechanism similar to that found in several neutrophilic sulfur bacteria like *Paracoccus versutus* (Kelly, 1999). Purified sulfide dehydrogenase from *Thioalkalimicrobium aerophilum* oxidized sulfide via a one-electron mechanism implying the formation of a sulfide radical as immediate product. Further oxidation may proceed via some kind of enzyme-bound [S-S] intermediate with a step-by-step oxidation of sulfur atoms to sulfate (Sorokin et al., 1998).

6.2. Growth characteristics of *Thioalkalivibrio* species

Optimal growth conditions

Strains of *Thioalkalivibrio* are obligately chemolithoautotrophs growing optimally at pH 10.0-10.2. Some strains are capable of growing down to 9-9.5 while others fail to grow below pH 10. Many strains of *Thioalkalivibrio* are halotolerant or extremely halotolerant, being able to grow between 0.6 and 4 M Na^+. Some strains of *Thioalkalivibrio versutus* are obligate halophilic and thermotolerant, growing only above 1 M Na^+ (optimum at 1-2 M) and up to 45-47°C (optimum at 40°C).

Growth kinetics

In general, the batch and continuous cultivation have shown that the maximum specific growth rate with thiosulfate is less than 0.2 h^{-1}. Unlike *Thioalkalimicrobium* species, the maximum molar yield on thiosulfate in *Thioalkalivibrio* strains is higher (up to 8 g protein mol^{-1}) (Sorokin et al., 2001a, b).

Sulfur oxidation potential and cytochrome composition

Thioalkalivibrio strains oxidize sulfide, thiosulfate, elemental sulfur, sulfite and polythionates with relatively low activities within the pH range 7.0 to11.0-11.5 (optimum pH 9-10). The rates of oxygen consumption with various inorganic sulfur substrates are ranging between 0.2 to 0.8 µmol O_2 (mg protein)$^{-1}$ min^{-1}. Sulfide and polysulfide oxidation by washed cells display a biphasic kinetics, a first phase attributed to fast oxidation to intermediate sulfur, and a second phase attributed to a slower oxidation of elemental sulfur to sulfate. Elemental sulfur is transiently produced at the beginning of the exponential phase in batch cultivation on thiosulfate at alkaline pH. Elemental sulfur when produced is stored in the periplasm. No sulfur formation was observed upon polysulfide oxidation in cell suspension. The end product of inorganic sulfur oxidation is sulfate. Tetrathionate is hydrolysed first to thiosulfate, elemental sulfur and sulfate in *Thioalkalivibrio versutus* strains. 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, 1999; Pronk et al., 1990).

Thioalkalivibrio thiocyanoxidans and *Thioalkalivibrio paradoxus*, isolated from sediments of Kenyan or Egyptian lakes, are capable of utilizing thiosulfate as energy source and thiocyanate (SCN^-) as sole source of energy, nitrogen and sulfur. Washed cells of

Thioalkalivibrio paradoxus were able to oxidize carbon disulfide, a unique property among *Thioalkalivibrio* species. Thiocyanate-oxidizing capacity is inducible in these strains. As a result of thiocyanate degradation, cyanate (OCN^-) is produced as intermediate compound. Cyanase activity, which liberates NH_3, is absent in *Thioalkalivibrio paradoxus* and present in few strains of *Thioalkalivibrio thiocyanoxidans*. Cyanate is stable under alkaline conditions and it is used as nitrogen source by haloalkaliphilic strains. Interestingly, *Thioalkalivibrio paradoxus* has an extremely low elemental sulfur oxidation activity and therefore, the oxidation of thiosulfate, sulfide, polysulfide and thiocyanate results in the accumulation of elemental sulfur. Sulfite dehydrogenase activity in *Thioalkalivibrio* is AMP-independent, unlike sulfite dehydrogenase acitivity in *Thioalkalimicrobium* implying that the organisms may have substrate level ATP production. The cytochromes spectra showed that *c*- and *b*- types cytochromes are dominating in the membranes. The cytochrome *c* oxidase activity is relatively high, with moderate sensitivity to cyanide and with spectroscopic properties of cytochrome *o*. *Thioalkalivibrio denitrificans* is the only *Thioalkalivibrio* species that contains cytochrome oxidase type aa_3. The major ubiquinone in this genus is Q-8.

Thioalkalivibrio denitrificans grows best anaerobically in the presence of thiosulfate as electron donor and nitrous oxide (N_2O) as electron acceptor. Nitrite in small concentrations can also be used as electron acceptor but growth is slower than with N_2O. *Thioalkalivibrio nitratis* and *Thioalkalivibrio nitratireducens* are capable of reducing nitrate to nitrite under microaerobic conditions. The cytochrome oxidase activity of nitrate-reducing strains was much lower than in other species. The activity of the cytochrome *c* oxidase in cell-free extracts of *Thioalkalivibrio* species other than *Thioalkalivibrio nitratis* is 5-50 times higher than in the members of *Thioalkalimicrobium*. This activity is also 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. The high activity of cytochrome oxidases in *Thioalkalivibrio* is however in contrast with their low maximum respiratory activity as compared with *Thioalkalimicrobium*. It is possible that the high oxidase activity may be an adaptation to low oxygen concentration in order to lower the overall apparent affinity constant (K_s) for oxygen rather than increasing the overall (apparent) maximum respiratory capacity (V_{max}).

6.3 Growth characteristics of *Thioalkalispira* species

Thioalkalispira strains use thiosulfate or sulfide as electron donors. Washed cells oxidize thiosulfate, sulfide, polysulfide and elemental sulfur to sulfate. It grows optimally under micro-oxic conditions (1–2% O_2 in the gas phase), whereas growth is inhibited under fully oxic conditions. Among nitrous oxides only nitrate is used as electron acceptor but without growth. The representatives of the species are alkaliphilic and moderately halophilic bacteria growing between pH 8 and 10.4 (optimum around pH 10) and at a salt concentration between 0.3 and 1.5 M Na^+ (optimum 0.5 M). The maximum growth rate (0.08 h^{-1}) of the organism was achieved in a thiosulfate-limited micro-oxic continuous culture at pH 10 (Sorokin et al., 2002).

7. Potential applications of haloalkaliphilic SOB

Several environmental problems are caused by sulfur compounds like sulfate (pollution of surface water, acid mine drainage), SO_2 (acid rain), H_2S (odor problems, high toxicity, acid rain) and methylated sulfur compounds (odor problems, toxicity, climate change). The aim of sulfur biotechnology is to prevent loss of sulfur compounds to the atmosphere and to avoid complete oxidation of sulfur compounds to sulfate. Current research is therefore focused on the production of a sulfur compound, which can be easily separated from the waste streams, stored and re-used for other purposes. One of the successful processes is the production of elemental sulfur from H_2S-containing gas streams by sulfur-oxidizing bacteria in the Thiopaq® process (Paques BV, Balk, The Netherlands) (**Fig. 5**). In this system gasses can be treated by the absorption of H_2S in a scrubber unit, subsequent biological oxidation of sulfide to elemental sulfur at neutral pH and separation of the sulfur and recycling of the percolation water to the scrubber (Janssen et al., 2001). A variety of gas streams (pressurized natural gas, synthesis gas, biogas and refinery gas) can be treated with this two-step process. Points for major innovation of this process are the enhancement of the stripping efficiency of H_2S in the scrubber (by elevating the pH) and the reduction of the bleed stream of the aerobic reactor (by maintaining high salt conditions). Moreover, since high CO_2 content is usual for H_2S-containing industrial gases, use of alkaline carbonates in the scrubber instead of organic or inorganic alkali (NaOH) is beneficial for the effectiveness of H_2S absorption.

The alkaliphilic sulfur-oxidizing bacteria that originate from soda lakes of Siberia (Russia) and Kenya can tolerate a very high pH (up to 10.6-11) and high salt concentrations (1-4 M Na^+), making them attractive for biotechnological sulfide removal.

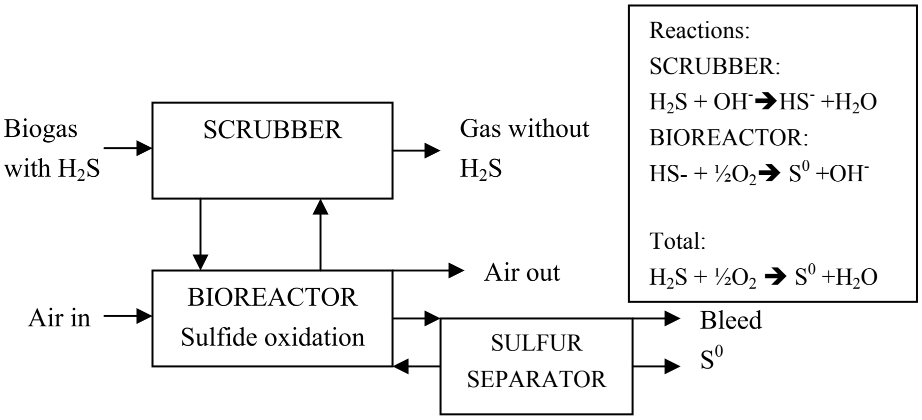

Figure 5. Block process diagram of the Thiopaq®-bioscrubber and reaction mechanisms involved

8. Scope and outline of the thesis

The present PhD thesis was initiated and supported by Dutch Foundation for Applied Research (STW) in the framework of a project on "*Production of S^0 and removal of heavy metals from S-containing industrial waste streams with inorganic biotechnology*". This project focused on the application of combinations of biological, physical and chemical processes to produce elemental sulfur from waste streams that contain sulfur compounds (e.g. from electrical power plants, mining industry, oil recovery plants and processes for the removal of heavy metals from polluted soil).

This research project aimed at the development of new S-processing unit operations utilizing microorganisms in combination with chemical and physical processes. These unit operations can be arranged in an optimal combination for a specific industrial application.
In particular, the scope of this thesis was to characterize the physiology and growth kinetics of the newly isolated haloalkaliphilic chemolithotrophic SOB with respect to their potential use in the biotechnology of H_2S removal at haloalkaline conditions. Living at high salt and high pH conditions implies special mechanisms that organisms have developed during the evolutionary selection. A close look to these adaptive mechanisms starting from population level down to biomolecules was another aim of this thesis.

The chemolithoautotrophic haloalkaliphilic SOB are a relatively new metabolic group isolated and characterized only in the past 5 years. Their taxonomy, metabolic diversity and the potential application in biological removal of toxic sulfur compounds were reviewed in **Chapter 1** (Introduction) of this thesis.

In **Chapter 2**, the growth physiology of representatives of the genus *Thioalkalimicrobium* and *Thioalkalivibrio* is compared. The competitive interaction between these two groups of organisms and their survival strategy with direct implications in their ecology is also described. The aim was to study what are the rationales for the environmental occurrence of one or another group in the soda lakes.

The aim of **Chapter 3** was to describe the sodium salt requirement for the growth and sulfur-oxidizing potential at alkaline conditions in *Thioalkalivibrio versutus* strains isolated from soda lakes. A clear distinction was made between the NaCl- and Na_2CO_3/$NaHCO_3$-requiring and tolerant strains.

Chapter 4 focuses on the effect of increasing salt concentration on the growth kinetics and possible implications on the cell energy metabolism in the alkaliphilic extremely salt-tolerant *Thioalkalivibrio versutus* strain ALJ 15. One of the goals of this research was to check whether in spite of the unusual high pH and salt concentration, this organism was capable of growing and oxidizing inorganic sulfur compound under environmental conditions similar towith those of neutrophilic sulfur-oxidizers. The second goal of the chapter was to characterize the growth of the organism on polysulfide as energy source, at high pH and high salt concentration, with respect to the potential industrial application and its ecological significance.

The growth physiology of an extremely salt-tolerant and facultative alkaliphile, *Thioalkalivibrio halophilus* sp. nov., is presented in **Chapter 5**. This organism was chosen as a model because it tolerated high concentrations of sodium carbonate and sodium chloride. It was also capable of growing well at pH 7.5 and 9.8. The hypothesis that two aqueous solutions with same Na^+ concentration but containing different anionic species (HCO_3^- /CO_3^{2-} and Cl^-), resembling two types of saline environments, would have different osmotic pressure is verified theoretically and experimentally. This difference might have a direct consequence on the production of organic compatible solutes in the same organism.
Life under extreme conditions of salt and pH requires certain physiological and biochemical adaptations.

The research concerning the salt-dependent compatible solutes production and membrane lipid composition of haloalkaliphilic sulfur-oxidizing bacteria is the subject of **Chapter 6** of this thesis. In addition to the lipid composition analysis, the estimation of the membrane surface potential was performed by using a fluorescent lipophilic and pH indicator probe.

In the last chapter (**Chapter 7**) general conclusions, remarks and a discussion are presented, pointing the uniqueness and importance of the chemolithoautotrophic haloalkaliphilic SOB for fundamental as well as for applied research.

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Growth physiology and competitive interaction of obligately chemolithoautotrophic, haloalkaliphilic, sulfur-oxidizing bacteria from soda lakes

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Abstract

Two different groups of haloalkaliphilic, obligately autotrophic sulfur-oxidizing bacteria belonging to the genera *Thioalkalimicrobium* and *Thioalkalivibrio* have recently been discovered in highly alkaline and saline soda lakes. To understand response to their extreme environment and different occurrence in soda lakes, the growth kinetics and competitive behavior of several representatives have been characterized in detail using batch and pH-controlled continuous cultivation. The bacteria belong to the true alkaliphiles, growing within the pH range 7.5-10.6 with maximum growth rate and maximum growth yield at pH 9.5-10. On the basis of their response to salt content, three groups can be identified. All the *Thioalkalimicrobium* strains and some of the *Thioalkalivibrio* strains belonged to the moderate halophiles. Some of the *Thioalkalivibrio* strains from hypersaline soda lakes were extremely salt-tolerant capable of growth in saturated soda brines. The *Thioalkalimicrobium* strains demonstrated relatively high specific growth rates, low growth yield, high maintenance and extremely high rates of thiosulfate and sulfide oxidation. In contrast, the *Thioalkalivibrio* strains, in general, were slowly growing and high-yield organisms with lower maintenance and much lower rates of oxidation of sulfide and thiosulfate. Moreover, the latter survived starvation much better than the *Thioalkalimicrobium*. Different growth characteristics and salt resistance appear to determine the outcome of the enrichment cultures from different soda lakes: the *Thioalkalimicrobium* dominated in the enrichments with freshly obtained samples from diluted soda lakes at low medium salinity, while the *Thioalkalivibrio* were the predominant organism in enrichments from aged samples and at hypersaline conditions. In mixed thiosulfate-limited chemostat cultures at low salinity *Thioalkalimicrobium* strain (μ_{max}=0.33 h^{-1}) outcompeted a *Thioalkalivibrio* strain (μ_{max}=0.15 h^{-1}) at D>0.02 h^{-1}. The overall results suggest that *Thioalkalimicrobium* and *Thioalkalivibrio* represent two different ecological strategies.

Introduction

Sulfur-oxidizing chemolithoautotrophic bacteria play an important role in natural sulfur cycling (Kuenen, 1975; Kelly, 1988; Jørgensen, 1989) and in the biological removal of toxic sulfur compounds in artificial systems (Kuenen and Robertson, 1992; Sorokin, 1994). Thus far, acidophilic and neutrophilic species of such bacteria have been thoroughly investigated (Kelly and Harrison, 1989; Pronk et al., 1990; Kuenen et al., 1992; Friedrich, 1997).

Our recent investigation of natural alkaline, saline environments (soda lakes) has revealed the presence of two previously unknown metabolic types of aerobic alkaliphilic sulfur-oxidizing bacteria, i.e. the obligately autotrophic and the obligately heterotrophic tetrathionate-forming types (Sorokin et al., 1996, 2000). The obligately autotrophic isolates from the soda lakes differ from the known sulfur-oxidizing species mainly by their ability to grow and oxidize sulfur compounds at extremely high pH values (>10.0) and high salt concentration. They belong to two different groups affiliated with separate lineages in the γ-*Proteobacteria,* and are represented by the new genera *Thioalkalimicrobium* and *Thioalkalivibrio* (Sorokin et al., 2001b). The groups differ genetically and also in many aspects of their growth kinetic parameters and sulfur-oxidizing potential. In batch cultures the representatives of *Thioalkalimicrobium* grow faster but have much lower growth efficiency. They are less versatile in their sulfur metabolism than the genus *Thioalkalivibrio,* which includes facultatively anaerobic (denitrifying) and thiocyanate-oxidizing representatives (Sorokin et al., 2001a, c). Although these properties are indicative of a possible different ecological strategy, ecophysiological and competition experiments with representatives of these two different alkaliphilic sulfur autotrophs are necessary to clarify their different behavior observed in the natural samples.

In batch cultures, the autotrophic alkaliphilic sulfur-oxidizing strains were able to grow well with thiosulfate in a mineral medium buffered with sodium carbonate within the narrow pH range from 9.5 to 10.2 where the specific buffering capacity of the carbonate system is maximal. Changing the buffer system to give a lower pH resulted in growth failure. In carbonate medium with initial pH values higher than 10.5 the pH rapidly dropped, even under sterile conditions, making the determination of the pH profiles for growth and the measurement of important growth parameters complicated, apart from the rapid decrease of pH caused by thiosulfate oxidation to sulfuric acid. Continuous cultivation under controlled conditions was required to prove the alkaliphilic nature of the new sulfur-oxidizing bacteria and to study their growth kinetics quantitatively in pure as well as in mixed cultures.

Growth and activity experiments with batch and pH-controlled continuous cultures demonstrated that the soda lake isolates of sulfur-oxidizing bacteria belong to true obligate alkaliphiles with moderate to extreme tolerance to sodium salts. The growth kinetic data and the results of competition experiments on the level of enrichment, pure and mixed continuous cultures indicate that the genus *Thioalkalimicrobium* represents an ecological strategy of the r-type, while the genus *Thioalkalivibrio* fits to a K-type.

Material and methods

Samples and strains

Samples of the surface sediments and water from twenty north-east Mongolian soda lakes (September 1999) were used to test the influence of various factors on the final composition of the enrichment cultures of sulfur-oxidizing alkaliphiles. Samples were integrated into five groups according to the total salts content of the lakes (g l^{-1}): 5-10, 15-30, 45-60, 200, 360. pH of the samples varied from 9.4 to 10.5.

Pure cultures of obligately chemolithoautotrophic, haloalkaliphilic, sulfur-oxidizing bacteria of the genera *Thioalkalimicrobium* and *Thioalkalivibrio* used in this work were obtained from various soda lakes, mostly in Central Asia and Kenya (Sorokin et al., 2000; 2001b). The type strains *Thioalkalimicrobium aerophilum* (strain AL 3) and *Thioalkalivibrio versutus* (strain AL 2), originally isolated from a Siberian soda lake and some strains from the Kenyan soda lakes (Sorokin et al., 2000, 2001b), routinely maintained in our laboratory, were used for the ecophysiological experiments.

Culture conditions

For routine batch cultivation a mineral medium buffered with a sodium carbonate/sodium bicarbonate mixture containing 0.6 M total Na^+ at pH 10.1 (after sterilization) was used (Sorokin et al., 2000, 2001b). It included (g l^{-1}): Na_2CO_3 – 22, $NaHCO_3$ – 8, NaCl – 6, K_2HPO_4 – 1, KNO_3 – 0.5, $MgCl_2 \cdot 6H_2O$ – 0.1-0.2, trace elements (Pfennig and Lippert, 1966) – 1 ml l^{-1}, and 40-80 mM thiosulfate as energy source. $MgCl_2$, trace elements and thiosulfate were added after sterilization from concentrated stock solutions. Cultures were incubated on rotary shaker at 200 rpm, 30°C. To perform enrichment and batch cultivation at different salt concentrations, a saturated soda mineral medium containing 1.7 M Na_2CO_3, 0.36 M $NaHCO_3$, 0.24 M NaCl (4 M total Na^+, pH 10.0 after sterilization) was mixed in different ratios with sterile distilled water adjusted to pH 10 by 0.1 M NaOH. All other nutrients were added as indicated above. The solid medium was prepared by 1:1 mixing of the complete alkaline base in double strength with 3.5-4% agar after sterilization at 50-60°C. Maximum possible soda content in solid medium was 2 M total Na^+. Above this concentration heavy salts precipitation was observed.

Continuous cultivation was performed in 1.5 l laboratory fermentors with a 1 l working volume, fitted with pH and oxygen controls (Applikon, Schiedam, The Netherlands). The pH was controlled by automatic titration with 2 M Na_2CO_3 and HCl. The dissolved oxygen concentration was controlled at the level of 50% air saturation by the stirring speed. The temperature was controlled at 30°C. For chemical stability, the medium was supplied from two reservoirs, containing acidic and alkaline solutions in double strength. The acidic solution contained: KH_2PO_4 – 1 g l^{-1}, $MgCl_2 \cdot 6H_2O$ - 0.2 g l^{-1}, 2 ml l^{-1} trace elements mixture and 1 ml/20 l silicone antifoam. The alkaline base included 1 g l^{-1} KNO_3 and 1 M total Na^+ (final 0.5 M) as NaCl for pH <8.0; 0.2 M NaCl + 0.8 M $NaHCO_3$ for pH 8-9.5; 0.2 M NaCl + 0.4 M Na_2CO_3 for pH values above 9.5. Thiosulfate was sterilized separately as 2 M solution and added to the alkaline base to give a final concentration of 40 mM.

Viable count and discrimination of different species

Different types of sulfur-oxidizing alkaliphiles can be discriminated by their colony morphology on agar media at pH 10. On alkaline mineral thiosulfate agar *Thioalkalimicrobium* strains produced large reddish colonies without sulfur; low-salt tolerant *Thioalkalivibrio*, like strain ALJ 7, formed smaller colonies with heavy sulfur deposition; colonies of extremely salt-tolerant autotrophs, such as strains ALJ 15 and ALJ 24, were large, with sulfur deposition at the beginning of growth, becoming transparent and yellow-pigmented after prolonged incubation. This allows to examine the competition results in enrichment cultures, when submaximum and maximum positive dilutions on liquid mineral medium with thiosulfate at pH 10 were plated on solid medium of the same composition. Although all strains were able to form colonies on alkaline thiosulfate agar, the plating efficiency varied substantially. On the other hand, growth on liquid soda minerals was more efficient and reproducible. Therefore, for competition experiments with pure mixed cultures, those strains were chosen which can be easily discriminated by their cell morphology. In particular, *Thioalkalimicrobium aerophilum* AL 3 is a small motile rod, while *Thioalkalivibrio versutus* ALJ 7 is a thin motile spirillum. The MPN method with dilution series on alkaline liquid medium (0.6 M total Na^+, pH 10) with 80 mM thiosulfate was used as the main method for examination of the competition results in chemostat experiments. The same MPN technique was used to study death rate during starvation experiments with pure cultures.

Respiration measurements

Cells were harvested by centrifugation, washed and resuspended in buffers containing 0.6 M total Na^+ at a pH corresponding to that of the growth medium with cell protein concentration 20 mg l^{-1}. For subsequent tests the concentrated suspension was diluted in respiration buffer up to 100-500 times. The respiration rates were measured at 30°C in a 5 ml cell mounted on a magnetic stirrer and fitted with a dissolved oxygen probe (Yellow Springs Instr., Ohio, U.S.A.). Thiosulfate and freshly prepared tetrathionate were added at final concentrations of 50-200 μM. Sulfide, sulfite and polysulfide (S_8^{2-}) (final concentrations 50 μM) were added as anaerobic solutions containing 5 mM EDTA to prevent autooxidation. Polysulfide was prepared by autoclaving a 0.1 M Na_2S solution with a large excess of powdered crystalline sulfur under anaerobic conditions, at 110°C during 20 min. Elemental sulfur (final concentration 70 μM) was supplied as a saturated acetone solution. Acetone, alone, did not inhibit thiosulfate oxidation at the concentration used. All buffers contained 0.6 M total Na^+ and 50 mM KCl. The following buffers were used: pH 6-8, 0.1 M HEPES-Na_2CO_3 + NaCl; pH 8, freshly prepared $NaHCO_3$; pH 9-11.3, sodium carbonate buffer. The rate values represent average results obtained from 3-5 independent experiments with standard deviations less than 10%.

Kinetics analysis

The kinetic constants $qO_{2\ max}$ and apparent K_s were measured with an oxygen electrode as described above using washed cells of the different bacteria grown either in batch or continuous cultures with thiosulfate at pH 10. To increase the sensitivity of the K_s measurements at 1-5 μM substrate level, the respiration experiments were run at 10 % air saturation. The K_m values were calculated from the VO_2-S plots based on three independent measurements. Maintenance coefficient (m_s) and Y_{max} values were determined graphically from q_s-D ($q_s=\mu/Y$) and 1/Y-1/D plots, respectively, obtained for chemostat cultures of autotrophic alkaliphilic strains AL 3 and ALJ 7 on the basis of the Pirt modification of the Monod growth model. For each dilution rate at least three steady state biomass concentrations were measured with an interval of 1 volume change. Each determination was done in triplicate; the data represent average values with standard deviation less than 10 %. The maximum specific growth rate for each pH value was determined experimentally as the dilution rate at which wash-out of the biomass and accumulation of thiosulfate started.

Chemical analysis

Micromolar thiosulfate and tetrathionate concentrations were determined by cyanolytic procedures (Kelly et al. 1969). Millimolar-range thiosulfate consumption in batch cultures was followed by standard iodimetric titration after neutralization of the medium with 50% acetic acid. Sulfide and sulfite were measured colorimetrically according to Trüper and Schlegel (1964). Elemental sulfur was assayed by cyanolysis after extraction from the cell pellet with acetone (Sörbo, 1957). Cell protein was measured by the Lowry method. When elemental sulfur was present, it was extracted with acetone from the biomass pellet before hydrolysis to avoid interference with Lowry assay. The accurate measurement of the dry weight was not possible because the bacteria lysed during washing of the filters with distilled water. A rough estimation from the data obtained with salt-containing washing buffer indicates that the biomass of sulfur-oxidizing alkaliphiles contains 65-70% (w/w) protein.

Results

The most important environmental parameters of soda lakes are the high pH and the presence of salts from moderate to very high concentration. Therefore, careful investigation of the influence of these factors on growth and activity of the soda lake autotrophic isolates was necessary to understand the adaptation of bacteria to this specific environment.

Influence of pH on growth and activity of pure cultures

Both type strains of the haloalkaliphilic genera *Thioalkalimicrobium* and *Thioalkalivibrio*, *Thioalkalimicrobium aerophilum* AL 3 and *Thioalkalivibrio versutus* AL 2, were able to grow in a thiosulfate-limited continuous culture within a pH range between 7.2-7.5 and 10.6. Their growth rate and growth yield were maximal at pH 9.5-10 which indicates the true alkaliphilic nature of these chemolithoautotrophic bacteria (**Fig. 1**).

Growth was significantly influenced by the presence of low concentrations of Mg^{2+}, which increased both the growth rate and the growth yield at the pH extremes. It should be mentioned here that Mg has a limited, but sufficiently high solubility in carbonate-containing alkaline media due to the formation of the soluble basic carbonate $Mg_2CO_3(OH)_2$. The Mg effect was especially evident at pH values lower than 9, where growth without Mg^{2+} was either impossible or much slower than in the presence of 0.5 mM of Mg^{2+}. In particular, below pH 8.5, the presence of Mg^{2+} prevented sphaeroplast formation and cell lysis.

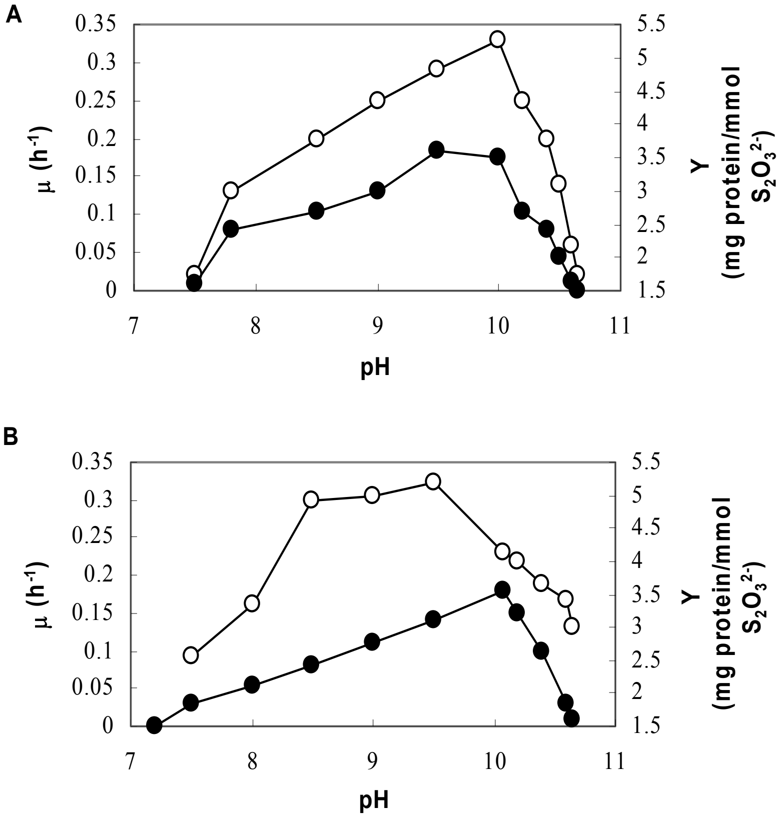

Figure 1. Influence of pH on growth of moderately halophilic and alkaliphilic sulfur-oxidizing autotrophs in thiosulfate-limited and pH-controlled continuous culture at 0.5 M total Na^+. **A** – *Thioalkalimicrobium aerophilum* AL 3, **B** – *Thioalkalivibrio versutus* ALJ 7. Open circles – maximum specific growth rate (μ), closed circles – maximum growth yield (Y).

As indicated in Methods, the μ_{max} values were determined by a wash-out curve. When wash-out began at pH values greater than 8.5, thiosulfate (AL 2) or thiosulfate+sulfite (AL 3) started to accumulate in the culture supernatant, and there was a decrease in the biomass concentration. At lower pH values, elemental sulfur accumulated before thiosulfate accumulation and wash out of the biomass started. The pH profiles for chemostat growth tested for another species of *Thioalkalimicrobium*, *Tm. sibiricum* AL 7, was essentially the same as for *Tm. aerophilum* AL 3. On the other hand, among three Kenyan isolates of *Thioalkalivibrio* (ALJ 10, ALJ 2 and ALJ 7) tested only the latter was able to grow at pH below 10 with the profile similar to strain AL 2. Growth of strains ALJ 2 and ALJ 10 at a pH below 10 was very unstable. At pH 9.8, after the first 3-4 volume changes, even at very low dilution rates, the cultures started to produce elemental sulfur. Subsequently, the oxygen consumption increased dramatically, thiosulfate accumulated and the biomass started to wash out. The reason for such behavior remains unclear. In batch cultures, these Kenyan strains did not stop growing when the pH dropped from 10.2 to 9.5 during the oxidation of 40 mM thiosulfate to sulfate. Similar to growth, the pH optimum for respiration activity of washed cells grown at pH 10 for all tested strains was at pH 9.5-10, while the pH limits were much broader than for growing cultures, especially at the highest ecologically relevant pH values. Usually, cells actively respired up to pH 11.0, and in some *Thioalkalivibrio* strains even up to pH 11.4 (**Fig. 2**).

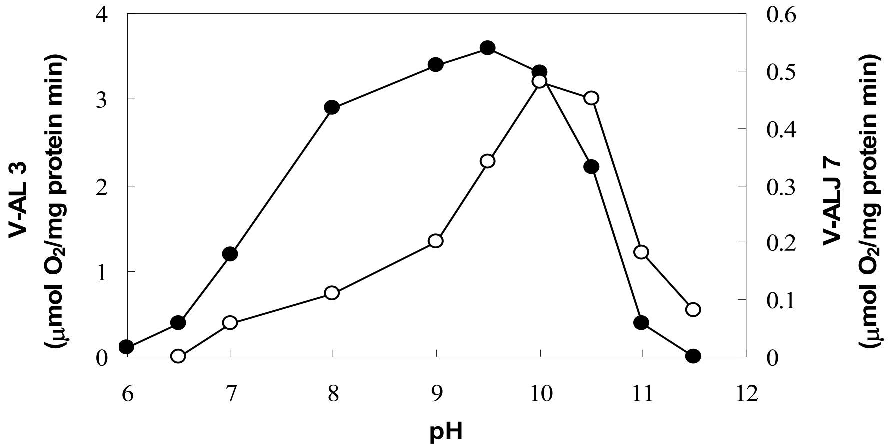

Figure 2. Influence of pH on the activity of thiosulfate-dependent respiration by washed cells of *Thioalkalimicrobium aerophilum* AL 3 (closed circles) and *Thioalkalivibrio versutus* ALJ 7 (open circles). Buffers contained 0.6 M total Na^+ as NaCl at pH 6-8 in 0.1 M HEPES and combinations of $NaHCO_3$/Na_2CO_3 at pH 8-11.5.The cells were grown at pH 10.

Influence of salts on growth and activity of pure cultures

All the *Thioalkalimicrobium* strains and some of the *Thioalkalivibrio* strains isolated mostly from low/moderately saline soda lakes on the medium containing 0.5-1 M total Na^+ belonged to the moderate halophiles. They were able to grow in the presence of 0.3-1.5 M Na^+/carbonates at pH 10. At higher salinity some growth occurred up to 1.8 M Na^+, but thiosulfate was oxidized incompletely with heavy elemental sulfur production. Thiosulfate-dependent respiration of washed cells of these bacteria was active up to 2-2.5 M total Na^+ (**Fig. 3**). Several strains that belong to the genus *Thioalkalivibrio* were isolated from the hypersaline soda lakes in Mongolia and Kenya. They represent two categories of extremely salt-tolerant alkaliphiles. In one category active growth and respiration were observed within a very broad salinity range between 0.3 and 4 M Na^+ (optimum 0.5-2 M). Growth at 3-4 M Na^+ was much slower than at lower salinity. The representatives of the second category differed from the other types by their inability to grow and oxidize thiosulfate at salinity <1 M Na^+. These strains developed optimally at 2 M Na^+ and were still very active up to 4 M Na^+ (**Fig. 3**).

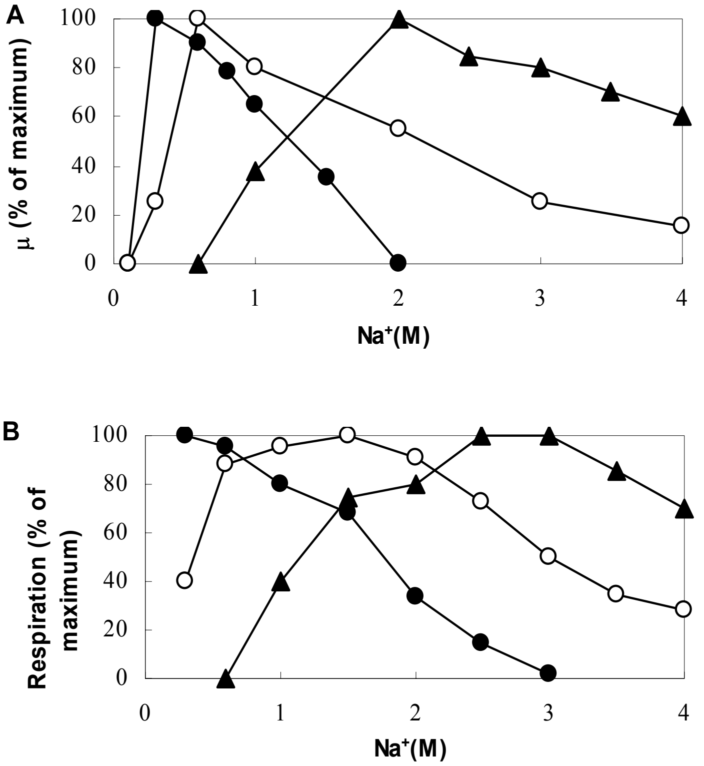

Figure 3. Influence of sodium carbonate/bicarbonate on growth with thiosulfate (**A**) and on thiosulfate-dependent respiration activity of washed cells (**B**) at pH 10 in three different types of autotrophic sulfur alkaliphiles. Closed circles – *Thioalkalimicrobium* and moderately salt-tolerant *Thioalkalivibrio* strains, open circles – extremely salt-tolerant *Thioalkalivibrio* strains, triangles – halophilic *Thioalkalivibrio* strains.

Given the ability of these isolates to grow up to 50°C, pH 10.5 and 4 M Na^+/carbonates, they apparently represent a unique type of triple extremophiles. Overall, the latter two types of *Thioalkalivibrio* clearly differed by their salt tolerance from the moderately salt-tolerant *Thioalkalivibrio* subgroup and from the *Thioalkalimicrobium* group.

Experiments with pure cultures demonstrated that while high pH is necessary for growth of all sulfur-oxidizing isolates from soda lakes, the salt content might determine the development of specific forms of haloalkaliphiles. This was confirmed in the specially conducted “competitive enrichment” experiments with five categories of Mongolian soda lake samples with a salt content ranging from 5 to 360 g l^{-1} (see in Methods) using mineral medium with pH 10 and salt content 0.6-4 M total Na^+.

Two types of sulfur-oxidizing haloalkaliphiles developed in the enrichments. On the low-saline medium (0.6 M Na^+) an absolute domination of the *Thioalkalimicrobium* group was observed with a cell density 10^4-10^6 cells ml^{-1}. On media containing >1.2-1.5 M Na^+, a yellow-colored, extremely salt-tolerant subgroup of the *Thioalkalivibrio* was dominant with viable cell density 10^3-10^5 cells ml^{-1} in the sample groups containing >50 g l^{-1} total salts. However, our numerous enrichment results from various soda lakes can not be explained only by the influence of salt concentration. In many cases, when low-salt medium was used for enrichments from relatively diluted soda lakes, a domination of either the *Thioalkalimicrobium* or the moderately salt-tolerant *Thioalkalivibrio* was observed. In this case storage time of the samples appeared to be important. In particular, prolonged storage (or a substrate starvation period) favored the selection of the *Thioalkalivibrio* over the *Thioalkalimicrobium*. Therefore it was suggested that competition between the two types of moderately salt-tolerant sulfur alkaliphiles might be determined by a different growth and survival strategy.

Growth kinetics parameters in moderately salt-tolerant sulfur-oxidizing alkaliphiles

Our previous experiments with batch cultures of numerous strains of the moderately salt-tolerant sulfur-oxidizing isolates from the sodas lakes demonstrated quantitative differences between *Thioalkalimicrobium* and *Thioalkalivibrio* groups (Sorokin et al, 2000; 2001c; 2002). Basically, *Thioalkalimicrobium* strains have a higher specific growth rate and a much higher respiratory activity with thiosulfate and sulfide, but lower growth efficiency than *Thioalkalivibrio*. This tendency was confirmed quantitatively using pH-controlled continuous cultivation at pH 10 at a salt content of 0.5-0.6 M total Na^+ with thiosulfate or sulfide as growth-limiting substrates. The summary of the kinetics data obtained with two representatives of the moderately salt-tolerant alkaliphiles is presented in **Table 1**.

Table 1. Kinetic parameters of moderately salt-tolerant haloalkaliphilic sulfur autotrophs (average data) grown with thiosulfate or sulfide on mineral sodium carbonate medium at pH 10 and 0.5-0.6 M total Na^+. Y – growth yield, K_s – affinity constant, m_s – maintenance coefficient, qS_{max}– maximum substrate-utilizing potential, $qO_{2\,max}$ - maximum respiration activity with thiosulfate, μ_{max} – maximum growth rate

Parameter	Method	*Thioalkalimicrobium*		*Thioalkalivibrio*	
		Batch	Chemostat	Batch	Chemostat
Y [mg protein ·(mmol $S_2O_3^{2-}$)$^{-1}$]	Biomass measurements	2.3 (20)1)	max –3.5 (2)	6.0 (15)	5.5 (5)
K_s ($S_2O_3^{2-}$; HS^-) (µM)	BOM at pH 10	3.5 ± 1.5 (5)		2.8 ± 0.8 (6)	
m_s [µmol $S_2O_3^{2-}$(mg protein h)$^{-1}$]	q - D plot	nd2	6 (1)	nd	2 (1)
qS_{max} [µmol $S_2O_3^{2-}$(mg protein h)$^{-1}$]	Experimental	nd)	94 ± 2 (1)	nd	25 ± 1(5)
	BOM3)	225 (20)	240 (2)	33 (15)	30 (5)
μ_{max} (h^{-1})	Experimental (wash-out kinetics)	0.23 (20)	0.33 (2)	0.10 (15)	0.12 (5)

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1) number in parentheses - number of strains tested;

2) nd – not determined;

3) BOM – respirometry in the biological oxygen monitor

Competition between pure cultures of the autotrophic sulfur alkaliphiles at moderate salinity (0.6 M Na^+)

For a further analysis of the competitive behavior under energy limitation, two representatives of the *Thioalkalimicrobium* and *Thioalkalivibrio* were used. Coculturing of the two types of autotrophic alkaliphiles in batch culture with high (80 mM) thiosulfate content at 0.6 M Na^+ and pH 10 resulted in a final domination of the fast-growing strain AL 3, although the slowly growing strain ALJ 7 was still able to increase its initial density by a factor of 100 and finally comprised up to 10% of the total viable cell count (data not shown). In thiosulfate-limited continuous culture at pH 10.05 (near-optimum) and 10.5 (near-maximum) and 0.5 M Na^+ the outcome of the competition between *Thioalkalimicrobium aerophilum* AL 3 and *Thioalkalivibrio versutus* ALJ 7 was determined by the dilution rate. Only at the lowest D-value used (0.02 h^{-1}), strain ALJ 7 was able to outcompete strain AL 3 (**Fig. 4 A**).

The course of the competition was complex. At first, within two volume changes, the density of strain ALJ 7 decreased, while the biomass of strain AL 3 remained relatively stable. Then the cell number of strain ALJ 7 started to increase and recovered its initial value after 3.5 volume changes. After 5.5 volume changes strain ALJ 7 took over completely. Strain AL 3 was completely eliminated from the reactor after 8 volume changes. At D≥0.05 h^{-1} the result of the competition was opposite with the total domination of strain AL 3 both at pH 10.05 and 10.5. Moreover the course of the competition was straightforward with rapid elimination of the loser (**Fig. 4 B**).

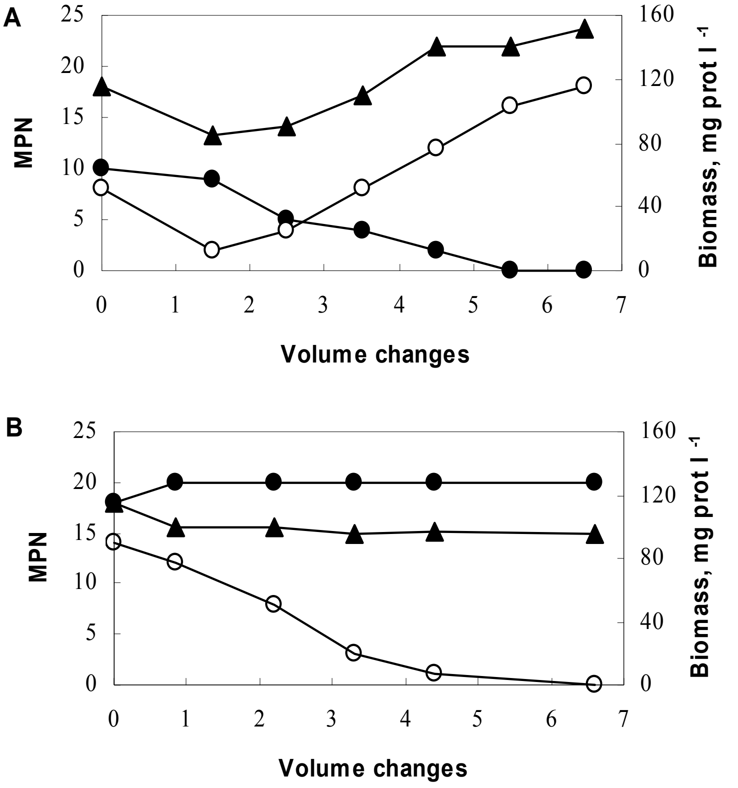

Figure 4. Competition between two different strains of obligately autotrophic alkaliphilic sulfur bacteria in thiosulfate-limited (40 mM) continuous culture at pH 10.05 and salt content 0.5 M total Na^+. **A** – D=0.02 h^{-1}, **B**– D=0.05 h^{-1}. MPN – viable counts on liquid medium with 80 mM thiosulfate at pH 10, x10^8 cells ml^{-1}. Open circles – *Thioalkalivibrio versutus* ALJ 7, closed circles – *Thioalkalimicrobium aerophilum* AL 3, triangles – biomass.

Resistance of sulfur-oxidizing haloalkaliphiles to starvation.

The results of the original enrichments with fresh and aged samples at low salinity, as well as the results of the chemostat competition experiments, indicated that resistance to starvation conditions might be important for selection of different types of autotrophic sulfur haloalkaliphiles. Therefore, the change in activity and survival of two representative strains of *Thioalkalivibrio* (strain ALJ 7) and *Thioalkalimicrobium* (strain AL 3) groups were examined under complete starvation conditions (**Fig. 5**).

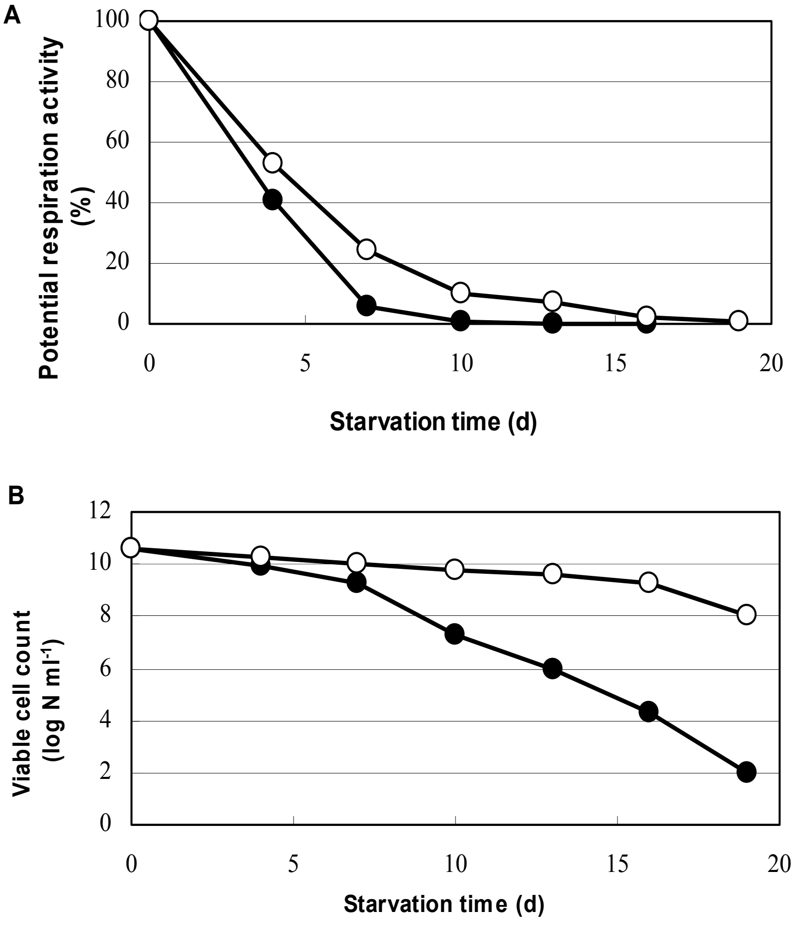

Figure 5. Comparison of the respiratory inactivation (**A**) and the death (**B**) rates in alkaliphilic sulfur bacteria *Tm. aerophilum* AL 3 (closed circles) and *Tv. versutus* ALJ 7 (open circles) at pH 10 and 30°C. Batch cultures were pregrown on mineral medium at pH 10 and 0.6 M total Na$^+$ until all thiosulfate was oxidized.

The bacteria were pregrown in batch culture with thiosulfate at pH 10, 0.6 M total Na^+. The respiratory activity decreased 5-10 times during the first 7-10 days of starvation in both organisms, although the decline was more rapid in strain AL 3 (**Fig. 5 A**). The actual cell death kinetics were much slower and substantially different in the two strains. The viable population of strain AL 3 decreased by a factor 10 within the first week and then rapidly dropped by several orders of magnitude. In contrast, the viability of strain ALJ 7 decreased only one order within 20 days of starvation (**Fig. 5 B**). Overall this experiment demonstrated a much higher resistance of the *Thioalkalivibrio* group to starvation in comparison with the *Thioalkalimicrobium* group.

Discussion

Among the nonhalophilic and halophilic alkaliphiles most attention has been focused on obligately heterotrophic bacteria and archaea (Horikoshi, 1999; Jones et al., 1998). Among the autotrophic population in soda lakes the anaerobic and aerobic phototrophic bacteria with haloalkaliphilic phenotype have been previously identified (Zavarzin et al., 1999). Until recently, however, the aerobic chemolithotrophic bacteria were missing in pure culture. In spite of a dramatic increase in the number of publications on heterotrophic alkaliphiles over the last five years, very few contained detailed and/or quantitative information on their growth physiology and particularly on the influence of pH and salts. Meanwhile, such data and their accuracy are extremely important for general biology because they extend the basic knowledge and understanding on the borders of life.

The two groups of SOB isolated from the soda lakes (Sorokin et al., 2000, 2001b) are the first representatives of chemolithoautotrophic alkaliphiles investigated in pure culture. These bacteria demonstrated properties unknown for the neutro- and acidophilic sulfur bacteria. In particular, the soda lake alkaliphiles grow optimally at pH values at which other sulfur oxidizers can not grow at all. The highest validated pH-limit demonstrated for heterotrophic alkaliphilic *Bacillus* strain under controlled conditions in continuous culture is 11.4 (Sturr et al., 1994). In the sulfur-oxidizing alkaliphiles the highest pH-limit was one unit lower, while the respiratory pH-limit was close to the *Bacillus* growth limit. The discrepancy between the relatively high respiratory activity and inability to grow at pH range from 10.6-11.0 might reflect specific problems of chemolithoautotrophic metabolism. In particular, at these pH values the alkaliphilic autotrophs may face a problem with the availability of their carbon source, which, according to the pH optimum of their inorganic carbon uptake is HCO_3^- (Sorokin et al., 2000). Given its very low concentration relative to CO_3^{2-} at pH values higher than 10.5, the autotrophic alkaliphiles could become carbon-limited in spite of the high total inorganic C-content in the medium. The presence of large numbers of carboxysome-like structures in the cells of most of the alkaliphilic strains grown at pH >10 (Sorokin et al., 2001b) might reflect their growth under carbon-limiting conditions, as has been shown in the neutrophilic *Halothiobacillus neapolitanus* (Beudeker et al., 1980). The same conclusion was drawn during an investigation of the role of the bicarbonate ion in photosynthesis at pH>10 in alkaliphilic cyanobacteria (Miller and Colman, 1980; Kaplan et al., 1982).

Salt content is another environmental factor important for growth and activity of the soda lake isolates. Three types were identified among the sulfur-oxidizing alkaliphiles according to their salt tolerance. Although only some of the representatives of the genus *Thioalkalivibrio* were able to grow at extremely high salt content, all soda lake isolates can be considered as haloalkaliphiles, e.g. organisms which need not only high pH but also high salt content for optimal growth with a minimal requirement of Na^+ for growth >0.2 M. This might imply the possible presence of specific Na^+-dependent metabolic adaptations in such bacteria different from the well known mechanisms of alkaliphily in nonhalophilic *Bacillus* species (Krulwich et al., 1998) which would be very worthwhile investigating. Interestingly, we observed that the nitrifiers, in contrast to the sulfur-oxidizing bacteria, were incapable of growing at extremely high salt content (Sorokin et al., 1998, 2001d). A possible explanation might be that oxidation of reduced inorganic sulfur species, in contrast to ammonia or nitrite oxidation provides sufficient basis to cope with the energetic burden of double extremophily.

Another important aspect of salt tolerance is its obvious role in the selection of different types of sulfur-oxidizing populations enriched from the soda lakes. The sodium salts content in soda lakes varies over a broad range from a few grams per liter up to saturation. It had a direct effect on the selection of different types of sulfur-oxidizing alkaliphiles already at moderate levels (1.2-1.5 M total Na^+). Only one specific subgroup of the genus *Thioalkalivibrio* can grow at salt concentrations above 1.5 M Na^+ which is quite common for the hypersaline soda lakes. On the other hand, the low-salt tolerant *Thioalkalivibrio* and *Thioalkalimicrobium* strains clearly survive at high salt conditions since they can be enriched from the hypersaline soda lakes as well, as had been observed in the Mongolian and the Kenyan lakes. We postulate that the latter type might be active during the rain seasons when the salt content of the shallow soda lakes can decrease several folds.

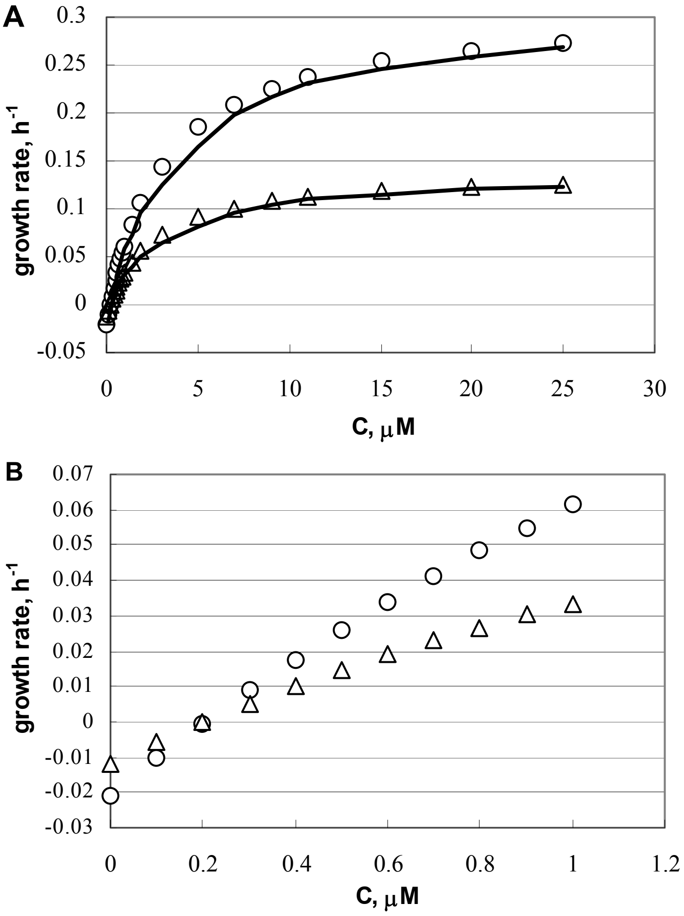

Figure 6. Simulation of the μ-Cs curves within coarse (**A**) and fine (**B**) scales for two types of the moderately halophilic sulfur-oxidizing alkaliphiles *Thioalkalimicrobium aerophilum* AL 3 (circles) and *Thioalkalivibrio versutus* ALJ 7 (triangles) based on Pirt's growth equation : $\mu = [qS_{max} \cdot (C_s/C_s+K_s) - m_s] \cdot Y$. Kinetic parameters used for calculations at fixed C_s values were as follow (AL 3/ALJ 7): Y=3.5/6.0 mg protein $\cdot$ (mmol $S_2O_3^{2-}$)$^{-1}$; qS $_{max}$ = 94/25 μmol $S_2O_3^{2-}$ $\cdot$ (mg protein h)$^{-1}$; K_s=3.4/2.75 μmol $S_2O_3^{2-}$; m_s= 6/2 μmol $S_2O_3^{2-}$ $\cdot$ (mg protein h)$^{-1}$.

Competition experiments have been efficiently employed previously for the investigation of possible mechanisms of competitive interactions in mixed population of neutrophilic sulfur-oxidizing bacteria under single and mixed substrate supply (Kuenen et al. 1977; Gottschal et al. 1979; Smith and Kelly 1979). The information obtained from these experiments has been used to predict the role of different obligately chemolithotrophic *Thiobacillus* versus *Thiomicrospira* spp. and of different metabolic types of sulfur bacteria in natural habitats (Kuenen et al., 1977; Gottschal and Kuenen, 1980; Beudeker et al., 1982). Among the specialized sulfur-oxidizing autotrophs, a high specific growth rate is usually linked to a relatively low affinity (μ_{max}/K_s) for substrate and *vice versa* (Kuenen et al., 1977). Therefore the slowly growing autotrophs may outcompete fast growing organisms under conditions of low substrate supply rates on the basis of higher specific growth rates at very low substrate concentrations. However, in the case of the obligately autotrophic sulfur haloalkaliphiles, both the slowly growing *Thioalkalivibrio* and fast growing *Thioalkalimicrobium* strains had very low affinity constants, which were close. *Thioalkalimicrobium* and *Thioalkalivibrio* had a K_s of 3.5 and 2.8 respectively (**Table 1**). However, their maintenance energy requirement differs by a factor 3. Competition experiences done under thiosulfate limitation showed that at D=0.02 h^{-1} the *Thioalkalivibrio* sp. outcompeted *Thioalkalimicrobium*. This confirmed the prediction made by modelling the competition on the basis of the Pirt equation (which includes the maintenance) within the experimental errors. At a K_s (*Thioalkalimicrobium*) of 4.5 and a K_s (*Thioalkalivibrio*) of 2.2 the crossing points of the μ-C_s-curves would be well above μ =0.02 h^{-1} (**Fig. 6**). Other differences important for the competitive ability and survival of different types of haloalkaliphilic sulfur autotrophs may include experimentally observed differences in the loss of viability during starvation and accumulation of storage material (*Thioalkalivibrio* strains can accumulate elemental sulfur in its periplasm).

Overall, it seems realistic to suggest that the observed success of the *Thioalkalivibrio* strain ALJ 7 over *Thioalkalimicrobium* strain AL 3 in substrate-limited chemostat at low D-values was determined by a better adaptation of *Thioalkalivibrio* to grow at low substrate supply rates. Two types of alkaliphilic autotrophs can grow simultaneously only in case of unlimited substrate supply in batch cultures. On the basis of its growth kinetic parameters (low growth efficiency at very high substrate consumption rate), the *Thioalkalimicrobium* group can be regarded as a typical *r*-strategist, the ecotype which becomes dominating in the system during short periods of excessive substrate supply, being able to outcompete the other forms on the basis of its high maximal specific growth rate (R). Translated to the natural situation, these data suggest that the observed *Thioalkalimicrobium* domination in enrichments from freshly collected samples (at low medium salinity) may be determined by the presence of sufficient amount of sulfide present in sediments. During prolonged storage of the samples under aerobic conditions sulfide disappears and *Thioalkalimicrobium* population rapidly declines. This and much lower decay rates would allow slowly growing *Thioalkalivibrio* to take over. Low growth rates and maintenance and the ability to survive prolonged starvation are characteristic properties of K-strategists. Considering substantial difference in the viability kinetics during starvation between the two types of haloalkaliphilic autotrophs (see **Fig. 5**), it is interesting to mention the data obtained in a special investigation of the potential of haloalkaliphilic sulfur autotrophs to produce resting forms under different stress situations, in particular during starvation (Loiko et al., 2002; 2003). These studies have demonstrated that *Thioalkalivibrio versutus* formed several orders of magnitude more cyst-like resting cells in starving population than the *Thioalkalimicrobium aerophilum*, which might be one of the reasons for much better survival of the former. On the other hand, the cyst-like resting cells of *Thioalkalimicrobium*, although few, were of “higher quality”, e.g. more protected from the imposed stress influence, which probably allows these bacteria to survive under unfavorable conditions at very low population number. In general these observations are in good agreement with the data obtained in the present study.In conclusion our data demonstrated that on their kinetic parameters the obligately autotrophic sulfur-oxidizing bacteria isolated from the soda lakes are well adapted for a life at high pH and salts content – the dominant extreme factors in such unusual environment. Moreover, despite narrow physiological specialization common for obligately autotrophic bacteria, the example of haloalkaliphilic sulfur-oxidizing representatives demonstrates presence of substantially different subtypes within the same physiological type of the chemolithoautotrophs.

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3

Sodium salts requirement for the growth and activity in *Thioalkalivibrio versutus* strains from soda lakes: halophiles *vs.* natronophiles

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Abstract

Six strains of alkaliphilic sulfur-oxidizing bacterium *Thioalkalivibrio versutus* isolated from soda lakes in Eastern Africa and Central Asia were tested for their salt requirement and tolerance for the growth and respiration, at extreme sodium salt concentration (4 M Na^+) and at highly alkaline pH. Based on their NaCl tolerance, two groups could be distinguished: low-chloride tolerant and requiring strains originating from Kenyan and Central Asian soda lakes, and high-chloride tolerant strains, requiring at least 0.1-0.5 M Cl^- for their growth. The latter group was represented by the Egyptian strains isolated from soda lakes with a high NaCl content. All strains were capable of growing at high SO_4^{2-} concentration (1.5 -1.75 M), at pH 10 and in the presence of at least 0.5 M soda and of a minimal Cl^- concentration for NaCl-requiring strains. The activity of the washed cells was less sensitive to salt variations than the growth. The different sodium salt requirement in the strains belonging to the same species allows us to suggest that there is a clear physiological difference among the alkaliphilic, high–salt organisms: "natronophiles" that require and tolerate high $Na_2CO_3/NaHCO_3$ but not NaCl and "halophiles" that require and tolerate high concentrations of NaCl for the growth.

Introduction

Soda lakes represent a specific type of salt lake, which contains an alkaline sodium carbonate/bicarbonate fraction among its dominant salts. Like most of the other inland salt lakes, the soda lakes are located in areas with a dry climate facilitating gradual salt accumulation in depressions. The main mechanism of their genesis is leaching of sodium-rich rocks by high CO_2-containing ground waters under condition of low Ca^{2+} and Mg^{2+} content (Eugster, 1970; Jones et al., 1977). The presence of sodium carbonate creates a uniquely stable natural alkaline habitat, although the total salt content and the sodium carbonate fraction in the soda lakes vary within a broad range, depending on the local conditions. In soda lakes, the presence of sodium carbonate ensures a stable high-to-extremely high pH (usually around 9.5-10.5) in the water. Thus, the soda lakes seem to be the only natural environment with appropriate conditions for the stable development of obligately alkaliphilic microorganisms, which usually grow optimally at a pH around 10. The well-known hypersaline soda lakes are located in the East African Rift Valley in Kenya and Tanzania (Bogoria, Magadi, Natron), in the Libyan Desert in Egypt (Wadi Natrun), in California and in Nevada. Another area where saline alkaline lakes are numerous is Central Asia, where the Transbaikal dry steppe stretches from south-east Siberia to north-east China. Here much smaller, shallow and usually less saline lakes predominate, with a relatively unstable water regimen and a freezing winter period causing frequent and substantial fluctuations of the water salinity and temperature. Recent microbiological analysis of the soda lakes by both traditional and culture-independent molecular techniques revealed the remarkable fact that, despite the doubly extreme conditions of salt and pH, fully structured and active microbial communities are present, even in saturated alkaline brines (Imhoff et al., 1979; Grant and Tindall, 1986; Jones et al., 1998; Zavarzin et al. 1999; Zavarzin and Zhilina, 2000; Humayoun et al., 2003; Sorokin et al., 2004). The microbial sulfur cycle seems to be one of the most active in the soda lakes, with anaerobic phototrophic purple sulfur bacteria and sulfate-reducing alkaliphiles as the main participants (Isachenko, 1951; Imhoff et al., 1979; Zhilina et al., 1997; Gorlenko et al., 1999). Only very recently, the presence and the role of chemolithotrophic sulfur bacteria in soda lakes have been studied (Sorokin et al., 2000; 2001).

In the present study we have used several strains of a single species (*Thioalkalivibrio versutus*) isolated from different locations situated at vast distances one from another (Kenya, Egypt, SE Siberia and Mongolia). The hypersaline soda lakes from which the strains were isolated have different CO_3^{2-}, HCO_3^-, Cl^- and SO_4^{2-} ratios. We aimed to determine the salt tolerance and salt requirement for the growth and respiration of these extremely salt tolerant strains. Another goal of this research was to prove that in strains belonging to the same species, there is a special requirement for high soda or high chloride concentrations depending on the origin of the strain.

Material and methods

Origin of samples

Surface sediment samples from hypersaline soda lakes in four different geographical areas (Kenya, Egypt, SE Siberia and Mongolia) were collected during our expeditions. The main characteristics of the samples are given in **Table 1**. The sampled hypersaline lakes can be divided into three categories: soda lakes with high CO_3^{2-}/HCO_3^- content (Lake Bogoria, Kenya), soda lakes with high Cl^- content (Egyptian lakes and Lake Hotontyn in Mongolia) and soda lakes with equal molar concentrations of CO_3^{2-}/HCO_3^- and Cl^- (Kulunda lakes in SE Siberia).

Enrichment, isolation and cultivation of alkaliphilic sulfur-oxidizing strains from soda lakes

In the present study we used six different strains belonging to a single species, *Thioalkalivibrio versutus* (Sorokin et al., 2001). For the enrichment of the high-salt alkaliphilic sulfur-oxidizing strains, a mineral base medium, containing 2-4 M of total Na^+ was used (see **Table 1**). The mineral soda medium (0.6, 2 or 4 M Na^+) included: Na_2CO_3, 22, 85 or 180 g/l; $NaHCO_3$, 8, 19 or 38 g/l; NaCl, 6 g/l; K_2HPO_4, 1 g/l; KNO_3, pH 10. After sterilization, the alkaline mineral base medium was supplied with 20-80 mM of thiosulfate, 1 ml/ l of trace elements solution (Pfennig and Lippert, 1966) and 1 mM of $MgCl_2 \bullet 6H_2O$.
For the inocula from the hypersaline lakes where NaCl was the dominant salt (Wadi Natrun), the amount of sodium carbonate in the base medium was reduced by substitution with NaCl with the equivalent amount of Na^+ (as molar) and a minimum amount of sodium carbonate/bicarbonate, equivalent to 0.5-1 M Na^+. Such mineral bases provided sufficient alkalinity to buffer the medium at pH 9.5-10.2, even when a dense sulfur-oxidizing culture producing high amounts of sulfuric acid from thiosulfate developed. The use of this medium provided a basis for the successful enrichment and isolation of many strains of obligately alkaliphilic sulfur bacteria with varying degrees of salt tolerance.

The isolation strategy was based on the following procedure. Positive enrichment cultures were subcultured several times with 1:100 dilutions to obtain a stable active culture. This was serially diluted and maximum positive dilutions were plated to obtain the dominant organism.

All isolated strains were grown in a medium containing 4 M Na^+ with different $NaHCO_3/Na_2CO_3/NaCl$ ratios. A minimum of 0.5 M soda was maintained for buffering capacity at around 9.8-10.0. In the experiment with Na_2SO_4, the total Na^+ concentration and the alkalinity were kept constant while the anion ratio was changed. Haloalkaliphilic strains were cultivated in batch cultures with thiosulfate as energy source, at 30°C, on a rotary shaker at 100 rpm.

Growth was monitored by measuring thiosulfate consumption (iodimetric titration in presence of acetic acid) and by microscopy.

Table 1. Origin of several haloalkaliphilic sulfur-oxidizing *Thioalkalivibrio versutus* strains, chemical characteristics of the soda lakes and composition of the enrichment media.

Strain	Origin	Area	S^a (g/l)	CO_3^{2-}/HCO_3^- (M)	Cl^- (M)	SO_4^{2-} (M)	pH	Enrichment
ALJ 15	L. Bogoria	Kenya	50-130	0.5-1	0.2	0.005	10-10.5	2 M soda
AKL 15	3 lakes in Salt Lake Steppe	Kulunda (SE Siberia)	140-180	1.2-1.5	0.7-0.8	0.3	9.5	2 M soda
ALMg 2	L.Hotontyn	Mongolia	360	0.9	2.8	0.2	9.8	2 M soda
ALE 20	L. Fazda	Wadi Natrun (Egypt)	250	0.46	2.2	0.5	9.7	1+3[b]
ALE 10	L. Hamra		200	0.23	Nd	Nd	9.6	1+1[c]
ALE 28	L. Zugm		350	0.75	3.9	0.33	9.8	1+3

[a] S – Total salinity;

[b] – 1 M Na carbonate + 3 M NaCl, pH 10;

[c] – 1 M Na carbonate + 1 M NaCl, pH 10;

Nd – Not determined

Respiration measurements

Cells collected from the batch cultures and stored at 4°C, were harvested by centrifugation, washed and re-suspended in buffers containing 4 M Na^+, pH 9.6. For subsequent tests the concentrated suspension was diluted in respiration buffer to 0.05-0.1 mg protein/ml. The effect of Na^+ concentration on the sulfur-oxidizing potential in the high salt tolerant strain ALJ 15 was studied in soda mineral buffers lacking nitrogen and phosphorus, at pH 10 and various Na^+ concentrations. For the experiments concerning the effect of different anions on the sulfur-oxidizing potential in *Thioalkalivibrio versutus* strains, respiration buffers consisted of 4 M Na^+ and with varying carbonate, chloride and sulfate ratio. Respiration rates were measured at 30°C in a 5 ml glass chamber mounted on a magnetic stirrer and fitted with an oxygen electrode (Yellow Springs Instr., Ohio, U.S.A.) connected to a chart recorder (Kipp & Zonen, model BD40). Different sulfur substrates were used at final concentrations of 25-50 μM.

Results

Effect of growth history and Na^+ requirement for the sulfur-respiration of extremely salt-tolerant alkaliphilic *Thioalkalivibrio versutus* strain ALJ 15

The obligately alkaliphilic *Thioalkalivibrio versutus* strain ALJ 15, isolated from hypersaline Lake Bogoria (Kenya), was chosen as model organism to study the Na^+ requirement and the sulfur-oxidizing potential at different Na^+ concentrations because of its extreme salt tolerance. The batch cultivation of this strain showed an optimal concentration for growth between 0.6 and 1 M Na^+ at pH 10. The strain was capable of growing between 0.5 M and 4 M Na^+ (as $Na_2CO_3/NaHCO_3$), at pH values up 10.6, and at a temperature up to 50°C (data not shown). When cells of strain ALJ 15 were pre-grown at three different Na^+ concentrations (0.6, 2 and 4 M), washed and suspended in mineral soda buffers at different concentrations of Na^+, at pH 10, the maximum specific rate of oxygen consumption shifted from 0.6 to 1.5 and 2 M Na^+ respectively (**Fig. 1**). The results of biological oxygen monitoring (BOM) experiments were confirmed by cell suspension experiment. The rate of thiosulfate consumption in the cell suspensions of the 0.6, 2 and 4 M Na^+-grown cultures showed an optimum at increasing Na^+ concentration; corresponding to the Na^+ concentration the cells were formerly grown. Thus, the experiments with the strain ALJ 15 showed that its substrate oxidation system is able to adapt and change its optimum for Na^+ concentration, depending on the growth history. Below 0.5 M sodium carbonate/bicarbonate, the buffering capacity of the growth medium was not sufficient to maintain the optimum pH. Another negative effect of decreasing sodium salt concentration in the growth medium is the partial or total lysis of cells which occurs at values lower than 0.3 M Na^+. Therefore, the minimal Na^+ requirement for growth in alkaliphilic strain ALJ 15 could not be precisely measured in whole-cell systems.

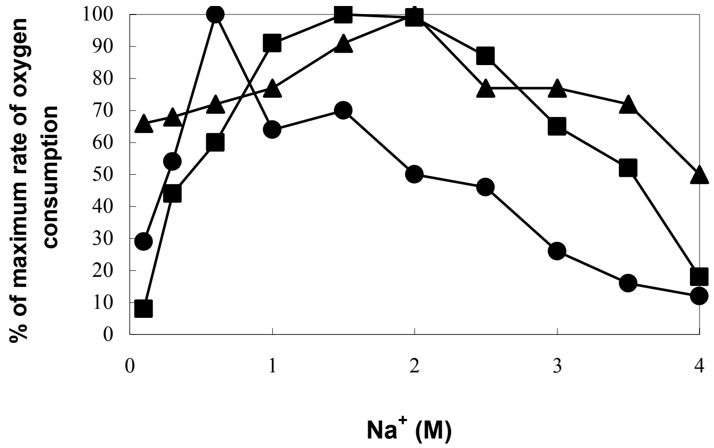

Figure 1. Effect of growth history on the oxygen consumption rate in washed cells of thiosulfate-grown *Thioalkalivibrio versutus* strain ALJ 15. Cells were pre-grown at pH 10, in mineral medium containing 0.6 M Na$^+$ (circles), 2 M Na$^+$ (squares) or 4 M Na$^+$ (triangles), washed and suspended at a concentration of 0.1 mg of protein/ml in soda buffers at variable Na$^+$ concentration, pH 10 and 50 µM thiosulfate. The maximum specific rates of oxygen consumption for the three cultures were: 0.44, 0.55 and 0.1 µmoles O_2/mg of protein min^{-1} respectively.

NaCl effect on growth and sulfur-oxidizing potential of *Thioalkalivibrio* strains at 4 M Na$^+$, pH 10.

Six different strains belonging to *Thioalkalivibrio versutus* were grown in a mineral medium with increasing concentrations of NaCl, at alkaline pH. **Fig. 2 A** shows the effect of varying Cl$^-$ concentration on the substrate consumption rate of strains ALJ 15, AKL 15 and ALMg 2 while **Fig. 2 B** presents the effect of Cl$^-$ concentration on the growth of the Egyptian strains ALE 20, ALE 10 and ALE 28. Based on their DNA-DNA homology, the Egyptian strains could be divided into two main clusters, one represented by strain ALE 10 and the other represented by the strains ALE 20 and ALE 28 (D.Y. Sorokin, unpublished data). All Egyptian strains used in this experiment required high salt concentration for optimal growth and grew poorly below 0.6 M Na$^+$. Strain ALE 10 was isolated in 2 M soda medium with 1 M NaCl. The original isolation of strains ALE 20 and ALE 28 had been successful at 4 M Na$^+$ in the presence of 3 M NaCl. The results of a growth experiment at various NaCl concentrations indicated a clear difference of the Kenyan-Asian strains from the Egyptian strains and less variation among the Egyptian strains.

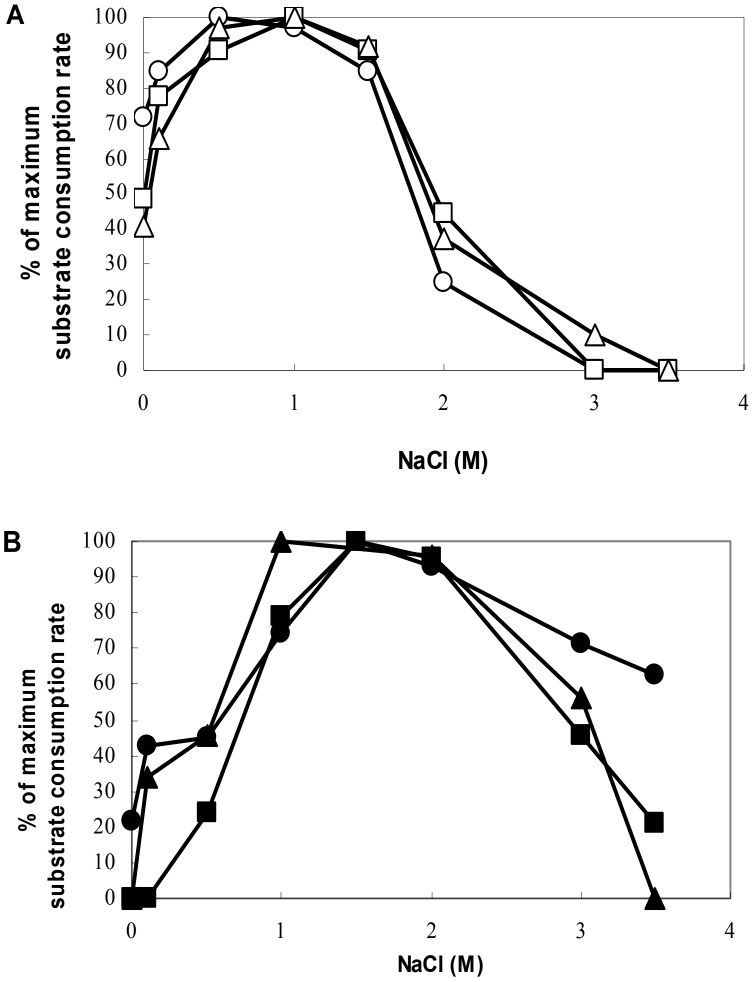

Figure 2. The effect of Cl^- on substrate consumption rate of alkaliphilic *Thioalkalivibrio* strains originating from soda lakes in Kenya, Mongolia and Siberia (**A**) and from Egypt (**B**) at 4 M Na^+ and pH 10. Symbols: open circles - strain ALJ 15; open triangles – AKL 15; open squares - strain ALMg 2; closed circles - strain ALE 20; closed squares – strain ALE 10 and closed triangles – strain ALE 28.

The three strains isolated from soda lakes in Kenya and Central Asia did not tolerate high concentrations of NaCl. They grew optimally at high soda concentration. They also grew well in pure soda medium. Most probably traces of NaCl were present as contaminant of the Na_2CO_3 and $NaHCO_3$ stocks. Strain AKL 15 was the most NaCl-tolerant in this group. In contrast, the three Egyptian strains grew optimally at high NaCl concentration

(1.5-2 M) requiring a minimum of 0.1 M NaCl (strain ALE 20 and ALE 28) or even higher NaCl concentrations (0.5 M – strain ALE 10). Strain ALE 20 had the highest NaCl tolerance growing well at minimum and at maximum NaCl concentration achieved under conditions of stable alkalinity (0.1 to 3.5 M NaCl). Strain ALE 10 showed the narrowest range of NaCl tolerance, growing well between 0.5 and 3 M NaCl while strain ALE 28 showed an intermediate pattern, growing between 0.1 and 3 M NaCl.

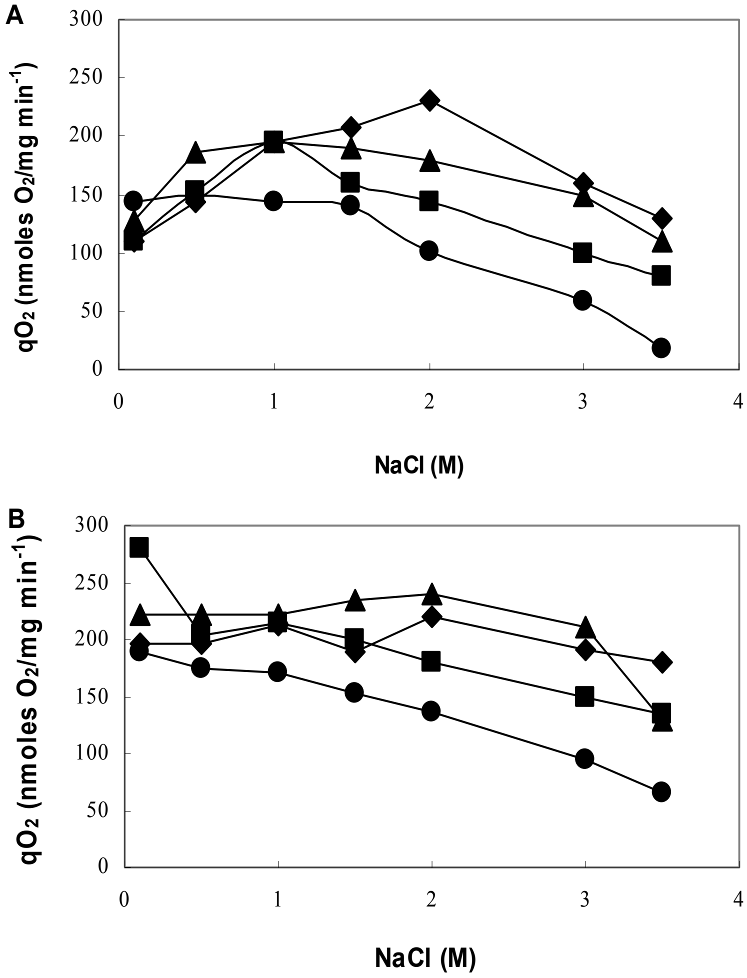

Figure 3. Effect of Cl^- concentration on thiosulfate-oxidizing (**A**) and on sulfide-oxidizing potential (**B**) in four *Thioalkalivibrio* strains, at 4 M Na^+ and pH 10. Symbols: circles - strain ALJ 15; triangles - strain AKL 15; squares – strain ALMg 2 and diamonds - strain ALE 20.

Washed cells of alkaliphilic strains from soda lakes were suspended in a buffer containing 4 M Na^+, at pH 10. Chloride and carbonate concentrations were varied considering a minimum concentration of 0.5 M Na_2CO_3/$NaHCO_3$, similarly to the growth conditions. The effect of Cl^- concentration on the thiosulfate- and sulfide-oxidizing capacity in the three Cl^--sensitive strains and one of the Cl^--resistant strains are presented in **Fig. 3 A** and **B**. The results indicated that the respiration was less sensitive to the NaCl variations than the growth. In general, thiosulfate-dependent respiration showed a maximum rate between 1 and 2 M NaCl. The sulfide-dependent respiration was even less sensitive to NaCl than thiosulfate respiration.

SO_4^{2-} effect on growth and sulfur-oxidizing potential of *Thioalkalivibrio* strains at 4 M Na^+, pH 10.

The effect of SO_4^{2-} on the growth of two low Cl^- strains, ALJ 15, ALMg 2 and one high Cl^- strain, ALE 20, is represented in **Fig. 4 A** and **B** respectively. At 4 M Na^+ and pH 10, in the presence of sodium carbonate/bicarbonate, strains ALJ 15 and ALMg 2 grew well up to 1-1.5 M SO_4^{2-}. In the case of high Cl^- strain ALE 20, its absolute requirement for Cl^- was confirmed by severe growth inhibition in the presence of SO_4^{2-} and absence of Cl^-. When the optimal NaCl concentration (1 M) was used in the growth medium of strain ALE 20, increasing SO_4^{2-} concentration stimulated the substrate consumption rate up to 1 M SO_4^{2-} (2 M Na^+).

The influence of high sulfate concentration was further tested in the activity (oxygen uptake) experiments. **Table 2** shows the values of maximum specific oxygen uptake rates ($qO_{2\,max}$) of strains ALJ 15, ALMg 2 and ALE 20 at different SO_4^{2-} concentrations, in the presence of a minimum of 0.5 M Na_2CO_3/$NaHCO_3$, at 4 M Na^+ and pH 10.

`All strains were capable of oxidizing thiosulfate and sulfide up to 1.75 M sulfate (equivalent to 3.5 M Na_2SO_4). Interestingly, sulfate stimulated sulfide respiration in strain ALJ 15. In strain ALJ 15, which also grew well at 1.75 M sulfate, thiosulfate respiration is less sensitive to sulfate concentration. Thiosulfate respiration capacity was inhibited by high sulfate concentration in strain ALE 20.

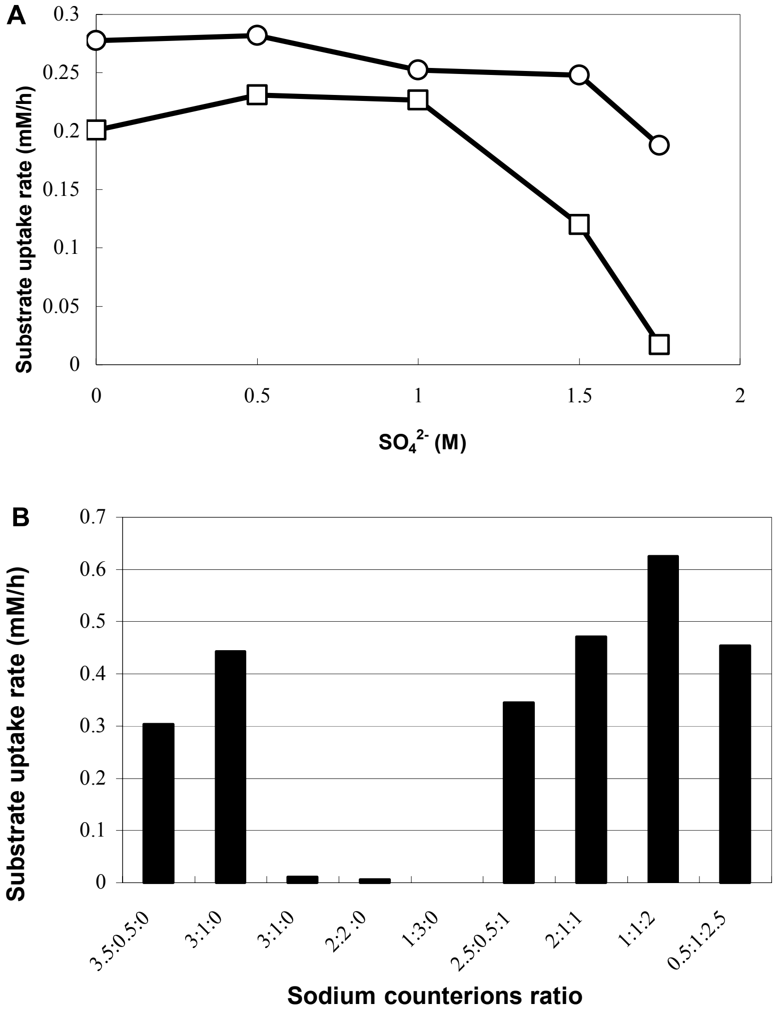

Figure 4. Effect of Cl^- and SO_4^{2-} concentration on substrate consumption rate of thiosulfate-grown *Thioalkalivibrio versutus* strains ALJ 15 (open circles) and ALMg 2 (open squares) (**A**) and of high chloride-tolerant strain ALE 20 (**B**), at 4 M Na^+ and pH 10. The ratios indicate soda: Cl^-: SO_4^{2-}.

Table 2. Effect of sulfate ions on maximum specific oxygen uptake rate ($qO_{2\ max}$) with thiosulfate and sulfide, in washed cells of three alkaliphilic strains of *Thioalkalivibrio*, at 4 M Na^+, 0.5 M $Na_2CO_3/NaHCO_3$ and pH 10.

Anion, M		$qO_{2\ max}$ (nmoles O_2/mg protein min^{-1})					
HCO_3^-/ CO_3^{2-}	SO_4^{2-}	ALJ 15		ALMg 2		ALE 20	
		$S_2O_3^{2-}$	HS^-	$S_2O_3^{2-}$	HS^-	$S_2O_3^{2-}$	HS^-
4.0	0	143	189	127	221	111	196
3.5	0.5	160	150	130	230	127	255
3.0	1.0	172	165	Nd	Nd	Nd	Nd
2.0	2.0	160	176	Nd	Nd	100	272
1.0	3.0	155	215	Nd	Nd	Nd	Nd
0.5	3.5	160	255	144	240	68	250

Nd - Not determined

Discussion

An important environmental factor in the selection of a particular type of sulfur-oxidizing bacteria type is the total salt content. Although all strains isolated from the soda lakes belonged to the haloalkaliphiles, three different subgroups can be identified on the basis of their salt tolerance and requirement. (1) All *Thioalkalimicrobium* and some of the *Thioalkalivibrio* isolates belong to a moderately salt tolerant type, being able to grow up to 1.2-1.5 M total Na^+. They originated mostly from the hyposaline lakes and were isolated on a medium containing a low salt concentration (Sorokin et al., 2000; 2001). (2) The largest group of the *Thioalkalivibrio* isolates was extremely salt-tolerant, able to grow in saturated soda brines (4-4.5 M Na^+). However, most of them grew optimally at moderate salt concentrations (0.5-1 M Na^+). Only a few isolates belonged to group (3), the true extreme halophiles unable to grow at salt concentrations below 1 M Na^+. We have studied six representatives of the latter two groups, which according to the present taxonomic criteria belong to one species: *Thioalkalivibrio versutus*. Three were low Cl^--organisms and three strains were high Cl^--tolerants.

All the extremely salt-tolerant *Thioalkalivibrio* strains were isolated from hypersaline soda lakes, mostly from Mongolia and Egypt. They were also the most thermotolerant, some being able to grow up to 50°C. The batch experiments presented in this paper, complemented by the continuous culture experiments with low Cl^- *Thioalkalivibrio versutus* strain ALJ 15 (see **Chapter 4** this thesis) demonstrated its excellent adaptation to doubly extreme conditions. Its growth rate and growth yield in soda brine at pH 10 and 4 M Na^+ were only 3 and 2 times lower, respectively, than found at 0.6 M Na^+. This strain showed a high versatility regarding the Na^+ concentration, being able to adapt the optimal activity of its respiration system to different sodium (soda) concentration (**Fig. 1**). The relatively high Cl^- and SO_4^{2-} tolerance and the potential to form cyst-like resting cells at suboptimal conditions are features that may give such bacteria a competitive advantage during the fluctuations of ion concentrations in their natural environment (Loiko et al., 2003).

Not only is the total sodium concentration important for optimal growth and activity, but also the anionic composition of alkaline brines. In particular, the ratio of carbonates/Cl- seems to be important. Based on their Cl^- tolerance the alkaliphilic sulfur-oxidizing *Thioalkalivibrio* strains from soda lakes can be divided into two major groups: low-chloride requiring/tolerant and high-chloride requiring/tolerant alkaliphiles. The first group is represented by Kenyan and Asian strains ALJ 15, AKL 15 and ALMg 2, isolated from soda lakes with sodium carbonates as the major salt species. They grew best in soda at low Cl^- concentration and their growth was inhibited at high Cl^- concentrations (above 3 M). These strains showed a true *natronophilic* (soda-loving) behavior, requiring high carbonate concentration instead of chloride for optimal growth. The second group is represented by the Egyptian strains ALE 10, ALE 20 and ALE 28 that had an optimal growth at high Cl^- concentration (1-2 M). They require a minimum of 0.1-0.5 M Cl^- for growth and can grow up to 3.5 M Cl^-. Above this Cl^- concentration, the alkalinity of the medium is not sufficient to keep optimum pH 10. The Egyptian strains have been isolated in high-chloride medium (1-2 M NaCl) showing a *haloalkaliphilic* behavior (sodium chloride-loving alkaliphiles).

Another interesting fact was that all tested strains were capable of growing up to 1.5 M sulfate. Natronophilic strain ALJ 15 had the highest tolerance for sulfate (it grew up to 1.75 M sulfate). The high salt versatility of this strain is known (Banciu et al., 2004; see also **Chapter 4**). In the haloalkaliphilic strain ALE 20, sulfate could not replace chloride ions. The strain was able to grow at high sodium sulfate concentration (1.25 M) only in the presence of a minimum of 0.5 M NaCl. Although the natronophilic strains were unable to grow at 3 M NaCl, at this concentration respire thiosulfate and sulfide under these conditions at more than 50% of the maximum. Sulfide respiration was less sensitive to chloride ions than thiosulfate respiration, a fact which might be explained by different localizations of the two enzymatic systems.

When relating the growth behavior and the chemical composition of the original environments of the natronophilic and haloalkaliphilic strains several observations can be drawn. At 4 M Na^+, well above the original conditions, the growth of extremely salt tolerant strain ALJ 15 was optimal around 0.5 M NaCl, close to the value in its original environment. However, the overall optimal growth for ALJ 15 was observed between 0.5 and 2 M Na^+, in the presence of 0.1 M NaCl. The other natronophilic strains, AKL 15 and ALMg 2 grew optimally at higher concentration of NaCl (1 M) in consistence with the higher NaCl content of their original environment. Despite the high NaCl concentration found in Lake Hotontyn, strain ALMg 2 behaved rather like a typical natronophile than like a halophile. From this point of view, the Egyptian strains ALE 10, ALE 20 and ALE 28, isolated from hypersaline alkaline lakes on NaCl-rich medium seems to form a compact group of halophilic alkaliphiles with optimal growth in the presence of 1-2 M NaCl. So, the isolation conditions are certainly important in selection of one or another subtype of salt-loving alkaliphiles. Interestingly, in all cases, lowering the total Na^+ concentration was linked with a lower Cl^- requirement for optimal growth, indicating a possible role of Cl^- in the osmoadaptation mechanisms rather than in the metabolism.

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4

Growth kinetics of the haloalkaliphilic sulfur-oxidizing bacterium *Thioalkalivibrio versutus* strain ALJ 15 in continuous culture

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Abstract

The chemolithoautotrophic sulfur-oxidizing bacterium *Thioalkalivibrio versutus* strain ALJ 15, isolated from a soda lake in Kenya, was grown in a continuous culture, with thiosulfate or polysulfide as growth-limiting energy source and oxygen as electron acceptor, at pH 10 and at 0.6, 2 and 4 M total sodium. The end product of the sulfur-compound oxidation was sulfate. Elemental sulfur and a cell-bound polysulfide-like compound appeared as intermediates during substrate oxidation. In the thiosulfate-limited culture, the biomass yields and maximum specific growth rates decreased two and three times respectively with increasing sodium concentration. The apparent affinity constant measured for thiosulfate and polysulfide were in the micromolar range (K_s= 6±3 μM). The maintenance requirement (m_s= 8±5 mmol $S_2O_3^{2-}$/g dry weight h^{-1}) was in the range of values found for other autotrophic sulfur-oxidizing bacteria. The organism had a comparable maximum specific rate of oxygen uptake with thiosulfate, polysulfide and sulfide while elemental sulfur was oxidized at a lower rate. Glycine betaine was the main organic compatible solute. The respiration rates with different species of polysulfides (S_n^{2-}) were tested. All polysulfide species were completely oxidized at high rates to sulfate. Overall data demonstrated efficient growth and sulfur compounds oxidation of haloalkaliphilic chemolithoautotrophic bacteria from soda lakes.

Introduction

Soda lakes are highly buffered saline and alkaline environments located mostly in arid areas of Eastern Africa, Central Asia and North America. Their brines contain high amounts of soda and NaCl (Grant and Tindall, 1986). A large number of heterotrophic haloalkaliphilic Archaea and Bacteria have been isolated from these lakes (Duckworth et al., 1996; Jones et al., 1998; Oren, 2002). The metabolic group of chemolithoautotrophic bacteria that appear to thrive in these soda lakes has only recently been discovered and studied. In particular, in our laboratory more than 100 strains of sulfur-oxidizing autotrophic bacteria have been isolated from soda lakes located in Central Asia (Mongolia, Southern Siberia) and Eastern Africa (Egypt, Kenya). *Thioalkalimicrobium* spp. and *Thioalkalivibrio* spp. were the first characterized aerobic chemolithoautotrophic haloalkaliphilic bacteria, that grow at pH 9-10 and at salt concentration ranging from 0.3 to 4 M of sodium (e.g. in a mixture of Na_2CO_3, $NaHCO_3$ and NaCl) using inorganic sulfur compounds as energy source (Sorokin et al., 2000, 2001a, b). They represent new genera in the γ subdivision of the Proteobacteria and are examples of double extremophily.

The sulfur-oxidizing haloalkaliphiles can be divided into three groups in relation to their salt tolerance: (i) low salt-tolerant alkaliphilic *Thioalkalimicrobium* species (growing up to 1.3 M Na^+), (ii) extremely salt-tolerant (growing between 0.5 and 4 M Na^+) and (iii) extremely halophilic alkaliphilic *Thioalkalivibrio* strains (which require a minimum of 1 M Na^+ for growth). Representatives of the first group have been found mostly in low-saline soda lakes in Central Asia. The extremely halotolerant *Thioalkalivibrio* strains were predominant in moderately saline soda lakes in Central Asia and Eastern Africa, while the extremely halophilic *Thioalkalivibrio* strains can be found only in permanently hypersaline and alkaline lakes in Eastern Africa (Sorokin et al., 2001a, b).

Although a large amount of data has been published about the halophilic and alkaliphilic bacteria surprisingly little information is available on the influence of the salt concentration on their growth kinetics and energy consumption (Oren, 1999). Such quantitative information can only be obtained by using the continuous culture approach. The growth of organisms in continuous culture has the advantage of strictly controlled and reproducible steady-state conditions, while parameters can be varied one by one. However, the use of highly saline media makes the continuous cultivation of haloalkaliphiles difficult. In an earlier study, continuous cultivation of *Thioalkalimicrobium* and *Thioalkalivibrio* was performed at low salt concentration (i.e. 0.6 M Na^+) and at variable pH, providing information on pH-dependent growth kinetics and competition for the growth limiting substrate. A clear conclusion could be drawn on the basis of these experiments:

Thioalkalimicrobium species are *r*-strategists, with high growth rate, low biomass yield and low resistance to starvation, whereas *Thioalkalivibrio* species are K-type strategists, with low growth rate, high yield and high resistance to starvation (Sorokin et al., 2003).

Polysulfides may represent the natural substrates of sulfur-oxidizing haloalkaliphiles from soda lakes and they are chemically or biologically produced from H_2S and elemental sulfur (S^0) both under oxic and anoxic conditions (Chen and Gupta, 1973; Giggenbach, 1974; Ginzburg et al., 1999; Gorlenko et al., 1999; Then and Trüper, 1983; Zavarzin et al., 1999). Polysulfides are released in haloalkaline environments and reach the layers populated by aerophilic sulfur-oxidizers. In natural systems free polysulfides can be found at high pH (Gun et al., 2000). The most common inorganic polysulfides found in aqueous solutions are tetra- and pentasulfides. Di- and trisulfides appear as a result of disproportionation of higher polysulfides, being more stable at very alkaline pH. Thiosulfate can be formed at alkaline pH as a product of chemical oxidation of polysulfides (Giggenbach, 1972).

The extremely salt-tolerant *Thioalkalivibrio versutus* strain ALJ 15 was chosen as a model organism because of its ability to grow well over a wide range of sodium concentration, on thiosulfate or polysulfide as energy source. The aim of this study was to find out to which extent the increasing concentration of sodium affects the growth of the model organism. The results presented in this paper demonstrate the unique potential of sulfur chemolithotrophic bacteria from soda lakes to grow efficiently over a wide range of salt concentration and at a high pH. The relevance of growth on polysulfide and oxidation of different polysulfide species by *Thioalkalivibrio versutus* strain ALJ 15 is discussed.

Material and methods

Strains and growth conditions

Thioalkalivibrio versutus strain ALJ 15, which is used in this study, was isolated from Lake Bogoria in Kenya (pH 10.1-10.2, conductivity 66 mS cm^{-1}) as described by Sorokin et al. (2001a). It is an obligate haloalkaliphilic, obligate chemolithoautotrophic, mesophilic Gram-negative bacterium able to use sulfide, polysulfide, thiosulfate, elemental sulfur and tetrathionate as energy source and oxygen as electron acceptor. It can grow at sodium concentrations up to 4 M, at pH values up 10.6, and at a temperature up to 50^oC.

Continuous cultivation was performed in 1.5 l laboratory fermentors with a 1 l working volume, fitted with pH and oxygen controls (Applikon, Schiedam, The Netherlands) (**Fig. 1**). The pH was controlled by automatic titration with 2 M NaOH and

HCl. The dissolved oxygen concentration was controlled at a minimum level of 50% air saturation by the stirring speed. The temperature was set at 35°C. Thiosulfate and polysulfide were sterilized separately as 2 M or 800 mM solution respectively, and added to the alkaline medium at approximatively 40 mM final concentration. The exact concentration has been determined as indicated below.

The mineral medium (0.6, 2 and 4 M Na^+) included: Na_2CO_3, 22, 85 or 180 g/l; $NaHCO_3$, 8, 19 or 38 g/l; NaCl, 6 g/l; K_2HPO_4, 1 g/l; KNO_3, 1 g/l; $MgCl_2 \cdot 6H_2O$, 0.1-0.2 g/l; trace elements (Pfennig and Lippert, 1966) – 1 ml/l, silicone antifoam, 1 ml/20 l, pH 10. As nitrogen source, nitrate was chosen over ammonia because is stable under alkaline conditions. NH_4^+ can be used as nitrogen source only at concentrations up to 2 mM (Sorokin et al., 2000).

The high concentration of sodium carbonate/bicarbonate did not cause corrosion in the fermentor during the whole period of cultivation (more than 40 days) although a salt crust formed on the metallic surface of the stirrer and the inlets. The continuous cultures were initiated with a short batch phase by addition of 100 ml of a dense inoculum to 900 ml of alkaline mineral medium supplied with 40 mM $Na_2S_2O_3$. When the substrate was completely consumed the continuous feeding of the culture was started. A steady-state culture was assumed to have been reached after at least 5 volumes changes.

The polysulfide-limited culture was started under the same conditions as for the thiosulfate-limited culture. For chemical stability, during the experiments using polysulfide as substrate, the medium was divided into acidic and alkaline solutions in double strength and supplied separately to the fermentors. To minimize the chemical oxidation of the labile polysulfide solution, the dropper was lengthened down to the surface of the culture. The alkaline stock solution containing polysulfide was continuously flushed with argon gas. Growth with polysulfide as energy source was performed at 2 M Na^+.

The sodium polysulfide solutions were prepared by dissolving known amounts of elemental sulfur in an argon-flushed stock solution of 200 mM Na_2S at room temperature, as shown in **Table 1** (Teder, 1967; Steudel et al., 1986). The mixtures were autoclaved at 120°C for 20 minutes and cooled down gradually. The concentrations of sulfane and sulfur atoms were measured colorimetrically using the methylene-blue method and by cyanolysis after acidic treatment (Giggenbach, 1972).

Table 1. Preparation and composition of various polysulfide compounds (tri-, tetra-, penta-hexa and octasulfide).

Compound	HS^- (mM)	Elemental sulfur added (mM)	Total sulfur (mM)	Fraction as sulfane	Fraction as zerovalent sulfur
$^-S\text{-}S\text{-}S^-$	200	100	300	0.75	0.25
$^-S\text{-}(S)_2\text{-}S^-$	200	200	400	0.5	0.5
$^-S\text{-}(S)_3\text{-}S^-$	200	300	500	0.4	0.6
$^-S\text{-}(S)_4\text{-}S^-$	200	400	600	0.333	0.666
$^-S\text{-}(S)_6\text{-}S^-$	200	excess	800	0.25	0.75

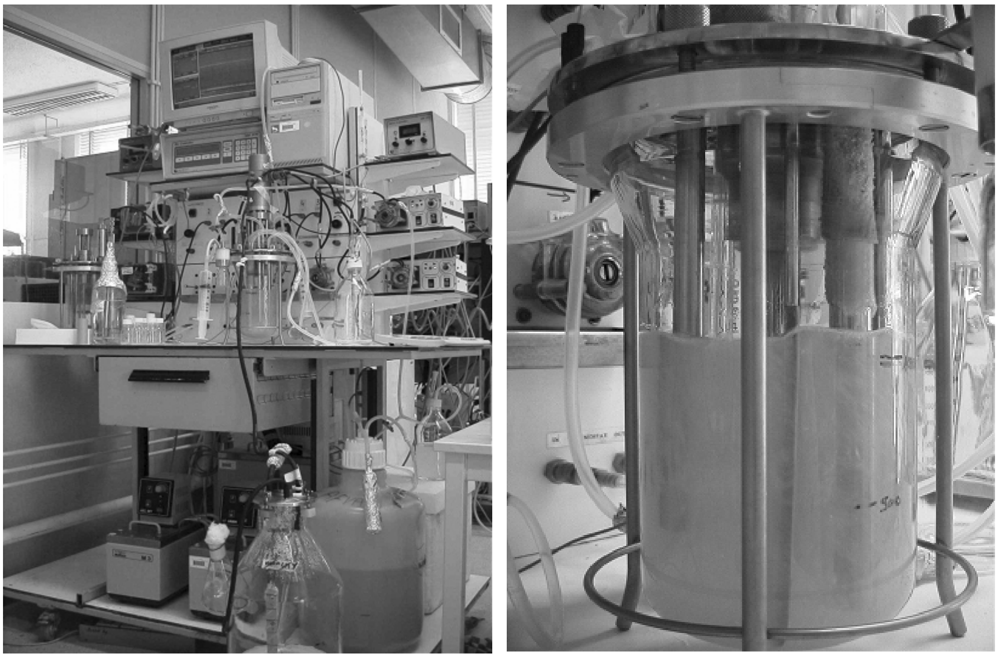

Figure 1. The experimental set up of continuous culture.

Respiration measurements

Cells were collected from the effluent on ice bath for a maximum period of 24 hours. Withdrawal of large volumes of sample directly from the chemostat would have disturbed the steady-state. No significant difference in the kinetic parameters was observed in the cells collected from the chemostat compared to the cells taken from the effluent stored within a period of several days. Cells were further harvested by centrifugation, washed and re-suspended in buffers containing 0.6, 2 or 4 M Na^+, pH 10. For subsequent tests the concentrated suspension was diluted in respiration buffer to 0.05-0.1 mg protein/ml. Respiration rates were measured at 35°C in a 5 ml glass chamber mounted on a magnetic stirrer and fitted with an oxygen electrode (Yellow Springs Instr., Ohio, U.S.A.) using different sulfur substrates at final concentrations of 34-50 µM. The buffers consisted of a mixture of carbonate and bicarbonate (0.1-4 M Na^+), pH 10 and 50 mM KCl. The rate values represent average results obtained from 3 to 5 independent measurements with standard deviations less than 10%.

Kinetics analysis

The kinetic constants, maximum specific oxygen uptake rate ($qO_{2\,max}$), and apparent affinity constants (K_s) were measured with an oxygen electrode using washed cells taken from the effluent of the cultures grown at different salt concentrations. To increase the sensitivity of the K_s measurements at 1-5 µM substrate level, the respiration experiments were run at 10% air saturation. The K_s values were calculated based on three independent measurements by plotting the oxygen uptake rate against the substrate concentration. The maintenance coefficient (m_s) was determined graphically from plotting the substrate uptake rate ($q_s = \mu/Y$) against dilution rate (D) and from reciprocal 1/Y-1/D plots, respectively, on the basis of the Pirt modification of the Monod growth model. For each dilution rate at least three steady-state biomass concentrations were measured with an interval of 1 volume change. Each determination was done in triplicate; the data represent the average values with standard deviation less than 10%. The maximum specific growth rate (μ_{max}) for each salt concentration was determined experimentally as the dilution rate at which washout of the biomass and accumulation of thiosulfate or free polysulfide started.

For the quantification of energetic efficiency in this organism we used the Gibbs free energy dissipation approach developed by Heijnen and van Dijken (1992) based on the "black box" concept. The Gibbs energy dissipation term (ΔG_{DIS}) is defined as the amount of free energy dissipated by the microbial system in order to produce 1 C-mol of biomass from the available C-source, electron donor and electron acceptor, and is expressed in kJ/C-mol biomass or kJ/g biomass. ΔG_{DIS} was corrected for pH, temperature and for the N-source.

The stoichiometric equation for *Thioalkalivibrio versutus* strain ALJ 15 grown in 2 M Na^+ with a biomass yield of 9.6 g dry weight mol^{-1} thiosulfate was considered as follows:

$$2.56\ S_2O_3^{2-} + CO_3^{2-} + 0.2\ NO_3^- + 2.36\ H_2O + 3.68\ O_2 \rightarrow 5.13\ SO_4^{2-} + CH_{1.8}O_{0.5}N_{0.2} + 2.93\ H^+$$

$$\Delta G^0\,(308\ K,\ pH\ 10) = -1711\ kJ\ /reaction$$

Chemical analysis

Micromolar thiosulfate concentrations were determined by cyanolytic procedures (Kelly et al., 1969). Millimolar-range thiosulfate consumption in batch cultures was followed by standard iodimetric titration after neutralization of the medium with 50% (v/v) acetic acid. Sulfide was measured colorimetrically according to Trüper and Schlegel (1964). Elemental sulfur was assayed by cyanolysis after extraction from the cell pellet with acetone (Sörbo, 1957). A certain fraction of intermediary sulfur compounds could not be extracted directly with acetone. This form of sulfur was cell-bound and could be detected only after acid treatment of the biomass (Sorokin et al., 1996). This fraction of sulfur behaves as polysulfide bound to the cell, which in acidic conditions liberates sulfide and extractable hydrophobic sulfur. Sulfate concentration was determined by a modified turbidimetric method (Kolmert et al., 2000). Cell protein was measured by the Lowry method (Lowry et al., 1951). When elemental sulfur was present, it was extracted with acetone from the biomass pellet before hydrolysis to avoid interference with the Lowry assay. Accurate measurement of the dry weight was difficult because of cell lysis during the washing procedures. Therefore 0.6 M and 2 M NaCl solutions were used for washing the cells. The total organic carbon was measured by using a non-dispersive infrared gas analyzer (Shimadzu TOC-5050A). The data represent average values obtained from 3 independent measurements. The standard error was ± 2%. The carbon composition of the biomass was further checked by "flash combustion" method using an Elementar Vario EL III elemental analyser, equipped with an integrated autosampler. The standard error of this method was ± 1%.

Determination of potassium and chloride ions was performed by INAA (Instrumental Neutron Activation Analysis) at Department of Chemistry, Delft University of Technology. The "Hoger Onderwijs Reactor" was used as source for neutrons. The gamma spectrometer used a germanium semiconductor as detector and a computer controlled sample changer. Intracellular compatible solutes were extracted and analyzed following a modification of the methods described by Galinski and Herzog (1990). HPLC separation used an isocratic system from Thermo Separation Products (CA), a 3 μm Grom-sil Amino-1PR column (Grom Analytik, Rottenburg-Hailfingen, Germany) and a Shodex refractive index detector (model RI17, Showa Denko KK, Tokyo, Japan). The mobile phase consisted of 80% (v/v) acetonitrile at a flow rate of 1 ml min^{-1}.

Natural abundance ^{13}C-NMR spectra of compatible solutes were recorded in the pulsed Fourier transform mode on a Bruker spectrometer (model Avance 3000 DPX) operating at 75.48 MHz (^{13}C) and at 300 MHz for the proton-decoupling channel relative to sodium trimethylsilylpropionate (TMSP).

Results

Influence of salt on the biomass yield

Thioalkalivibrio versutus strain ALJ 15 was grown successfully and reproducibly in the chemostat at alkaline pH and high salt concentration. During cultivation thiosulfate or polysulfide could not be detected in the supernatant indicating that the culture was substrate-limited.

The growth yield of *Thioalkalivibrio versutus* strain ALJ 15 decreased with increasing sodium concentration (**Table 2**). Differences in yield values were anticipated since batch experiments had indicated an optimum growth between 0.6 and 1 M Na^+, while at higher sodium concentrations the yield was lower. However, as can be seen in **Table 2**, the protein yield is only 25% or 50% lower when the organism is grown in 2 M or 4 M Na^+ respectively, compared with the culture grown at 0.6 M Na^+.

Table 2. Maximum specific growth rate (μ_{max}) and maximum yield (Y_{max}) of *Thioalkalivibrio versutus* strain ALJ 15 grown in continuous culture, under energy limitation at different salt concentration and pH 10.

Salinity (M Na^+)	Growth substrate	μ_{max} (h^{-1})	Y_{max}* (g dry weight/ mol substrate)	Y_{max}* (g protein/ mol substrate)
0.6	$S_2O_3^{2-}$	0.29	13.5	7.9
2	$S_2O_3^{2-}$	0.21	9.6	6
2	S_8^{2-}	0.21	8.5	5
4	$S_2O_3^{2-}$	0.11	6.1	4

* - *Yields were expressed in g of biomass per mol of* $S_2O_3^{2-}$ *or per mol of [S] respectively.*

The maximum specific growth rate (μ_{max}) determined experimentally was also influenced by the salt concentration. The μ_{max} of the culture grown on thiosulfate at 4 M Na^+ was three times lower than that of the culture grown at 0.6 M Na^+.

Determination of compatible solutes in *Thioalkalivibrio versutus* strain ALJ 15 grown at different salt concentrations

Growth at high salt concentration requires several structural adaptations of the organism. With a few exceptions, halophilic bacteria are known to control their osmotic pressure with organic compatible solutes. The analysis revealed that *Thioalkalivibrio versutus* strain ALJ 15 has glycine betaine as the main organic compatible solute. This is in agreement with the finding that *Thioalkalivibrio* sp. is closely related with *Ectothiorhodospira* sp. (Sorokin et al., 2001a), a group in which glycine betaine is one of the main compatible solutes (Galinski, 1995). In *Thioalkalivibrio versutus* strain ALJ 15, glycine betaine represents 5-9% of total dry weight at 2 and 4 M Na^+. Glycine betaine is involved in salt adaptation of strain ALJ 15 since at 2 M Na^+ and D= 0.2 h^{-1}, the concentration of betaine was at least four times higher than at 0.6 M Na^+ at the same D. A further increase of sodium concentration to 4 M resulted in little change of the glycine betaine content. The minor component, sucrose (2.5-0.5%), an energetically more expensive osmolyte, decreased with increasing growth rate (**Fig. 2**).

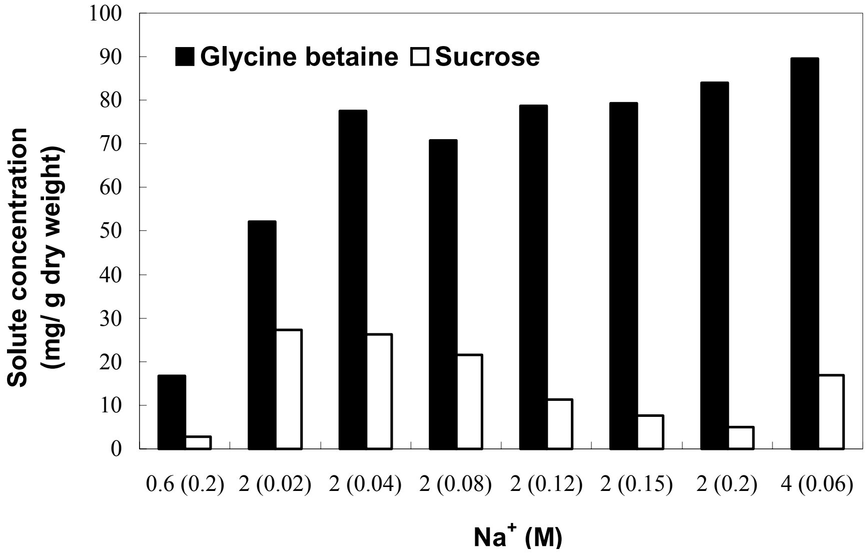

Figure 2. Compatible solutes composition of *Thioalkalivibrio versutus* strain ALJ 15 grown in continuous culture at 0.6, 2 and 4 M Na^+, pH 10. The numbers in the parentheses represent the dilution rates (D, h^{-1}).

Experiments in batch culture indicated that a dosage of 0.25-1 mM glycine betaine to the culture increased the growth rate of strain ALJ 15 at 4 M Na^+ with more than 20% (data not shown). The addition of glycine betaine precursors, glutamate or choline, did not affect the growth rate. This indicates that the organism has the capacity to import glycine betaine from its environment as well as for *de novo* synthesis.

The analysis of internal concentration of K^+ and Cl^- revealed levels of 1-2% of the dry weight for potassium and 0.1% of the dry weight for chloride ions. The error limits were between 5-20% for K^+ and 1.7-3.4% for Cl^-.

Growth rate dependence of biomass production, maintenance requirement and Gibbs free energy dissipation (ΔG_{DIS})

During growth of strain ALJ 15 at different dilution rates, the biomass yield values did not change significantly. At 0.6 and 2 M Na^+, already at a low specific growth rate (0.02-0.04 h^{-1}), the yields were high and remained constant up to the near-maximum growth rate, which is usually not the case in neutrophilic sulfur-oxidizing bacteria. The steady-state values of biomass yields and their standard deviations measured in strain ALJ 15 grown at 2 M Na^+ are presented (**Fig. 3**).

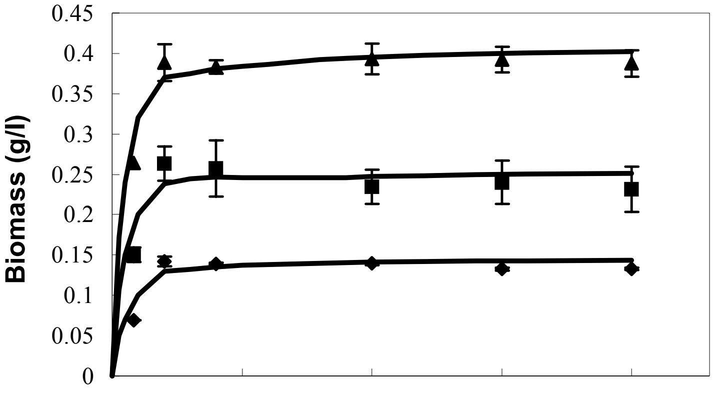

Figure 3. Steady-state biomass concentration as a function of dilution rate in *Thioalkalivibrio versutus* ALJ 15 grown in thiosulfate-limited chemostat at 2 M Na^+, pH 10 and 35°C. Symbols: triangles, dry weight; squares, protein; diamonds, total organic carbon.

At 2 M sodium concentration, the total protein content and the total organic carbon analysis of the biomass collected at a number of steady-states gave mean values of 62 % and 35% of the dry weight, respectively. Calculation of the maintenance coefficient (m_s) based on Pirt's model, in the thiosulfate-limited culture at 2 M Na^+, gave a value of 8±5 mmol $S_2O_3^{2-}$/g biomass h^{-1}.

Comparative values of ΔG_{DIS} calculated for different chemolithotrophic sulfur-oxidizing bacteria are presented in **Table 3**. The value of ΔG_{DIS} for *Thioalkalivibrio versutus* strain ALJ 15 is relatively low. This reflects a relatively high energetic efficiency of the organism grown at high salt concentration (2 M Na^+) and at pH 10.

Table 3. Optimal growth conditions, maximum dry weight yields (Y_{max}) and Gibbs free energy dissipation (ΔG_{DIS}) of chemolithotrophic sulfur-oxidizing bacteria grown on thiosulfate as energy source.

Organism	pH_{opt}	T_{opt} (°C)	N-source	Y_{max} (g dry weight/ mol substrate)	ΔG_{DIS} (kJ/ g dry weight)	Reference
Acidiphilium acidophilum	3	30	NH_4^+	5.7	123.8	a
Halothiobacillus halophilus	7.1	30	NH_4^+	6.4	113.1	b
Acidithiobacillus ferrooxidans	2	30	NH_4^+	6.3	105.1	c
Paracoccus versutus	7.8	30	NH_4^+	8.3	83.8	d
Halothiobacillus neapolitanus	6.8	30	NH_4^+	8.6	80.2	d
***Thioalkalivibrio versutus* ALJ 15**	**10**	**35**	NO_3^-	**9.6**	**69.6**	**this study**
Thermithiobacillus tepidarius	7.1	43	NH_4^+	10.7	69.3	e
Thermothrix thiopara	6.8	65	NH_4^+	18.0	32.5	d
Thiobacillus denitrificans	7	30	NH_4^+	20.6	21.1	f

[a] - Heijnen and van Dijken (1992)
[b] - Wood and Kelly (1991)
[c] - Hazeu et al. (1986
[d] - Mason et al. (1987)
[e] - Wood and Kelly (1986)
[f] – Kelly (1999)

Substrate affinity in thiosulfate and polysulfide-limited cultures

One of the most important kinetic parameter determined during the chemostat experiments was the apparent affinity constant (K_S) for thiosulfate and polysulfide. From the previous chemostat experiments with other representatives of *Thioalkalivibrio*, values within a range of 1-10 µM were determined. The measurement of K_s for thiosulfate and polysulfide in *Thioalkalivibrio versutus* strain ALJ 15 gave values of 6±3 µM. The K_s values for thiosulfate slightly increased with specific growth rate and finally, at maximum growth rate, a threshold of 2 µM thiosulfate was found. Below this value no respiration was observed.

Oxidation capacities for various sulfur substrates

During growth at different salt concentrations under thiosulfate and polysulfide limitation, cells of *Thioalkalivibrio versutus* strain ALJ 15 produced elemental sulfur (**Fig. 4 A**). The sulfur production was higher at 0.6 and 2 M Na^+, while at 4 M Na^+ the sulfur production decreased. An increase in sulfur production was observed transiently each time the dilution rate was changed. However, the steady-state concentration of sulfur represented only a small fraction of the loaded substrate. These amounts did not exceed 9% of the total sulfur (data not shown).

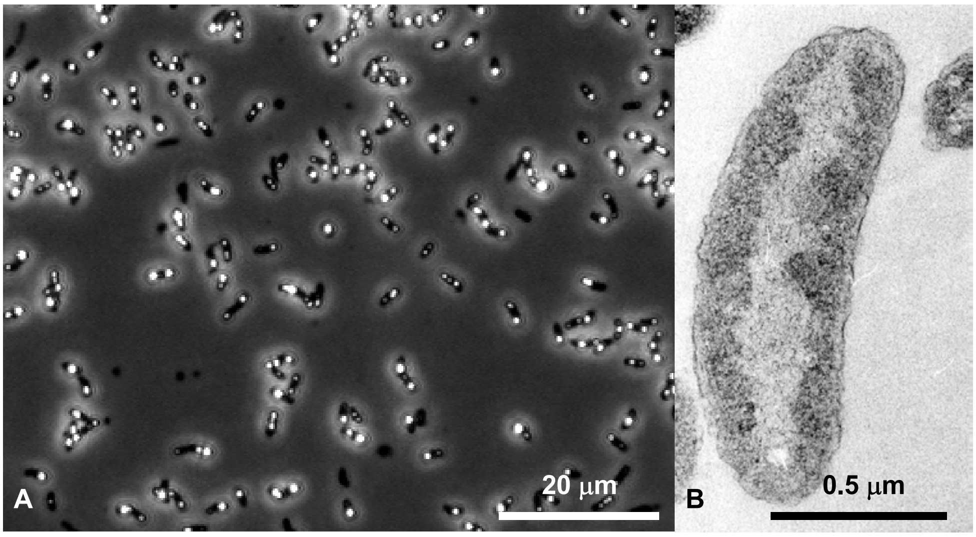

Figure 4. Phase-contrast micrograph of *Thioalkalivibrio versutus* strain ALJ 15 grown in continuous culture under thiosulfate limitation at 2 M Na^+ and pH 10, during elemental sulfur accumulation (A); electron-micrograph aspect of the strain ALJ 15 grown under same conditions but devoid of sulfur (B).

The cultures grown at different salt concentration and substrate limitation were tested for their capacity of sulfur substrates utilization. The values of maximum specific rates of oxygen consumption ($qO_{2\ max}$) with different sulfur substrates and their standard deviations are presented in **Table 4**. Thiosulfate and polysulfide were oxidized at the highest rate independent of the salt concentration. Compared to thiosulfate, sulfide or polysulfide, the rates of elemental sulfur oxidation were lower. The highest values for sulfur compound oxidation were found in cells grown at 0.6 M Na^+. The cells cultivated in polysulfide-limited chemostat showed higher rates of polysulfide oxidation than those found in the culture grown under thiosulfate limitation. During the oxidation of thiosulfate and polysulfide, small amounts of elemental sulfur and cell-bound polysulfide could be detected as minor transient intermediates. The formation of intermediary sulfur and cell-bound polysulfide was reported for several sulfur-oxidizing autotrophs (Hazeu et al., 1988; Moriarty and Nicholas, 1970a, b). Sulfate was the final product of thiosulfate, sulfide and polysulfide oxidation.

Table 4. Maximum specific rate of oxygen consumption ($qO_{2\ max}$) of *Thioalkalivibrio versutus* strain ALJ 15 grown in continuous culture under energy limitation at different salt concentration, at pH 10 and D= 0.04 h^{-1}. The rates were measured with 50 μM thiosulfate, sulfide or polysulfide or 34 μM sulfur. The results are the averages of three independent experiments and are shown ± standard deviation.

Salt concentration (M Na^+)	Growth substrate	$qO_{2\ max}$ (μmoles O_2/ mg protein min^{-1})			
		$S_2O_3^{2-}$	HS^-	S_8^{2-}	S^0
0.6	$S_2O_3^{2-}$	0.74 ± 0.06	0.54 ± 0.04	0.71 ± 0.06	0.30 ± 0.02
2	$S_2O_3^{2-}$	0.65 ± 0.05	0.40 ± 0.03	0.62 ± 0.04	0.21 ± 0.02
2	S_8^{2-}	0.55 ± 0.05	0.60 ± 0.05	0.75 ± 0.05	0.23 ± 0.01
4	$S_2O_3^{2-}$	0.28 ± 0.02	0.22 ± 0.03	0.30 ± 0.02	0.03 ± 0.002

Oxidation of different polysulfide species by polysulfide-limited culture

It was of particular interest to investigate the fate of polysulfide in cultures and cell suspension of the haloalkaliphiles because the alkaline environment provides unique conditions for the chemical stability of polysulfides. When a culture of *Thioalkalivibrio versutus* strain ALJ 15 was grown at 2 M Na^+, with polysulfide (S_8^{2-}) as electron donor, the sulfur and cell-bound polysulfide concentration was even lower than in thiosulfate-limited culture. The washed cells of polysulfide-limited culture oxidized polysulfide (S_8^{2-}) at highest rate observed in this organism at 2 M Na^+ (qO_{2max}=0.75 µmoles O_2/mg protein min^{-1}).

The oxidation of polysulfides of different length (S_n^{2-}) was studied with washed cells of *Thioalkalivibrio versutus* strain ALJ 15 grown under polysulfide limitation at 2 M Na^+ and pH 10. The oxidation proceeded in two phases. The rate of each phase was plotted against total sulfur. The first phase of polysulfide oxidation was proportional to the sulfane atoms concentration, while the second phase was constant (**Fig. 5**). From this point of view, trisulfide was the fastest oxidizable polysulfide substrate.

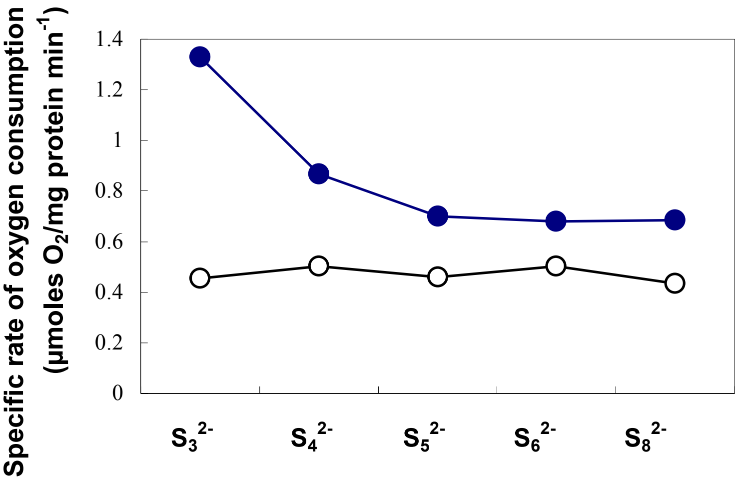

Figure 5. Oxygen consumption rates as a function of polysulfide length (S_n^{2-}) of washed cells of *Thioalkalivibrio versutus* strain ALJ 15 pre-grown under polysulfide- (S_8^{2-}) limiting conditions, at 2 M Na^+, pH 10 and D= 0.04 h^{-1}. Polysulfide concentration was 50 µM total sulfur. Symbols: closed circles, first stage of oxidation; open circles, second stage of oxidation.

The oxidation of HS^- alone occurred at a lower rate than the first phase of polysulfide oxidation, which means that the organism oxidized the sulfane atoms faster than the sulfide molecule itself. Still, the rate of sulfide oxidation was higher than the second phase of polysulfide oxidation. The rate of second phase was comparable with that of elemental sulfur oxidation.

Hence it can be assumed that the oxidation of sulfane atoms of the polysulfides corresponds to the first (fast) rate of oxygen consumption, while the second (slow) phase resulted from the internal zerovalent sulfur atoms oxidation. Moreover, the stoichiometry calculated for the first phase as well as measurable sulfur formation at the end of first phase (data not shown) supported this hypothesis.

Discussion

Our knowledge about the physiology of halophiles is mostly limited to heterotrophic microorganisms living in moderate to high concentrations of NaCl. Soda lakes are strongly buffered natural environments consisting of a mixture of sodium carbonate, sodium bicarbonate and sodium chloride. The organisms living in carbonate-rich media may have a different strategy in high concentration of salts than those found in chloride-rich environments. A first striking property of *Thioalkalivibrio versutus* strain ALJ 15 is its ability to grow well at highly alkaline pH and over a wide range of salt concentration. This behavior was characterized by a high specific growth rate sustained by a relatively high growth yield, not much different from those found in neutrophilic chemolithotrophic sulfur-oxidizing bacteria. It is clear that the organism can cope successfully with the high salt concentrations by proper adaptation of its energy metabolism.

Haloalkaliphilic strain ALJ 15 has a maximum specific growth rate within the range known for other sulfur-oxidizing chemolithoautotrophs (**Table 5**). It is worthy of note that these values (0.29, 0.21 and 0.11 h^{-1}) are obtained under extremely saline and alkaline conditions. At the same time, the yield determined experimentally at 0.6 and 2 M is even higher in this haloalkaliphilic organism than in most of its neutrophilic counterparts.

The complete oxidation of thiosulfate and polysulfide yields 8 and 6.25 electrons per mol of sulfur according to the equations shown below. From the maximum yield values (**Table 2**) we calculated that 1.2 g and 1.3 g of protein are produced per electron with thiosulfate or polysulfide respectively, as energy source at 2 M Na^+.

$$4\,S_2O_3^{2-} + 20\,H_2O \rightarrow 8\,SO_4^{2-} + 40\,H^+ + 32\,e^- \qquad 8\,e^-/\text{mol [S]}$$

$$S_8^{2-} + 32\,H_2O \rightarrow 8\,SO_4^{2-} + 64\,H^+ + 50\,e^- \qquad 6.25\,e^-/\text{mol [S]}$$

Thus, the use of either thiosulfate or polysulfide yielded similar amount of biomass per electron transferred. This is consistent with a common metabolic pathway for both substrates.

Another important feature of the organism is its capacity of reaching near maximum biomass yield at relatively low specific growth rates. The maintenance requirement calculated based on Pirt's model is also comparable with the maintenance values calculated for other sulfur-oxidizing bacteria grown on thiosulfate as energy source (Mason et al., 1987).

Table 5. Maximum growth rate (μ_{max}) on thiosulfate or sulfide, the molar growth yield and sodium requirement of neutrophilic chemolithotrophic sulfur-oxidizing bacteria.

Organism	Growth substrate	μ_{max} (h^{-1})	Y (g dry weight/ mol substrate)	Optimal NaCl concentration (M)	Reference
Thermithiobacillus tepidarius	$S_2O_3^{2-}$	0.4	10.7	nd	a
Halothiobacillus halophilus	$S_2O_3^{2-}$	0.072	6.4	0.86-1	b
Thiobacillus W5	HS^-	0.2	6.4	nd	c
Thiobacillus W1	HS^-	0.18	5.5	nd	d
Thiobacillus o	$S_2O_3^{2-}$	0.3	4.2	nd	d
Thiomicrospira thyasirae	$S_2O_3^{2-}$	0.038	2.5	nd	e
Halothiobacillus kellyi	$S_2O_3^{2-}$	0.45	nd	0.45	f
Halothiobacillus hydrothermalis	$S_2O_3^{2-}$	0.6	2.5	0.43	g

[a] – Wood and Kelly (1986)
[b] – Wood and Kelly (1991)
[c] – Visser (1997)
[d] – Stefess (1993)
[e] – Wood and Kelly (1989)
[f] – Sievert et al. (2000)
[g] – Wood and Kelly (1988)

In the energetic balance of the halophiles one must take into account the production of compatible solutes (**Fig. 6**). According to Oren (1999), an organism grown in 4 M NaCl would need 8 M compatible solutes in order to balance the osmotic pressure. He predicted that for 0.2 g of dry material of structural cell components, 0.85 g of glycine betaine would be required, representing approximately 80% of the total dry weight (assuming that 1 cm^3 of biovolume contains 0.2 g dry material and 0.9 ml of water). Such hypothetical concentrations have never been shown experimentally, on the contrary, compatible solute concentrations are always lower. In our case, in the strain ALJ 15 grown at 4 M Na^+, the betaine (9% of the total dry weight) and the sucrose (1.5%) concentrations were relatively low. A significant contribution by potassium ions in balancing the predicted osmotic pressure is excluded since the intracellular K^+ concentration did not exceed 2% of the total dry weight. The measurement of sodium ions could not discriminate between their internal or external location. Still, a possibility remains that other unknown compounds contribute to the cell's overall osmotic adjustment. However the relatively low concentrations of organic and inorganic compatible solutes in *Thioalkalivibrio versutus* strain ALJ 15 implies that the energy requirement of adaptation in 4 M sodium carbonate/bicarbonate is not as high as one may expect. Therefore we assume that a large part of energy generated by oxidation of inorganic sulfur compounds is invested in growth and active biomass. This may explain why these chemolithoautotrophic extremophiles can grow well at high salt concentrations.

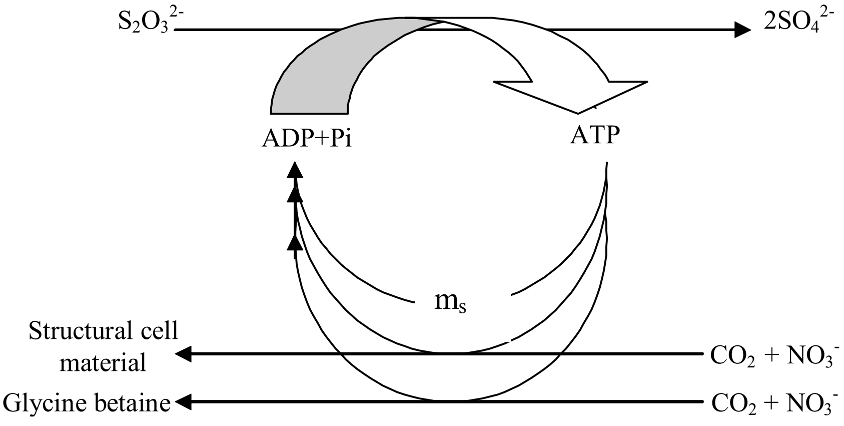

Figure 5. Schematic representation of energy requirements in the haloalkaliphilic sulfur-oxidizing *Thioalkalivibrio versutus* strain ALJ 15.

From the results obtained with polysulfides of different degrees of polymerization, it is clear that the sulfane atoms from polysulfide chain are oxidized first. It is possible that different forms of sulfur atoms from polysulfide molecule are preferred substrates for different enzymes. At this moment however it is not clear whether the same enzyme (sulfide dehydrogenase) was responsible for the initial attack on sulfide and on the sulfane atoms of polysulfide.

The unusual conditions of life in the soda lakes (high pH, high salt concentration) selected for haloalkaliphilic sulfur-oxidizing chemolithoautotrophs, such as *Thioalkalivibrio versutus* strain ALJ 15. This probably can explain the permanent presence of populations of *Thioalkalivibrio* in the sediments of many soda lakes, revealed both by cultivation techniques (up to 10^6 cells/g sediment) and directly by molecular methods (Baumgarte, 2003; Humayoun et al., 2003; Ma et al., 2003; Rees et al., 2003; DY Sorokin, unpublished results).

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Thioalkalivibrio halophilus sp. nov., a novel obligately chemolithoautotrophic facultatively alkaliphilic and extremely salt-tolerant sulfur-oxidizing bacterium from a hypersaline alkaline lake

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Abstract

A new chemolithoautotrophic, extremely salt-tolerant and facultative alkaliphilic and sulfur-oxidizing bacterium was isolated from an alkaline hypersaline lake in Altai steppe (Siberia, Russia). According to 16S rDNA analysis and DNA-DNA hybridization, strain HL 17^T was identified as a new species of the genus *Thioalkalivibrio* belonging to the γ subdivision of the *Proteobacteria* for which the name *Thioalkalivibrio halophilus* is proposed. Strain HL 17^T is an extremely salt-tolerant bacterium growing at sodium concentrations between 0.2 and 5 M with an optimum at 2 M Na^+. It grew at high concentrations of NaCl and of $Na_2CO_3/NaHCO_3$ (soda). Strain HL 17^T is a facultative alkaliphile growing at pH range 7.5-9.8, with a broad optimum between pH 8.0 and 9.0. It used reduced inorganic sulfur compounds (thiosulfate, sulfide, polysulfide, elemental sulfur and tetrathionate) as energy source and electron donor. In continuous culture, under energy limitation, thiosulfate was stoichiometrically oxidized to sulfate. In sodium carbonate medium under alkaline conditions the maximum growth rate was similar while the biomass yield was lower as compared with the NaCl-grown culture. The maximum sulfur-oxidizing capacity measured in washed cells was higher in the soda buffer independent of the growth conditions. The compatible solute content of the biomass

was higher in the sodium chloride-grown culture than in the sodium carbonate/bicarbonate-grown culture. The data suggest that the osmotic pressure differences between soda and NaCl solutions might be responsible for the difference observed in compatible solutes production. This may have important implications in overall energetic metabolism of high salt adaptation.

Introduction

The saline lakes are the result of complex interactions of the geological, climatic and biogeochemical conditions. Accordingly, acidic, neutral or alkaline saline lakes with different mineral composition can be formed. The neutral saline lakes with a pH between 6 and 8.5 usually contain Na^+ and Mg^{2+} as major cations and Cl^- and SO_4^{2-} as major anions, resulting in neutral salts with low buffering capacity (Grant et al., 1998). The alkaline saline or soda lakes (pH 9-11) contain large amounts of sodium carbonates (Na_2CO_3+ $NaHCO_3$) with high buffering capacity (Grant and Tindall, 1986). The (hyper)saline lakes are populated mostly with halophilic neutrophilic organisms, while the alkaline saline lakes are the habitats of haloalkaliphilic species. The organisms living in such environments possess special adaptation mechanisms that make them interesting not only for fundamental research but also for industrial application (Margesin and Schinner, 2001).

In the past decades the studies revealed an impressive diversity of organisms that thrive in highly saline and alkaline lakes (Duckworth et al., 1996; Humayoun et al., 2003; Jones et al., 1998; Oren, 1994, 2002; Zavarzin et al., 1999). So far, the biological oxidation of inorganic sulfur compounds was only known to occur under neutrophilic and acidophilic conditions (Kuenen et al., 1992). Recently, a new group of obligately chemolithoautotrophic sulfur-oxidizing bacteria living at high pH and high salt concentration has been discovered. The group so far includes three new genera, *Thioalkalimicrobium* spp., *Thioalkalivibrio* spp. (Sorokin et al., 2001) and *Thioalkalispira* (Sorokin et al., 2002b) which all belong to the γ subdivision of the *Proteobacteria*. These organisms play a crucial role in the natural sulfur-cycle in saline alkaline environments. A large number of sulfur-oxidizing haloalkaliphilic strains have been isolated and characterized in our laboratory (Sorokin et al., 1996, 2000, 2002 a, c). The first data regarding the strategy of growth and inorganic sulfur oxidation at such extreme conditions have been published only recently (Sorokin et al., 2003; Banciu et al., 2004). They have indicated the unique potential of haloalkaliphilic sulfur-oxidizing chemolithotrophic bacteria from soda lakes to grow efficiently over a wide range of soda (Na_2CO_3/$NaHCO_3$) concentration and an extremely high pH.

The aims of the present study were to identify and characterize the first known facultatively alkaliphilic and halophilic chemolithoautotrophic sulfur-oxidizing bacterium (strain HL 17^T) isolated from a hypersaline lake with low soda content, and to find out whether its growth in different sodium salts might induce different physiological and molecular adaptations. The results showed that in strain HL 17^T the production of compatible solutes was enhanced in NaCl containing culture medium compared to soda-containing medium. This may be the direct consequence of the difference in physico-chemical properties of the two salt solutions. The distinct physiological properties of strain HL 17^T correlated with its genetic difference from the known haloalkaliphilic species justify the description of a new species: *Thioalkalivibrio halophilus*.

Material and methods

Enrichment procedure and isolation of pure culture

Strain HL 17^T was isolated from the hypersaline (380 g/l total salts) and slightly alkaline (pH 9.05, total carbonate alkalinity 0.5 M) Stamp Lake in South-Kulunda Steppe (Altai, Russia). The enrichment was started by inoculating a sample in 4 M NaCl mineral medium containing 30 mM thiosulfate as energy source at pH 8, followed by incubation on a rotary shaker at 100 rpm and 30°C. Development of the culture was checked by monitoring the thiosulfate consumption. $NaHCO_3$ was periodically supplied for pH adjustment and as carbon source (20-50 mM).

A pure culture of autotrophic sulfur-oxidizing halophile was obtained by making serial dilution from the enrichment cultures after complete thiosulfate consumption. The capacity of growing at alkaline pH and at high concentrations of soda was tested by transferring the halophilic strain at gradually increasing carbonate concentration and pH.

Growth conditions and viability count

The 4 M NaCl mineral medium contained: NaCl 240 g/l; K_2HPO_4 1.5 g/l; NH_4Cl 0.5 g/l; pH 7.5. After sterilization, 50 mM of filter sterilized $NaHCO_3$ was added. The final thiosulfate concentration was 30 mM. The following salts were added: 1 mM $MgCl_2$, 1 mM $MgSO_4$ and 1 ml/l of trace element solution (Pfennig and Lippert, 1966) from a sterile concentrated stock solution. The final pH was 7.8-8.0. During growth in batch culture the pH was periodically adjusted with sterile 1 M $NaHCO_3$ solution. The high soda medium (4 M Na^+) was prepared by mixing 7 volumes of 4 M soda medium (pH 10.0) with one volume of 4 M NaCl (pH 8) medium. The final concentration of Na^+ as carbonate/bicarbonate salt was 3.5 M while the NaCl concentration was 0.5 M. The medium was strongly buffered at pH 9.6. The 4 M soda medium contained: Na_2CO_3 180 g/l; $NaHCO_3$ 38 g/l; NaCl 6 g/l; K_2HPO_4 1 g/l; KNO_3 1 g/l, pH 10. After sterilization all other salts were added as mentioned above. The electrical conductivity (mS/cm) was determined by using a pH and conductivimeter Jenway, model 4330. The osmotic pressure (osm/kg) of the growth media was determined using a vapor pressure osmometer (KNAUER, Berlin-Zehlendorf, Germany).

Continuous cultivation was performed in 1.5 l laboratory fermentors with a 1 l working volume, fitted with pH and oxygen controls (Applikon, Schiedam, The Netherlands). The pH was controlled by automatic titration with NaOH or HCl. The NaCl medium was titrated with 4 M NaOH to elevate the pH to 9.6 while the soda medium was titrated with 4 M HCl to maintain the pH at the same value. The dissolved oxygen concentration was controlled at a maximum level of 50% air saturation by the stirring speed. The temperature was set at 35°C. Thiosulfate was sterilized separately as 2 M stock solution and added to the medium at approximatively 40 mM final concentration. The exact concentration of thiosulfate has been determined as indicated below. The continuous culture in 4 M NaCl medium was initiated with a batch phase by addition of 100 ml of a dense cell suspension to 900 ml of 4 M NaCl mineral medium supplied with 40 mM $Na_2S_2O_3$. When the substrate was completely consumed the continuous feeding of the culture was started. The continuous culture in soda medium was started with 100 ml inoculum originating from a 4 M NaCl batch culture. A steady state culture was assumed to be reached after at least 5 volumes changes.

The number of viable cells in the chemostat cultures was determined by serial dilution (from 10^{-1} to 10^{-10}) in 20 ml tubes with 5 ml mineral medium and thiosulfate as substrate, at pH 9.6. The tubes were incubated with slow shaking at 35°C until turbidity appeared. The highest dilution where growth was observed was considered as the number of viable cells per millilitre of the chemostat culture.

Respiration measurements and kinetics analysis

Cells collected from the chemostat effluent and stored at 4°C, were harvested by centrifugation, washed and re-suspended in buffers containing 4 M Na^+, pH 9.6. For subsequent tests the concentrated suspension was diluted in respiration buffer to 0.05-0.1 mg protein/ml. Respiration rates were measured at 35°C in a 5 ml glass chamber mounted on a magnetic stirrer and fitted with an oxygen electrode (Yellow Springs Instr., Ohio, U.S.A.) connected to a chart recorder (Kipp & Zonen, model BD40). Different sulfur substrates were used at final concentrations of 34-50 µM.

The kinetic constants, maximum specific oxygen uptake rate ($qO_{2\ max}$) and apparent affinity constants (K_s) were determined in the washed cells collected from the effluent of continuous cultures. These parameters were calculated from the rates of oxygen consumption measured with an oxygen electrode as mentioned above. To increase the sensitivity of the recorder for the K_s measurements at 1-5 µM substrate level, the respiration experiments were run at 10 % air saturation. The K_s values were calculated based on three independent measurements by plotting the oxygen uptake rate against the substrate concentration. The maintenance coefficient (m_s) was determined graphically from plotting the substrate uptake rate ($q_s=\mu/Y$) against dilution rate (D) and from reciprocal 1/Y-1/D plots, respectively, on the basis of the Pirt modification of the Monod growth model. For each dilution rate at least three steady state biomass concentrations were measured with an interval of 1 volume change. Each determination was done in triplicate; the data represent the average values with standard deviation less than 10%. The maximum specific growth rate for each salt concentration was determined experimentally as the dilution rate at which washout of the biomass and accumulation of thiosulfate started.

Pigment analysis

Pigments were extracted overnight from freeze-dried biomass with methanol and centrifuged at 3000xg. The supernatant was recovered and the absorption spectra were recorded with a HP 8453 UV-Vis spectrophotometer.

Chemical analysis

Micromolar thiosulfate concentrations were determined by cyanolytic procedures (Kelly et al., 1969). Millimolar-range thiosulfate consumption in batch cultures was measured by standard iodimetric titration after neutralization of the medium with 50% (v/v) acetic acid. Elemental sulfur was analyzed by cyanolysis after extraction from the cell pellet with acetone (Sörbo, 1957). A certain fraction of intermediary sulfur compounds could not be extracted directly with acetone. This form of sulfur was cell-bound ("bound polysulfide") and could be detected only after acidic treatment of the biomass (Sorokin et al., 1996). Sulfate concentration was determined by a modified turbidimetric method (Kolmert et al., 2000). Cell protein was measured by the Lowry method (Lowry et al., 1951). For dry weight determination cells were harvested and washed twice with an isotonic solution (3 M NaCl). The biomass was frozen overnight at –80°C and lyophilized. The dry weight was corrected for 22% salt content as determined by instrumental neutron activation analysis (INAA) (Alfassi, 2001). INAA was performed at Department of Chemistry, TU Delft. The "Hoger Onderwijs Reactor" was used as source for neutrons. The gamma spectrometer used a germanium semiconductor as detector and a computer controlled sample changer. The standard error of ions determination was less than 5%.

At steady state, samples in duplicates were collected and analyzed for total organic carbon, elemental composition and compatible solutes. The total organic carbon was measured by using a non-dispersive infrared gas analyzer (Shimadzu TOC-5050A). The data represent average values obtained from 3 independent measurements. The standard error was ± 2%. The elemental composition of the biomass was analyzed by "flash combustion" method using an elemental analyser (Elementar Vario EL III) equipped with an integrated autosampler. The standard error of this method was ± 1%.

Intracellular compatible solutes were extracted and analyzed following a modification of the methods described by Galinski and Herzog (1990). HPLC separation was performed using an isocratic system from Thermo Separation Products (CA), a 3 µm Grom-sil Amino-1PR column (Grom Analytik, Rottenburg-Hailfingen, Germany) and a Shodex refractive index detector (model RI17, Showa Denko K.K., Tokyo, Japan). The mobile phase consisted of 80% (v/v) acetonitrile at a flow rate of 1 ml min^{-1}. Natural abundance ^{13}C-NMR spectra of compatible solutes were recorded in the pulsed Fourier transform mode on a Bruker spectrometer (model Avance 3000 DPX) operating at 75.48 MHz (^{13}C) and at 300 MHz for the proton-decoupling channel relative to sodium trimethylsilylpropionate (TMSP).

SDS-PAGE

Polyacrylamide gel electrophoresis of total proteins was performed under denaturing conditions using gel concentrations of 12%. Cells grown in chemostat with thiosulfate at pH 9.6 were resuspended in distilled water and sonicated. The resulting extracts were adjusted to a protein concentration 1 mg ml^{-1} and boiled in sample buffer (5x concentrated) with 2-mercaptoethanol and dithiothreitol (20 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 and phylogenetic analysis

The isolation of the DNA and subsequent determination of the DNA G+C content and DNA-DNA hybridization were performed by thermal denaturation method according to standard procedures (De Ley et al., 1970; Marmur, 1961).

Genomic DNA was obtained with the DNA Plant Extraction kit from MoBiol Laboratories. Subsequently, the 16S rRNA gene was amplified using primers for the 16S rDNA of bacteria. PCR products were purified from low-melting agarose using the Wizard PCR Prep kit (Promega) according to the manufacturer's instruction and sent for sequencing. Comparative sequence analysis was performed using the ARB software program. The sequence was aligned to sequences in the databases. A phylogenetic tree was constructed using the Neighbour Joining algorithm with Jukes Cantor correction. The GenBank accession number of the 16S rDNA sequence of strain HL 17^{T} is AY346464.

Results

Phylogenetic analysis

Comparative analysis of the 16S rRNA sequence of strain HL 17^T with sequences from other bacteria showed that it belonged to the genus *Thioalkalivibrio* in the γ subdivision of the *Proteobacteria* (**Fig. 1**) with as closest relative to *Thioalkalivibrio nitratis* (96.88% similarity). The DNA-DNA hybridization was used for further discrimination of strain HL 17^T from the other species of *Thioalkalivibrio*. A low similarity was found between strain HL 17^T and *Thioalkalivibrio versutus* (29%) and *Thioalkalivibrio jannaschii* (34%). The mol% G+C content of DNA from strain HL 17^T was 65.1 ± 0.5%. Since the differences were larger than the formal 2% in 16S rRNA sequence along with a minimum of 70% difference in DNA-DNA similarity (Rosselló-Mora and Amann, 2001), strain HL 17^T can be regarded a distinct species of the genus *Thioalkalivibrio* for which the name "*Thioalkalivibrio halophilus*" is proposed.

Growth characteristics and optimal growth conditions

Batch cultivation of *Thioalkalivibrio halophilus* HL 17^T and respiration experiments with washed cells collected from the chemostat cultures, showed that the optimum salt concentration for growth and respiration was 2 M Na^+ (**Fig. 2**) indicating the halophilic nature of this organism. The organism grew in a broad range of salt concentration (0.2 to 5 M Na^+). The minimal salt requirement for growth and respiration was 0.2 M Na^+. Strain HL 17^T, unlike other species of *Thioalkalivibrio* sp., was not able to grow in Cl^--free soda medium since it required at least 0.2 M Cl^- for growth. At a low Na^+ concentration (0.2 - 0.5 M NaCl), the growth was followed by production of elemental sulfur.

The organism grew between pH 7.3 and 9.8. The optimum pH for growth and respiration was between pH 8 and 9, indicating that strain HL 17T belongs to the facultative alkaliphiles (**Fig. 3**). While the pH effect on the substrate consumption rate had clear optimum between pH 8 and 9, the growth yield measurements indicated a rather constant value between pH 7.5 and 9.0. At pH higher than 9.5, an increase of yield was observed despite the obvious lower substrate consumption rate. In the chemostat culture at 4 M Na^+, such yield difference between soda and NaCl medium cultures was not observed at pH 9.6. The viability count revealed the presence of 5 times higher number of viable cells in soda culture as compared to NaCl culture ($5x10^9$ versus $\leq 10^9$ cells/ml).

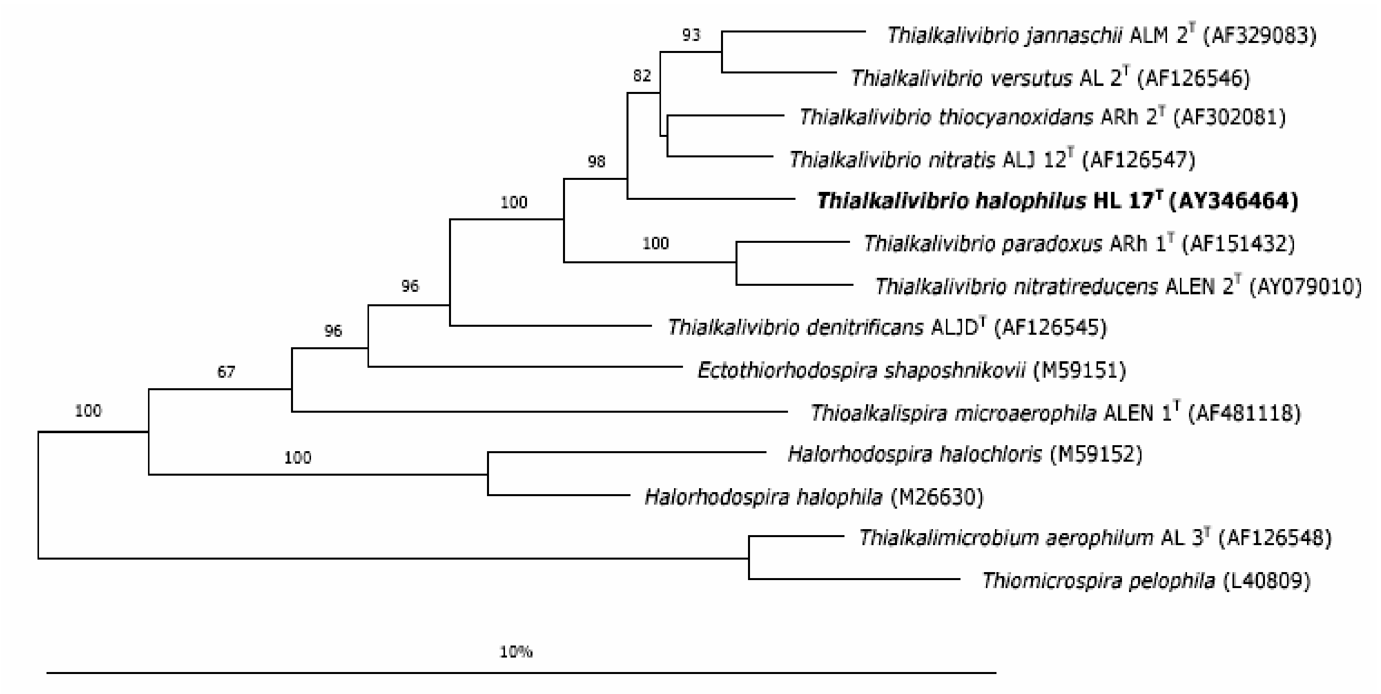

Figure 1. Neighbour Joining tree showing the phylogenetic affiliation of *Thioalkalivibrio halophilus* strain HL 17^T. *Paracoccus denitrificans*, *Rhodobaca bogoriensis* and *Sulfitobacter mediterraneus*, all belonging to the α subdivision of the *Proteobacteria*, were used as an out-group, but were pruned from the tree.

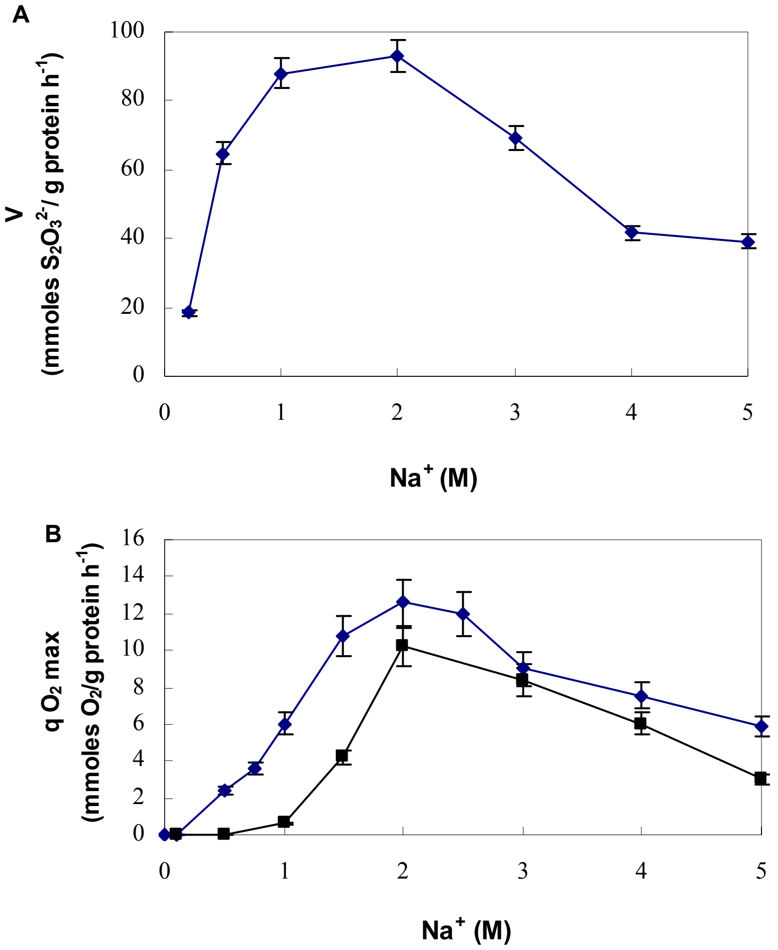

Figure 2. Optimal salt concentration for growth and respiration in strain HL 17^T. (A) Salt effect on growth of strain HL 17^T in batch culture at different concentrations of NaCl, pH 7.8 and 50 mM NaHCO$_3$ as carbon source. (B) Salt effect on thiosulfate respiration of washed cells grown in continuous culture under thiosulfate limitation at 4 M NaCl, pH 9.6 (dilution rate, D = 0.02 h^{-1}). Cells were incubated in Tris-HCl buffer with pH 7.5 (squares) or 9.6 (diamonds) at different concentrations of NaCl. **V**, specific rate of substrate consumption; **qO_2**, specific rate of thiosulfate-dependent respiration.

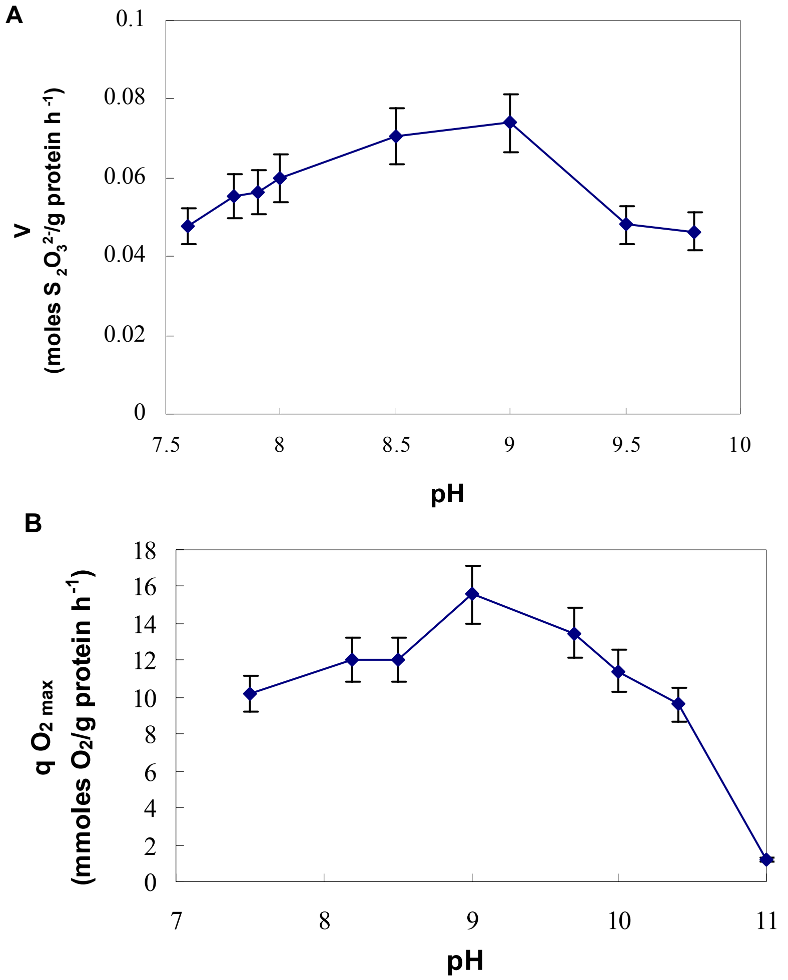

Figure 3. Optimal pH for growth and respiration of strain HL 17^T. (A) pH effect on growth of strain HL 17^T in batch culture, at 4 M Na$^+$. At low pH (6.5 - 8.0), 50 mM NaHCO$_3$ was added to buffer the medium containing 4 M NaCl. At alkaline pH, 0.5 M of NaHCO$_3$ + Na$_2$CO$_3$ was added to 3.5 M NaCl. (B) pH effect on thiosulfate respiration of washed cells grown in continuous culture under thiosulfate limitation at 4 M NaCl, pH 9.6 (D = 0.02 h^{-1}). Cells were incubated in 2 M NaCl buffered with Tris-HCl (pH7.5 - 9.8) and by addition of small quantities of Na$_2$CO$_3$ (pH 10.0-11.0). Abbreviations – as in **Fig. 2**.

Growth yield and maximum growth rates in continuous culture

Strain HL 17^T was successfully grown in thiosulfate-limited continuous culture, at 4 M Na^+ and at pH 9.6. The cultures were maintained in steady state for more than 50 days under such conditions. Micromolar elemental sulfur concentrations were detected in the continuous culture of strain HL 17^T cultivated at 4 M NaCl. In continuous culture of strain HL 17^T at 3.5 M Na_2CO_3 /$NaHCO_3$ + 0.5 M NaCl the sulfur formation was more prominent than that of 4 M NaCl culture. The cultivation at high NaCl concentration caused corrosion problems in the reactor. These problems were absent in the reactor with high soda medium. In both cultures thiosulfate was oxidized to sulfate in a proportion of 95%. **Table 1** summarizes the data on the growth kinetic parameters of strain HL 17^T in continuous culture in comparison with the obligately alkaliphilic *Thioalkalivibrio (Tv.) versutus* strain ALJ 15, grown under similar conditions (Banciu et al., 2004; see **Chapter 4**).

Table 1. Maximum specific growth rates (μ_{max}), maximum growth yields (Y_{max}) and maintenance coefficients (m_s) of *Thioalkalivibrio* strains grown in continuous culture at 4 M Na^+ and alkaline pH

Parameters	Organism		
	Strain HL17^T, chloride culture[a]	Strain HL17^T, soda culture[b]	*Tv. versutus* ALJ 15, soda culture[c]
μ_{max}, h^{-1}	0.033	0.035	0.11
Y_{max} (g protein/ mol $S_2O_3^{2})^-$	2.6	1.8	4
Y_{max} (g dry weight/ mol $S_2O_3^{2-}$)	6	4	6.1
m_s, (mol $S_2O_3^{2-}$/ g protein h^{-1})	$2.6 \bullet 10^{-3}$	$5.3 \bullet 10^{-3}$	nd

[a] Strain was grown at 4 M NaCl + 50 mM $NaHCO_3$, pH 9.6

[b] Strain was grown at 3.5 M Na_2CO_3/$NaHCO_3$ + 0.5 M NaCl, pH 9.6

[c] Strain was grown at 3.9 M Na_2CO_3/$NaHCO_3$ + 0.1 M NaCl, pH 10.0

The protein yield of strain HL 17^T grown in soda medium was 30% lower than that of chloride culture, while the maximum growth rate was similar. Compared with *Tv. versutus* strain ALJ 15, strain HL 17^T had a much lower μ_{max} and protein yield. The dry biomass yield, after subtracting the salt contribution, was 6 g dry biomass/ mol thiosulfate in the sodium chloride-grown culture and 4 g dry biomass/ mol thiosulfate for the soda-grown culture. The protein content of the biomass was 44 ± 1%. The total organic carbon of strain HL 17^T originated from both cultures had an average value of 37% of dry biomass, a value confirmed by elemental analysis. The maintenance coefficient (m_s) of the strain HL 17^T grown at 4 M NaCl was 2.6 mmol thiosulfate/ g protein h^{-1}, while the m_s of the soda-grown culture was two times higher (5.3 mmol thiosulfate/ g protein h^{-1}).

Substrate affinity and oxidation of inorganic sulfur compounds

The determination of the apparent affinity constant (K_s) for thiosulfate in the strain HL 17^T grown in 4 M NaCl gave a value of 1.6±0.6 µM. This value was two times lower than the value found in *Tv. versutus* strain ALJ 15 at the same Na^+ concentration (H. Banciu, unpublished results). The biological oxygen measurement in washed cells of the strain HL 17^T grown at 4 M Na^+, in chloride or soda buffers, revealed that thiosulfate, sulfide and polysulfide are oxidized at much higher rates than elemental sulfur (**Table 2.**). Washed cells of strain HL 17^T were also able to oxidize tetrathionate at low rates (data not shown). The oxidation rates of inorganic sulfur compounds in cell suspension of both cultures were in all cases higher in 4 M Na^+ soda buffer than in 4 M Na^+ chloride buffer, at pH 9.6. The elemental sulfur oxidation was exceptionally stimulated (9 to 10 times) in soda buffer compared to NaCl buffer at pH 9.6.

Table 2. Maximum specific oxygen uptake rates ($qO_{2\ max}$) of washed cells of strain $HL17^T$ grown in continuous culture at 4 M Na^+ and pH 9.6

Substrate	$qO_{2\ max}$, (mmoles O_2/g of protein h^{-1})				
	Strain $HL17^T$, chloride culture		Strain $HL17^T$, soda culture		*Tv. versutus* ALJ 15 soda culture
	4 M NaCl	4 M Soda buffer	4 M NaCl	4 M Soda buffer	4 M Soda buffer
$S_2O_3^{2-}$	13.8	17.4	10.2	15.1	15
HS^-	20.4	24.6	7.2	13.8	13.2
$^-S(S)_6S^-$ (polysulfide)	10.8	19.8	9.6	15	12
S^0	0.6	5.76	0.6	5.16	1.08

In order to determine the minimum salt requirement for respiration, the washed cells from chloride and soda cultures were suspended in buffers containing different types and concentrations of salts. The soda-grown cells started to respire at a low rate at 0.75 M KCl in the absence of Na^+ (0.75 M KCl + TrisHCl buffer, pH 9.6), while chloride culture was incapable of respiration under such conditions. Addition of small amounts (25 or 50 mM) of NaCl to KCl buffer stimulated the respiration of soda-grown cells by a factor of 2 and 3, respectively. The minimal Na^+ requirement for respiration in chloride-grown cells was 0.4 M Na^+ (in 0.4 M NaCl + 0.35 M KCl, TrisHCl buffer, pH 9.6). The results show that when

Cl^- ions are in excess, a minimal amount of Na^+ (25 – 50 mM) for soda-grown culture and 0.4 M for chloride-grown cells) is necessary for respiration process. Soda grown cells were also more responsive to the presence of Na_2SO_4. They started to respire in the absence of chloride at 0.2 M Na^+ (0.1 M Na_2SO_4 + Tris/Hepes, pH 9.6) while the chloride-grown cells started to respire only at a much higher Na^+ concentration (1 M Na^+ and 0.5 M SO_4^{2-}, pH 9.6). When soda-grown cells were incubated at 0.4 M Na^+ + 0.2 M SO_4^{2-}, the addition of 0.1 M KCl or 0.1 M NaCl stimulated respiration by 50% and 90% respectively compared with the Cl^- -free buffer. Overall it was noticed that the respiration at high pH required higher Na^+ and Cl^- concentrations in chloride-grown cells than in soda-grown cells. At excess of Na^+ ions, minimal amounts of Cl^- ions were necessary for stimulation of respiration. K^+ and SO_4^{2-} as cations and anions can create a favorable ionic environment but this is not sufficient for achieving the maximum respiration rate. The difference in ion requirement for soda- and chloride-grown culture may be explained by activation of specific metabolic mechanisms that required a certain concentration of sodium or chloride. The carbonate/bicarbonate anions are not required for respiration but their addition clearly stimulated the process.

Pigment and protein composition of cell-free extract

The spectra of the methanol extracts suggested that soda-grown cells of strain HL 17^T produced a high amount of a yet-unknown yellow pigment with absorption maxima at 426 nm (main) and 457 nm. The chloride-grown culture probably also produced pigments, but the quantity was so low that it was impossible to obtain a reasonable spectrum. The sonication of washed cell suspension revealed translucent colored supernatants. The chloride-grown cell free extract had a dark reddish appearance while the soda-grown cell free extract was bright yellow. The absorption spectra of cell-free extracts prepared from chloride-grown cells indicated the presence of c-type cytochromes in high concentration (data not shown).

The analysis of total protein from the cell-free extract by 12 % SDS-PAGE and 8-25% gradient SDS-PAGE revealed several bands which were overexpressed in one or another culture (**Fig 4**). A further two-dimensional gel electrophoresis would be necessary to shed light upon the fine differences in the total protein profile from both cultures.

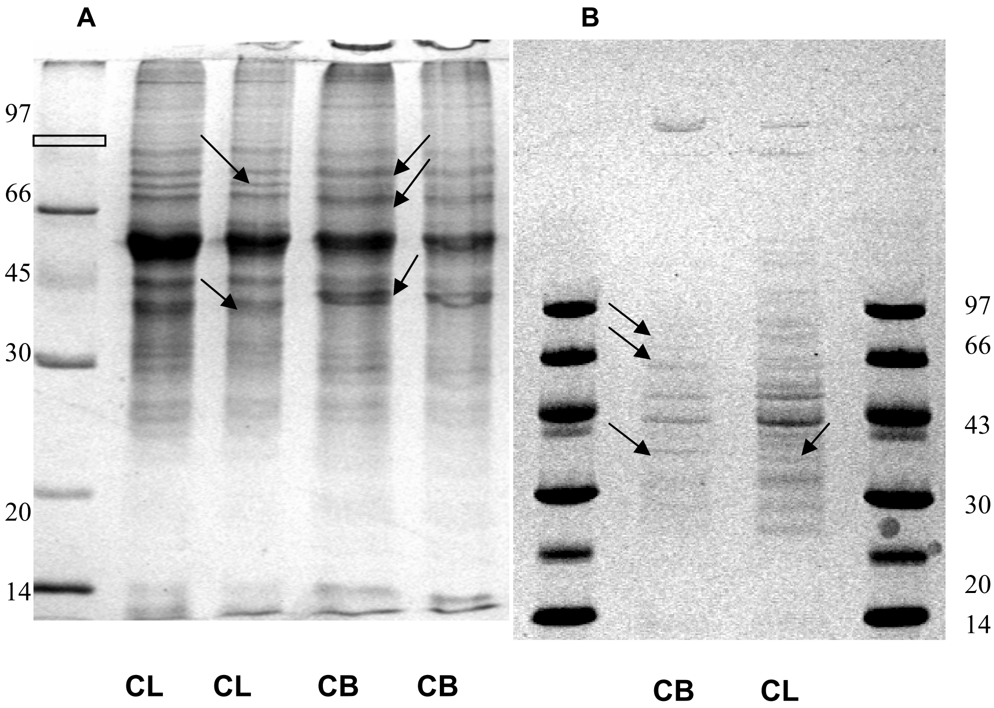

Figure 4. Total protein analysis of crude cell extract using (A) 12% SDS-PAGE and (B) 8-25% gradient SDS gel. The arrows indicate the bands which appear to be over-expressed in one or another culture. Symbols: CL chloride-grown culture, CB soda (carbonate)-grown culture.

Osmotic pressure determination and compatible solutes production

The direct measurement of osmotic pressure in the growth media containing NaCl or $Na_2CO_3/NaHCO_3$ revealed that in the 4 M NaCl medium the value (9.3 osm/kg) is much higher than in 4 M soda medium (5 osm/kg).

The lyophilized biomass of the NaCl and soda cultures was analyzed for compatible solutes composition. Glycine betaine was found in appreciable amounts in both cultures. This organic compatible solute has also been detected in other *Thioalkalivibrio* species (Banciu et al., 2004) and in *Halorhodospira* sp. (Galinski and Trüper, 1982), the closest relative of members of the *Thioalkalivibrio* genus. The NaCl-grown biomass of strain HL 17^T contained 19.8% (w/w) glycine betaine, while soda-grown biomass contained 12.4 % glycine betaine.

Discussion

Strain HL 17^T was isolated from the hypersaline lake that can be regarded as an intermediate between saline and soda lakes, more saline though, because the ratio of carbonates/Cl^- is very low. The carbonate concentration in such lakes is sufficient to maintain a constant alkaline pH around 9. These chemical characteristics seem to favor a domination of facultative haloalkaliphiles rather than neutrophilic halophiles thriving in hypersaline lakes with pH≤8.5 (D.Y. Sorokin, unpublished results). On the other hand, obligately haloalkaliphilic species of sulfur bacteria dominate only in those saline lakes which have permanently higher alkalinity and pH>9.5.

It is known that the production of organic compatible solutes during growth at high NaCl concentrations is energetically more expensive than the uptake of inorganic osmolytes (e.g. K^+) (Oren, 1999). Most of the halophilic organisms are able to tolerate significant variations of NaCl concentration and adopt the strategy of organic compatible solutes. The chemolithoautotrophic *Thioalkalivibrio* species, many of them being extremely salt tolerant organisms, accumulate the organic osmolyte glycine betaine (Banciu et al., 2004; see **Chapter 4**). It is also important to mention that compatible solutes have not yet been analyzed in any known haloalkaliphilic chemolithotrophic bacteria. The actual data mostly concern the bacterial adaptation to high NaCl concentration, while the strategy of adaptation to high soda (Na_2CO_3/$NaHCO_3$) concentration has never been considered. The ideal model organism for studying the difference in the energetics of NaCl versus Na_2CO_3/$NaHCO_3$ adaptation must be capable of growing at high concentrations of both salts. Strain HL 17^T proved to be a good candidate for this experiment. Grown at different sodium salt compositions, strain HL 17^T revealed an important difference in the organic compatible solutes production. Our experimental data with strain HL 17^T showed that growth at high NaCl concentration is optimal for this bacterium. In the NaCl-free carbonate medium the bacterium did not grow, suggesting that the organism required a minimum Cl^- concentration for growth, while carbonate is needed as the carbon source. A lower biomass yield found in soda-grown culture might indicate a less efficient coupling of substrate oxidation with the anabolic system as compared to NaCl environment. As NaCl medium proved to be optimal for the growth of strain HL 17^T, the high concentration of sodium carbonates may represent stressful condition.

The higher pigment production in soda cultures along with changes in protein expression is other indicatives for molecular changes in the soda-grown cells in comparison with the NaCl culture. The physiological function of the membrane-bound (yellow) pigments, which have recently been discovered in several haloalkaliphilic and halophilic sulfur-oxidizing bacteria (Sorokin, unpublished results), is not yet fully understood. Beside their possible roles in the oxidative (Miller et al., 1996) and UV radiation stress (Becker-Hapak et al., 1997) it is believed that they might contribute to the regulation of membrane fluidity or rigidity by replacing the cholesterol in the eukaryotes as membrane spanning agents (Chattopadhyay et al., 1997; Subczynski et al., 1992). Further detailed studies of the pigments and the total protein composition are necessary to reveal the nature of molecules that are associated with the changes in the salt composition in facultative haloalkaliphiles.

In order to explain the differences in the compatible solutes production one must look at the physico-chemical differences between NaCl and $Na_2CO_3/NaHCO_3$ media (**Table 3**). A decisive parameter that influences the osmolytes production could be the osmotic pressure of the solution. The experimentally determined osmotic pressure in the growth media with 4 M NaCl was 1.8 times higher than that of 4 M soda. The theoretical calculation of osmotic pressure used the relation between osmotic pressure (Π) and the osmotic coefficient (ϕ) (van't Hoff law):

$$\Pi = \phi \bullet (\nu m R T) \quad (1)$$

where ν is the number of ions from one molecule of salt ($\nu = 2$ for NaCl and $\nu = 3$ for Na_2CO_3); m the moles solutes per 1000 g solvent (molality); R is the gas constant (8.3143 J/ mol·K); T is the absolute temperature (K).

This equation was used to calculate the osmotic pressures of aqueous solutions of 4 M NaCl and 2 M Na_2CO_3 at 25^oC (**Table 3**). The values of the vapour pressures (p) and the water activities (a_1) can be used for calculation of the osmotic coefficients (ϕ).

Both measured and calculated values of osmotic pressure indicated a significant difference between aqueous solutions of NaCl and Na_2CO_3 with a Na^+ concentration of 4 M.

Based on these observations we consider that the higher osmotic pressure in the 4 M NaCl brine as compared to that of soda brine might be, at least partly, responsible for a higher glycine betaine content of chloride-grown cells.

Our results showed that, unlike other *Thioalkalivibrio* species that are soda-loving organisms, strain HL 17^T is optimally growing at high concentrations of NaCl being also able to withstand high concentrations of soda. Another exceptional feature of strain HL 17^T is its facultative alkaliphily. Having these qualities, strain HL 17^T would outcompete the other known obligate alkaliphilic *Thioalkalivibrio* species at a pH below 9.5 and at more than 1 M of NaCl. Together with the special physiological features, the genetic data indicate that strain HL 17^T is a new species, *Thioalkalivibrio halophilus* - the first halophilic facultative alkaliphilic chemolithoautotroph able to grow in NaCl and $Na_2CO_3/NaHCO_3$ brines.

Table 3. Physical properties of aqueous solutions of NaCl and Na_2CO_3 with 4 M Na^+ at 25° C (298° K).

Parameter	4 M NaCl	2 M Na_2CO_3	Reference
Actual molality (m) (moles/ kg)	4.58	2.36	-
Solubility (moles/ kg)	6.15	2.75	[c, d]
Density (ρ) (kg/ l)	1.145	1.182	[e]
Absolute viscosity (η) (cP)	1.34	2.40	[e]
Ionic strength (I) [a]	4	6	-
Vapour pressure of saturated aqueous solution (p) (kPa)	2.401	2.933	[c, d]
Water activity of saturated aqueous solution (a_1)	0.758	0.926	[c, d]
Electrical conductivity (mS/ cm)	175	95	[f]
Osmotic coefficient (ϕ)	1.157	0.521	[c, d, e]
Calculated osmotic pressure (Π) (osmole/ kg)[b]	11.6	4	-
Measured osmotic pressure (osmole/ kg)	9.3	5	[f]

[a] Calculated from equation : $I = \frac{1}{2} \Sigma(c_i \cdot z_i^2)$; where c_i is the concentration of the ion i and z_i is the valence of the ion i; it applies in the case of a complete dissociation;

[b] Calculated with the equation (1) considering 4 M NaCl or Na_2CO_3 solutions respectively

[c] Apelblat and Korin (1998)

[d] Apelblat and Manzurola (2003)

[e] Lobo and Quaresma (1989)

[f] Our determination

Description of *Thioalkalivibrio halophilus* sp.nov.

Thioalkalivibrio halophilus (ha.lo'phi.lus; Gr. adj. *halo*s, salt; Gr. adj. *philus*, loving; N.L. masc. adj. *halophilus*, salt-loving).

Gram-negative non-spore-forming, aerobic, obligately chemolithoautotrophic, halophilic and facultatively alkaliphilic bacterium. Cells are, 0.3-0.4 x 1-2 µm, motile by a single polar flagellum. At low salinity often produces extracellular sulfur from thiosulfate. Produces yellow, membrane-associated pigment during growth at high salinity, with a main absorption maximum at 426 and a minor one at 457 nm in the methanol extract. Requires NaCl for growth and tolerates up to 5 M Na^+ with an optimum at 2 M. The pH range for growth is 7.5-9.8 with an optimum between 8.0 and 9.0. Oxidizes sulfide, thiosulfate, polysulfide, sulfur and tetrathionate to sulfate. The G+C content in DNA is 65.1 ± 0.5 mol% (*T*m). Other properties are the same as for the genus.

The type strain is HL 17^T (DSM15791, UNIQEM225). Isolated from the surface sediments of alkaline hypersaline Stamp Lake (Kulunda steppe, Altai, Russia). The GenBank accession number of the 16S rDNA sequence is AY346464.

Emended description of genus *Thioalkalivibrio*

The genus *Thioalkalivibrio* (Sorokin *et al*., 2001) includes obligately and facultatively alkaliphilic strains. Most of the strains grow optimally in soda-rich media. Some strains have a chloride-dependent growth and can grow up to saturating concentrations of NaCl.

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Membrane properties and compatible solute composition of obligately chemolithoautotrophic alkaliphilic sulfur-oxidizing bacteria from soda lakes

(Manuscript in preparation)

Abstract

Salt adaptation in the chemolithoautotrophic alkaliphilic sulfur-oxidizing strains belonging to genera *Thioalkalimicrobium* and *Thioalkalivibrio* have been studied by the determination of salt-dependent changes in fatty acid and compatible solute composition. An estimation of the membrane surface potential was performed by using a lipophilic coumarin dye. In both alkaliphilic groups, represented by *Thioalkalimicrobium aeropilum* strain AL 3^T and *Thioalkalivibrio versutus* strain ALJ 15, unsaturated fatty acids predominate over the saturated ones. In low-salt tolerant strain AL 3^T, C18:1, C16:0 and C16:1 were the dominant fatty acids. In the extremely salt tolerant strain ALJ 15, the concentrations of C18:1 and cyc-C19 are salt-regulated in an inverse proportional relationship. This may suggest that the increase of the Na^+ concentration could stimulate the activity of a cyclopropyl-synthetase. Squalene, an apolar lipid, has been found in substantial amounts only in the strain ALJ 15. The analysis of the compatible solutes revealed that in both *Thioalkalimicrobium aerophilum* strain AL 3^T and *Thioalkalivibrio versutus* strain ALJ 15, ectoine and glycine betaine, respectively, are the main osmolytes. The production of ectoine and glycine betaine was dependent on salt concentration in the growth medium. The utilization of the fluorescent coumarin probe showed that at same salt concentration, in both alkaliphilic strains the estimated membrane surface potential is more negative than in the neutrophilic *E. coli*. The results suggest that the salt and alkaline pH adaptation of chemolithoautrotrophic sulfur-oxidizing bacteria from soda lakes is achieved, at least partially, by a highly negative charge of the membrane surface, by the presence of apolar lipids and by the production of compatible solutes.

Introduction

Soda lakes represent extreme environments characterized by moderate to high concentration of salts (as $Na_2CO_3/NaHCO_3$) and alkaline pH (9-10.5). The abundance of sodium carbonates leads to a high buffering capacity in such environments and, therefore, a permanent alkalinity occurs (Grant and Tindall, 1986). The organisms living in soda lakes are in general obligately haloalkaliphilic, requiring the presence of Na^+ and at least pH 9 for growth. Their diversity and biology have been extensively reviewed in the past (Baumgarte, 2003; Duckworth et al., 1996; Grant et al., 1990; Oren, 2002). Also, the physiology, biochemistry and molecular mechanisms that allow the adaptation of halophiles at high salt and of alkaliphiles at high pH are well documented (Krulwich et al., 1996; Oren, 1999; Ventosa et al., 1998). However, there are still questions concerning the adaptative features of double extremophiles such as the haloalkaliphiles.

Obligately chemolithoautotrophic sulfur-oxidizing bacteria that live in soda lakes have been recently discovered. They have been classified into three genera belonging to the γ-Proteobacteria. *Thioalkalimicrobium* spp., *Thioalkalivibrio* spp. and *Thioalkalispira* spp. are all capable of autotrophic growth at moderate to high salt concentration and at alkaline pH, utilizing inorganic sulfur compounds as electron donors and energy source (Sorokin et al., 2001, 2002). The genetic and metabolic diversity, ecology and growth physiology of representatives of *Thioalkalimicrobium* and *Thioalkalivibrio* have been studied and important differences have been found. Species of *Thioalkalimicrobium* were mostly isolated from low salt soda lakes in Eastern Africa and Siberia. *Thioalkalivibrio* group is represented by moderate to high salt tolerants and extreme halophiles isolated from moderate to high salt soda lakes in Eastern Africa, Central Asia and North America. The genetic and phenotypic diversity of this group is much higher than that of *Thioalkalimicrobium*. Yet, little is known about the mechanisms of salt adaptation in these sulfur-oxidizing bacteria.

Fundamental properties of the cell such as membrane transport, energy transduction, cell-cell interactions and cellular signalling, depend on the electriochemical state of the membrane. In general the biological membranes have a net negative charge favoring the adsorption of protons and cations at their surface. The electric potential at the membrane surface (the surface potential, Ψ_0) is determined by the biochemical nature of the lipid bilayer (electric charges carried by phospholipids and membrane proteins) and by the chemical association (binding and absorption) of different ions with the membrane surface. The salt concentration of the suspending medium also influences the surface potential.

The Gouy–Chapman theory states the existence of a diffuse electric double layer at the surface of a charged membrane. The relationship between the electrical potential at the surface (Ψ_0), the surface charge density (σ_{GC}) and the ion concentration of the bulk environment (c) is expressed by the Gouy-Chapman equation: $\Psi_0 = \frac{2kT}{ze}\sinh^{-1}(\frac{\sigma_{GC}}{\sqrt{8\varepsilon_0\varepsilon kTc}})$ *(1)* , where k is Boltzmann constant, T the absolute temperature, e the electronic charge, ε the aqueous dielectric constant, ε_0 the permittivity of free space and z is the ion valence.

Several methods have been used for the measurement of the membrane surface potential: (1) electrophoretic mobility of liposomes, cells and organelles; (2) ^{31}P-NMR and electronic spin resonance (ESP) techniques; (3) measurements of membrane conductance caused by small amounts of ionic and zwitterionic surfactants (Cafiso et al., 1989); (4) the use of phosphorescence and fluorescence probes reviewed by Rottenberg (1989). A resonance Raman dye probe was applied to study the potential of the purple membrane surface in *Halobacterium salinarum* (Ehrenberg and Berezin, 1984). This method has the advantage that the charged styryl dye molecules aggregate at the surfaces of the membrane and it can therefore be used to probe separately each side of the membrane. Experimentally, the estimate of the surface membrane potential can be performed with the help of probes of molecular size that embedded in the membrane would not disturb the system itself.

As fluorescent probes, the positively charged acridines have been used to estimate the surface potential in chloroplasts (Chow and Barber, 1980). 2-(p-toluidinyl)naphthalene 6-sulfonate (TNS) and its analogue, 1-anilinonaphtalene 8-sulfonate (ANS) are anionic fluorescent molecules considered as a good probes for estimation of the surface potential at the membrane-solution interface (Cafiso et al., 1989; Rottenberg, 1989). They were applied successfully in the artificial phospholipid membranes, but many complications have to be overcome when used in the biological membranes (e.g. mitochondrial membranes).

Finally, surface pH indicators such as 4-hepdadecyl-7-hydroxycoumarin (HDHC) have been used because the surface charge would change the concentration of protons near the surface. The pH indicator that is located at or near the surface can report these changes. Such indicators were used to estimate the surface potential in liposomes (Fernandez, 1981; Fromherz, 1983) and stomatitis virus (Pal et al., 1983). HDHC is a weak acid, a phenolic derivative with a double ring, which bears a long alkyl chain that can intercalate into lipid bilayers (**Fig. 1**). The polar head of the dye, with the hydroxyl (–OH) group, is located close to the head group of phospholipids (e.g. phosphate group). This means that the binding site of protons is directly influenced by the electric charge of the immediate surroundings (Fromherz, 1989). The probe is deprotonated in water with a pK=7.75. In its acidic form, at an emission wavelength of 450 nm, it shows a fluorescence excitation peak at 330 nm, while the basic form shows a peak at around 370 nm. The fluorescence emission of both species is maximal at 450 nm (**Fig. 2**).

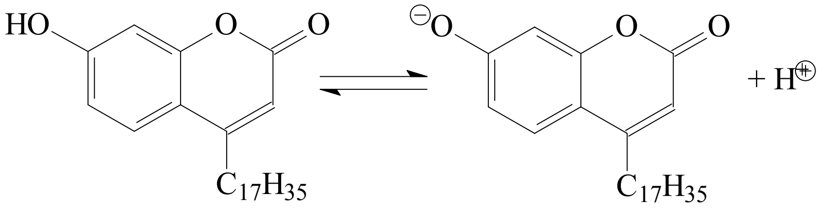


Figure 1 Chemical formula of HDHC and transition between protonated-deprotonated forms of the probe (after Fromherz, 1973).

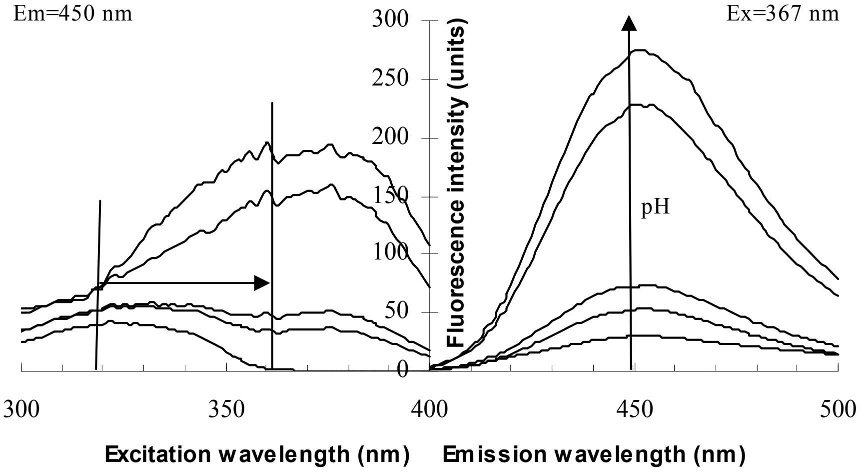


Figure 2 Fluorescence excitation and emission spectra of the lipophilic probe HDHC during the pH titration. The probe (1 μM) was solubilized in 0.1 mM Triton X-100 in a weakly buffered 5 mM potassium phosphate solution (pH 7). The fluorescence spectra at pH 5, 7, 8, 9.5 and 10.5 were recorded at the excitation and emission wavelengths indicated. The Ex and Em slits width were set at 2.5 nm.

The excitation spectrum reflects the acid-base equilibrium of the ground state. By exciting the base near 370 nm the fluorescence intensity reflects the dissociation degree

The pK of the probe corresponds to a dissociation degree of 0.5 (50%), when the acid and base concentrations are equal. The surface potential of a membrane is pH-dependent if interfacial acids and bases are present. If their pK is in the range of the pK of the probe, the titration curve of the dye may also reflect the pK of the acid-base transition state at the membrane surface level.

Fromherz (1989) developed an equation from which the surface potential (Ψ_0) can be calculated based on the pK of the charged dye (pK_{ch}) versus pK (pK_0) of uncharged dye: $pK_{ch} = pK_0 - \frac{\Psi_0 F}{2.3RT}$ from which it results $\Psi_0 = -2.3\frac{RT}{F}(pK_{ch} - pK_0)$ *(2)*

It was found that in a series of neutral systems (uncharged micelles like Triton X-100 or uncharged phospholipids like egg lecithin or phosphatydylcholine), the pK_0 of HDHC is rather invariant (8.6-9.0). The solubilization of the dye in charged micelles shifts the pK to a higher value (e.g. in negatively charged micelles of SDS) or lower values (e.g. positively charged micelles of CTAB). A consensus value for pK_0 is 8.8 (Fromherz, 1989).

The probe HDHC has been used for determination of surface potential in liposomes as well as in biological membranes like vesicular stomatitis virus (Pal et al., 1983), mycoplasmas (Schummer and Schiefer, 1984) and spiroplasmas (Schummer and Schiefer, 1988). These are in general easily accessible membranes, devoid of rigid cell walls.

Starting from the assumption that HDHC is a small molecule and a strong lipophilic dye, we have tried to measure the pK_{ch} of the dye bound to the Gram-negative membranes, in neutrophilic and alkaliphilic bacteria. By measuring the pK_0 of the dye in the presence of Triton X-100 micelles in the same conditions used for determination of pK_{ch} (0.5 M Na^+) and replacing the experimental values in the equation *(2)*, we calculated Ψ_0. An estimation of the accessible fraction of the fluorophore is made using a fluorescent quencher for HDHC.

The aim of this study was to reveal the biochemical-biophysical basis that contributes to the salt and high pH adaptation in the two representatives of alkaliphilic sulfur-oxidizing bacteria from soda lakes: the extremely salt-tolerant *Thioalkalivibrio versutus* strain ALJ 15 and the low-salt tolerant *Thioalkalimicrobium aerophilum* strain AL 3. These two alkaliphilic organisms, representing two distinctive ecological groups, have been found to differ biochemically and physiologically in spite of their similar nutrition and adaptation to high salt concentration and high pH (Sorokin et al., 2001). The results of the membrane lipid composition, of the estimated surface potential and the ways chosen for osmoregulation were compared with the data in the literature. Moreover, the search for molecular markers that could help for *in situ* detection of these organisms was another scope of this research.

Material and methods

Strains and growth conditions

Two models of autotrophic, alkaliphilic sulfur-oxidizing strains: the low-salt tolerant *Thioalkalimicrobium aerophilum* strain AL 3^T and the extremely-salt tolerant *Thioalkalivibrio versutus* strain ALJ 15 have been used in this experiment.

Thioalkalimicrobium aerophilum strain AL 3^T, isolated from a soda lake in Siberia (Russia), is a mesophilic, low-salt tolerant and alkaliphilic strain that grows optimally under aerobic conditions, in the presence of 0.6 M Na^+ and at pH 10. For lipid and compatible solute analysis as well as for the surface potential estimation, strain AL 3^T was grown in batch culture on alkaline mineral medium and thiosulfate as energy source. The mineral medium (0.6 M Na^+) contained: 20 g/l Na_2CO_3, 6 g/l NaCl, 1 g/l K_2HPO_4, 1 g/l KNO_3 and 8 g/l $NaHCO_3$. Separately sterilized 40 mM thiosulfate, 1 mM $MgCl_2 \cdot 6H_2O$ and 1 ml/l of trace elements solution (Pfennig and Lippert, 1966) were added to the alkaline medium. The final pH was 10. For the growth at 0.2 and 1.2 M Na^+, the medium contained less or more Na_2CO_3 and $NaHCO_3$.

The extremely salt-tolerant and alkaliphilic *Thioalkalivibrio versutus* strain ALJ 15 was chosen because of its ability to grow over a wide range of sodium concentration, at alkaline pH. It was isolated from a soda lake in Kenya and it grew optimally at 1-2 M Na^+ and at pH 10. For the analysis of fatty acid composition, strain ALJ 15 was grown in batch culture on mineral medium, at 0.6, 2 and 4 M Na^+, pH 10, with thiosulfate as energy source. For the estimation of membrane surface potential, strain ALJ 15 was grown on 0.6 M Na^+ medium. Basically the growth medium for strain ALJ 15 had the same composition as presented for strain AL 3^T except the Na_2CO_3 and $NaHCO_3$ concentrations, which were varied to give final concentrations of 0.6, 2 and 4 M Na^+. Both strains were cultivated aerobically, on a rotary shaker at 30^0C (strain AL3^T) and at 35^0C (strain ALJ 15).

For determination of salt-dependent production of compatible solutes in strain ALJ 15, the substrate-limited continuous cultivation was used. Continuous cultivation was performed in 1.5 l laboratory fermentors with a 1 l working volume, fitted with pH and oxygen controls (Applikon, Schiedam, The Netherlands). The pH was controlled by automatic titration with 2 M NaOH and HCl. The dissolved oxygen concentration was controlled at a minimum level of 50% air saturation by the stirring speed. The temperature was set at 35^oC. The mineral media had identical composition with those used in batch cultivation except for the thiosulfate concentration, which was the limiting nutrient.

The growth of haloalkaliphilic strains was followed by thiosulfate consumption in the media. Micromolar thiosulfate concentrations were determined by cyanolytic procedures (Kelly et al. 1969). Millimolar-range thiosulfate consumption in batch cultures was followed by standard iodimetric titration after neutralization of the medium with 50% (v/v) acetic acid.

Escherichia coli was grown in batch culture, on LB medium, on a rotary shaker, at 37°C and pH 7.5.

Lipid analysis

For lipid analysis as well as for compatible solutes determination, cells grown under different sodium concentration, were harvested at 10,000xg for 20 min. Biomass was washed twice in isoosmotic saline solutions (e.g. 0.6, 2 and 3 M NaCl) to avoid cell lysis. The pellet was frozen overnight at -80^0C and freeze-dried. Lipids were ultrasonically extracted from the freeze-dried cell material using methanol, methanol/dichloromethane (1:1, v/v) and dichloromethane (x3). An aliquot of the total lipid extract, named as free lipids, was methylated with diazomethane in diethyl ether and filtered over a small SiO_2 column with ethyl acetate as the eluent. Another aliquot of the total lipid extract (free+bound lipids) was subjected to base hydrolysis (1 M KOH in 90% methanol under reflux for 1 h) and was subsequently processed in the same way. Alcohols in the ethyl acetate eluate of both fractions were converted to their trimethylsilyl ether derivatives with bis(trimethylsilyl)trifluoracetamide (BSTFA) in pyridine. Individual fatty acids methyl esters and trimethylsilyl derivatives of alcohols were quantified by gas chromatography (GC) and identified by GC-mass spectrometry (MS) as described in detail elsewhere (Schouten et al., 2000).

The analysis of organic and inorganic compatible solutes

Determination of potassium and chloride ions was performed by INAA (Instrumental Neutron Activation Analysis) at Department of Chemistry, TU Delft. The "Hoger Onderwijs Reactor" was used as source for neutrons. The gamma spectrometer used a germanium semiconductor as detector and a computer controlled sample changer.

Intracellular compatible solutes were extracted and analyzed following a modification of the methods described by Galinski and Herzog (1990). HPLC separation used an isocratic system from Thermo Separation Products (CA), a 3 µm Grom-sil Amino-1PR column (Grom Analytik, Rottenburg-Hailfingen, Germany) and a Shodex refractive index detector (model RI17, Showa Denko KK, Tokyo, Japan). The mobile phase consisted of 80% (v/v) acetonitrile at a flow rate of 1 ml min^{-1}. Natural abundance ^{13}C-NMR spectra of compatible solutes were recorded in the pulsed Fourier transform mode on a Bruker spectrometer (model Avance 3000 DPX) operating at 75.48 MHz (^{13}C) and at 300 MHz for the proton decoupling channel relative to sodium trimethylsilylpropionate (TMSP).

Loading of the fluorescent probe

After complete substrate consumption, cells of alkaliphilic strains were harvested and washed twice with 0.6 M NaCl. They were re-suspended in 0.4 M NaCl + 40 mM Na_2CO_3 + 20 mM $NaHCO_3$, pH 10. Cells were checked for activity by measuring the respiration rate and for integrity under microscope. Cells of *E. coli* were washed twice in 10 mM NaCl + 50 mM phosphate buffer, pH 7.5. The cells were re-suspended in the same buffer and checked for integrity and respiratory activity.

The probe (4-heptadecyl-7-hydroxycoumarin, Fluka) was dissolved in 96% ethanol at a concentration of 5 mM and stored at 4°C. The loading of the probe onto the cells was performed as follows: cell suspensions of haloalkaliphilic strains were diluted to 1 mg of protein/ ml with 0.5 M Na^+ buffer (pH 10). The ethanolic solution of the probe was added to a final concentration of 100 μM (0.1 μmoles dye/ mg protein). Cells were incubated for 0.5-2 hours on a rotary shaker at 30°C. The cell suspension of *E. coli* was diluted in 0.5 M NaCl + 20 mM Tris-HCl buffer, pH 8. Under the microscope, no major damage of *E. coli* cells was observed under these slightly hypertonic conditions. The dye concentration and incubation conditions were the same as for haloalkaliphilic strains. After incubation, cells were washed thoroughly (three times) with the same buffer. The supernatants of the first and second wash were saved for the estimation of unbound dye.

After incubation, cells containing the fluorescent dye were checked for respiratory activity and integrity. The addition of the probe to the cell suspension did not lead to damage of the cells and did not affect their respiratory activity. As control, cells of all studied strains were incubated without probe.

Titration of the probe.

Washed cells, with or without bound probe, were resuspended at a concentration of 0.1 mg protein/ ml in 0.5 M NaCl + 5 mM phosphate buffer (KPi), pH 7. Titration consisted in the addition of small volumes of concentrated solutions (2-4 M) of KOH or HCl to the weakly buffered medium.

Negatively charged and neutral micelles were prepared in a weakly buffered solution, from sodium dodecylsulfate and Triton X-100, respectively. When the probe was titrated in the presence of uncharged micelles of Triton X-100, a molar ratio of 1/100 or 1/200 of dye to detergent was used. The solution consisted of 0.1 mM Triton X-100 (Serva) + 5 mM phosphate buffer, pH 7. The addition of NaCl to Triton X-100 buffer did not affect significantly the pK values of the uncharged dye. The pH was measured with a glass combined minielectrode (Ingold-Mettler Toledo). Permanent attention was paid to the calibration of the electrode. However, a deviation of ±0.02 units of pH was considered acceptable.

Fluorescence measurements

Determination of fluorescence intensity was performed with a Varian/Cary Eclipse spectrofluorimeter, at room temperature (20^{o}C). The probe was excited at 367 nm (Ex) and fluorescence intensity (*I*) was recorded at emission wavelength (Em) of 450 nm. The final values were corrected for the background fluorescence. The chemical control consisting of cell-free buffer with NaCl, KOH or HCl gave negligible fluorescence. When the fluorescence signal was very strong, the sensitivity of the apparatus (the slit width) was lowered manually. Unless otherwise specified, the measurements were performed with the excitation and emission slits set at 10 nm and 5 nm width respectively.

The estimation of accessible probe

Theoretically the fluorescent probe HDHC is equally distributed on the inner and outer sides of the membrane. A chance exists that the dye would bind unspecifically. This ratio is influenced by the asymmetry of the cell membrane. The utilization of an impermeable compound that is capable of quenching the fluorescence of the dye would indicate the fraction of fluorophore which is located on the outer surface of the membrane. DPX (p-xylene-bis-pyridinium bromide, Sigma) was used as an impermeant quencher. DPX was dissolved in MilliQ water at a final concentration of 1 M. When used, the quencher was added to the reaction at variable concentrations in such a way that the added volume would be minimal. The cells preincubated with the fluorescent probe were suspended at a final concentration of 0.1 mg/ml in a strongly buffered solution containing 0.5 M Na^+ and pH 10.8. At this pH value, the fluorescence intensity is maximal. The cells were titrated with DPX up to 14 mM and the fluorescence quenching at emission wavelength of 450 nm was recorded. No toxic or damaging effect of the quencher was observed at the highest concentration used. The valinomycin and nigericin stocks were prepared as ethanolic solutions at 1 mM final concentration.

Results

Lipid and fatty acid composition

The results of fatty acid analysis in *Thioalkalimicrobium aerophilum* strain AL 3^T grown at 0.6 M Na^+ (pH 10) and in *Thioalkalivibrio versutus* strain ALJ 15 grown at different sodium concentration (pH 10) are presented in **Tables 1** and **2**. From these results several differences between the two strains (both grown at 0.6 M Na^+ and pH 10) could be outlined. In strain AL 3^T the concentration of monounsaturated fatty acids, and in particular C18:1 (80.4%), is higher than in strain ALJ 15 (49.5%). A particular monounsaturated branched fatty acid C19 named “C19:1” was detected in strain AL 3^T but not in strain ALJ 15.

Table 1. Fatty acid composition in *Thioalkalimicrobium aerophilum* strain AL 3^T grown at optimal salt concentration (0.6 M Na^+) and at pH 10.

Fatty acid	% free	% free+bound
C16:1	5.5	4.1
C16:0	16.9	12.7
C18:1	74.2	80.4
C18:0	1.7	1.3
"C19:1"	1.7	1.4

Table 2. Fatty acid composition in *Thioalkalivibrio versutus* strain ALJ 15 grown at different salt concentration and at pH 10.

Fatty acid	% free			% free+bound		
	Na^+			Na^+		
	0.6 M	2 M	4 M	0.6 M	2 M	4 M
C16:1	-	-		0.4	0.4	0.5
C16:0	20.3	16.2	18.0	22.2	17.2	17.0
C17:0	-	-		0.7	0.7	0.6
C18:1	65.2	64.4	51.9	49.5	50.9	45.4
C18:0	5.8	6.0	5.8	6.7	6.4	6.1
Cyc-C19	8.8	13.5	24.3	19.7	24.1	29.9
C20:1	-	-	-	0.5	0.2	0.3
C20:0	-	-	-	0.2	-	-

In *Thioalkalivibrio versutus* ALJ 15, the dominant fatty acids are saturated C16:0, the monounsaturated C18:1 and the saturated cyclopropyl-C19 (cyc-C19). The salt dependence of fatty acid distribution in strain ALJ 15 was best observed in the change of C18:1 and cyc-C19. The proportion of the C18:1 fatty acid remained relatively unchanged in cells grown at near-optimal salt concentration (0.6-2 M Na^+) but was 10% lower at 4 M. At the same time cyc-C19 showed an increase of approx. 20% from 0.6 to 2 M and approx. 50% from 0.6 to 4 M Na^+. These changes were even more pronounced with the relative proportion of free fatty acids (80% and 300% increase, respectively).

Overall, in both sulfur-oxidizing strains the total concentration of unsaturated fatty acids is higher than that of the saturated fatty acids.

Squalene, a nonpolar lipid (**Fig. 3**), was found in high amounts in cells of strain ALJ 15 grown over the whole range of Na^+ concentration but could not be related with the salt adaptation. The relative squalene concentrations (% of squalene related to sum of squalene and all quantified fatty acids) in *Thioalkalivibrio versutus* strain ALJ 15 were: 41% (in 0.6 M Na^+-grown cells) 52% (at 2 M Na^+) and 28% (at 4 M Na^+). The relative proportion of squalene is highest at near-optimum salt concentrations (0.6-2 M Na^+) than at marginal salt concentration (4 M Na^+).

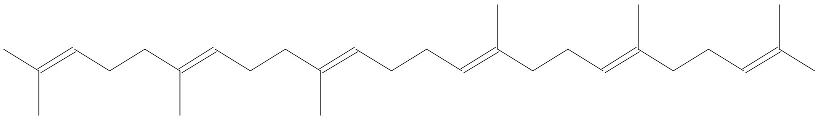
Another finding was that the relative concentration of the diglycerides in strain ALJ 15 is highest in 4 M Na^+-grown culture (data not shown).

Compatible solute composition

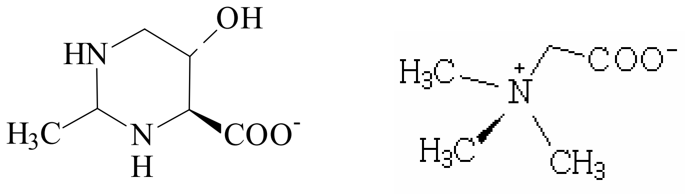
The main compatible solute in *Thioalkalimicrobium aerophilum* strain AL 3^T was ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) (**Fig. 3**) which counted for 0.9-8.7% of the total dry weight depending on the growth conditions.

There was a direct dependence of ectoine production on Na^+ concentration at which the organism was grown (**Fig. 4**). This result may suggest that ectoine is involved in the osmoregulation of *Thioalkalimicrobium aerophilum* strain AL 3^T. Another organic compatible solute that could be detected in strain AL 3^T in minor amounts was glutamate. The glutamate production decreased with increasing salt concentration (data not shown).

In *Thioalkalivibrio versutus* strain ALJ 15 grown in continuous culture under substrate limitation, glycine betaine (N,N,N-trymethylglycine) (**Fig. 3**) was the main organic compatible solute. It showed a salt-dependent production: 1.5%, 7.5% and 9% of total dry weight at 0.6, 2 and 4 M Na^+ respectively. Sucrose was produced as a minor secondary organic compatible solute in this organism and its concentration was highest in cells grown at 2 M Na^+. The sucrose counted for 0.3%, 2.5% and 1.7% of the total dry weight at 0.6, 2 and 4 M Na^+ respectively. The quantitative analysis of the potassium and chloride ions, which are potentially involved in the osmoregulation process (e.g. *Archaea*, bacteria belonging to *Halanaerobiales* and *Salinibacter* sp.), showed that their contribution to salt adaptation in the two haloalkaliphilic sulfur-oxidizing bacteria is minor.



Squalene



Ectoine Glycine betaine

Figure 3. Chemical structures of the C30 lipid squalene and of the main organic compatible solutes in the haloalkaliphilic sulfur-oxidiying bacteria.

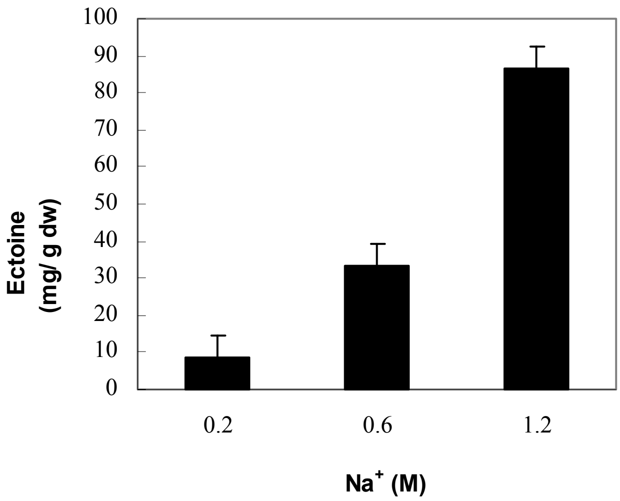


Figure 4. The effect of increasing sodium concentration on ectoine production in *Thioalkalimicrobium aerophilum* strain AL 3^T grown at pH 10.

Estimation of the membrane surface potential

Accumulation and retention of HDHC

Bacterial cells incubated at different time intervals (30, 60, 120 and 180 minutes) with the fluorescent dye bound the probe at their maximum capacity after the first 30 minutes. When the supernatant of the cell suspension was separated and assayed for the excitation and emission fluorescence spectra for detection of the unbound probe, extremely low HDHC concentrations could be detected in *Thioalkalimicrobium aerophilum* AL 3^T cells as well as in *Thioalkalivibrio versutus* strain ALJ 15 and in *Escherichia coli* (data not shown). Based on the calibration curve of the free probe in the same buffer, it was assumed that more than 90% of the fluorescent probe was incorporated in the bacterial cell system (around 90 nmoles dye/ mg of protein). The binding of the probe to the cells was checked again by solubilization of HDHC-loaded cells in 0.1 mM Triton X-100 (+25 mM KPi buffer at pH 8.25). The probe was released in the bulk solution producing an increase of the fluorescence intensity (data not shown).

To verify whether the dye is tightly bound to the cell membranes, the washed cells were incubated at room temperature and on ice at a variable time intervals. No significant loss of dye was recorded either in the sample incubated on ice for 48 hours or in the bound probe incubated at room temperature for 3 hours.

Titration of free and bound HDHC and determination of Ψ_0

The free fluorescent probe HDHC was titrated both in uncharged and in negatively charged solutions in the presence of 0.5 M NaCl and 5 mM KPi.. The fluorescence values (*I*) were then used to determine the dissociation degree (α) according to the following equation: $\alpha = \frac{(I - I_{\min})}{(I_{\max} - I)}$ *(3)*, where I_{min} and I_{max} are the minimum and the maximum fluorescence intensity recorded at Em=450 nm. At a dissociation degree (α) of 0.5, the pK of the probe is equal to the pH at which the probe was titrated: $pK = pH - \log\frac{\alpha}{1-\alpha}$ *(4)*. The dissociation degrees of the probe HDHC solubilized in the presence of charged and uncharged micelles as well as in the presence of the cells are presented in **Fig. 5**.

The pK of the fluorescent probe was significantly shifted in the charged systems compared to the pK of the probe solubilized in uncharged micelles. Based on this approach the calculated pK values are: 8.85 (1), 9.35 (2), 9.45 (3), 10.15 (4) and 11.07 (5). The pK values of the probe solubilized in Triton X-100 (1) and SDS (2) are highly comparable with the values reported in the literature (Fernandez and Fromherz, 1977; Zuidam and

Barenholz, 1997). Inserting the experimental pK values of the bound probe in the equation *(2)* and assuming that pK_0 is 8.85 and 2.3RT/F is equal to 59 mV, the following ΔpK and surface potentials (Ψ_0) can be calculated. The values were 0.5 (ΔpK) and -29.5 mV (Ψ_0) for *E. coli*, respectively; 0.6 and -35.4 mV for strain AL3^T; 1.3 and -76.7 mV for strain ALJ 15.

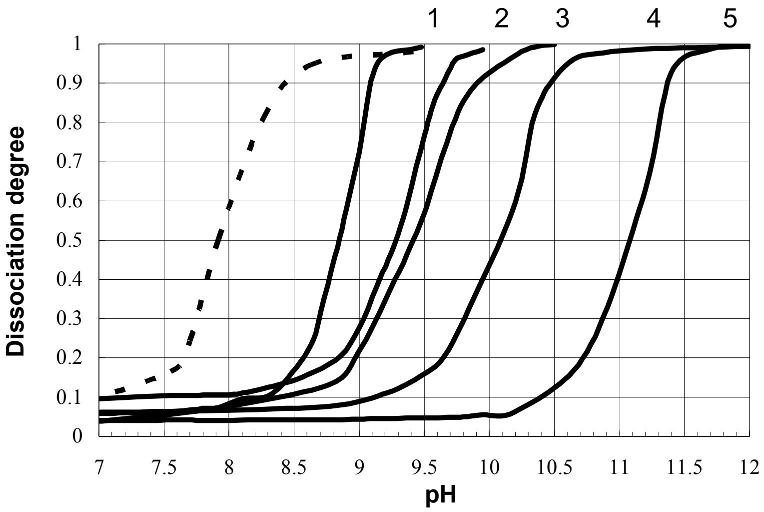


Figure 5. Dissociation degree of the lipophilic probe HDHC *vs.* bulk pH. The figure compares the titration of the dye in the presence of neutral (Triton X-100, 1) and negatively charged micelles (1 mM SDS, 5). The curves 2, 3, 4 are the dissociation curves of the dye bound to cells of *E. coli*, *Thioalkalimicrobium aerophilum* strain AL 3^T and *Thioalkalivibrio versutus* strain ALJ 15 respectively. The dotted line represents the dissociation curve of the probe (0.1 µM) in water (5 mM KPi).

Determination of the accessible fraction of the HDHC probe

The lipophilic fluorescent probe HDHC has been proven to be located at the head-group region of the lipid bilayer (Pal et al., 1985). However, once the probe binds to the lipid bilayer, some uncertainty remains regarding its accessibility for accurate determination of the fluorescence changes. Theoretically, the probe binds on both sides of the lipid bilayer in an equal proportion. Practically the accessibility of the probe could be determined by adding an impermeant quencher that affects directly the fluorescence of the probe located on the outer side of the cell membranes (Tarba and Hinkle, 1996).

In principle, the bound probe is titrated with the quencher in the absence and presence of a mixture of ionophores (valinomycin and nigericin). The quenching of the fluorescence in the absence of ionophores indicates the fraction of the probe on the outer side of the bilayer. The quenching of the fluorescence in the presence of valinomycin and nigericin (when cells are permeabilized) might indicate the fraction of the probe located both on the outer and the inner side of the lipid bilayer.

The relation for fluorescence changes with concentration of impermeant quencher was given by Lehrer (1971) as follows: $\frac{F_0}{\Delta F} = \frac{1}{K_{eff}[Q]} + \frac{1}{f}$ *(5)* where F and F_0 are the fluorescence values in the presence or absence of the quencher; ΔF is $(F-F_0)$; K_{eff} is the effective quenching constant for the accesible probe; $[Q]$ is the quencher concentration and f is the fraction of accessible probe.

The experimental values as obtained with the strain AL 3 are presented in **Table 3** and **Fig. 6**. The calculation of f indicated that 75-80% of the probe is accessible for the fluorescence measurement. The fraction of probe that is presumably located on the outer side of the membranes was estimated at around 75%.

Table 3. Fluorescence quenching of HDHC bound to cells of strain AL 3 by DPX. Cells were suspended (0.1 mg/ml) in a solution containing 0.5 M Na^+ and buffered at pH 10.8 to obtain the maximum fluorescence. The mixture of ionophores consisted of 7 μM valinomycin + 3.75 μM nigericin.

In the absence of ionophores					In the presence of ionophores				
F_0= 182					F_0=241				
$[Q]$, mM	$\frac{1}{[Q]}$	F	ΔF	$\frac{F_0}{\Delta F}$	$[Q]$, mM	$\frac{1}{[Q]}$	F	ΔF	$\frac{F_0}{\Delta F}$
1	1	132.5	49,5	3.676	1	1	165	76	3.171
2	0.5	113	69	2.637	2	0.5	130	111	2.171
4	0.25	90	92	1.978	4	0.25	102	139	1.733
8	0.125	69.5	112.5	1.617	8	0.125	80.5	160.5	1.501
10	0.1	63	119	1.529	10	0.1	77	164	1.469
14	0.071	57	125	1.456	14	0.0714	69.5	171.5	1.405
1/f=1.32; **f=0.757**					1/f=1.26; **f=0.793**				

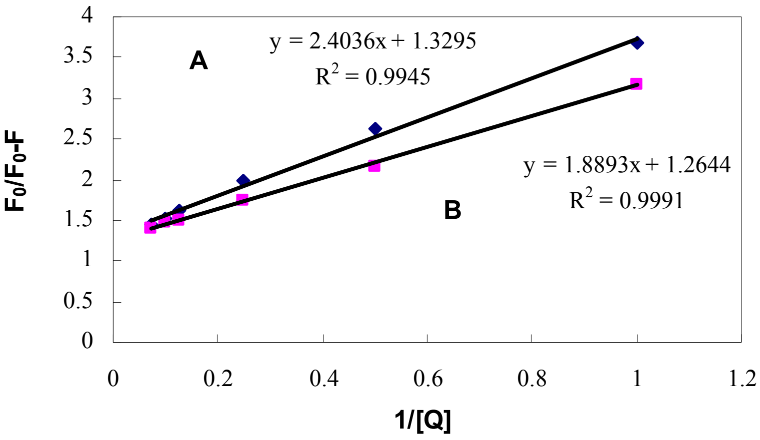


Figure 6. The fluorescence quenching of cell-bound HDHC by DPX in strain AL 3^T, in a buffer containing 0.5 M Na^+ and pH 10.8. The fluorescence values used in this figure were obtained in the absence (A) or presence (B) of ionophores.

Discussion

The extremely halotolerant *Thioalkalivibrio versutus* strain ALJ 15 has a very similar fatty acid composition as *Ectothiorhodospira* (Imhoff and Thiemann, 1991) and *Halomonas* species (Valderrama et al., 1998; Romano et al., 2001). The C16:0, C18:1 and cyc-C19 fatty acids were also the dominant fatty acids in *Ectothioshodospira* sp. and *Halomonas* sp. The relative abundances of the C16:0 and C18:1 fatty acids in strain ALJ 15 is in the same range (14-20% and 60-70% respectively) as in *Ectothiorhodospira* while the fatty acid C18:1 concentration is two times higher than that found in *Halomonas salina*. However, the concentration of cyc-C19 in strain ALJ 15 was 5 times higher than that found in *Ectothiorhodospira* and *Halomonas salina*. Small amounts of saturated C17:0 and C20:0 fatty acids have been found in *Thioalkalivibrio versutus* strain ALJ 15 grown at high salt concentration that are absent in *Ectothiorhodospira* but present in *Halomonas salina* only when grown at 3 M Na^+. The observation that increasing salt concentration induced the decrease of monounsaturated C18:1 and concomitant increase of cyc-C19 was also made in *Halomonas* species (Valderrama et al., 1998; Monteoliva-Sanchez et al., 1988) and in *Lactobacillus* strains (Gilarova et al., 1994). This is consistent with the knowledge that *cis* monounsaturated fatty acids like C18:1 and cyc-C19 can be interconverted. This may suggest a salt-dependent activation of cyclopropane synthetase, an enzyme involved in the regulation of membrane lipid composition and fluidity. The increase of cyclopropane fatty acid content at the expense of unsaturated fatty acids would contribute to an increase of the membrane rigidity (Russell, 1993). A stimulation of cyclopropane synthetase activity by addition of organic compatible solutes (e.g. glycine betaine) was observed in the moderately halophilic bacterium "*Pseudomonas halosaccarolytica"* (Monteoliva-Sanchez et al., 1993). Cyclopropane fatty acids such as cyc-C19 are considered as biomarkers for Gram-negative bacteria as the branched fatty acids are biomarkers for the Gram-positives. They are commonly found in anaerobes such as sulfate-reducing bacteria and have been associated with the oxygen stress in *E. coli* and with low redox potential in soils (Fang and Barcelona, 1998).

Squalene (C30 isoprenoid) may also play an important role in the osmoadaptation of the halotolerant strain ALJ 15. As an apolar lipid, squalene is likely to be located within the lipid bilayer, in the most hydrophobic part of the membranes. Here, this lipid can act as a barrier decreasing the membrane permeability for ions (Clejean et al., 1986). The constant presence of squalene in the cells grown at variable salt conditions may indicate that squalene is constitutively produced as part of salt (and pH) adaptation in the obligate alkaliphilic and extremely salt–tolerant strain ALJ 15.

We have not determined the phospholipid composition, but the presence of high amounts of diglycerides together with substantial amounts of C16:1 and C18:1 fatty acids may suggest a significant concentration of negatively charged cardiolipin (diphosphatidylglycerol) in the membranes of haloalkaliphilic strain ALJ 15. The alkaliphilic bacteria were shown to contain high concentrations of squalene and anionic phospholipids, especially cardiolipin (Clejean et al., 1986). The permeability studies in the membrane vesicles prepared from obligate or from facultative alkaliphiles revealed that increasing squalene concentration has lowered the membrane permeability, an opposite effect with that obtained by increasing the diacylglycerol concentration (Clejean and Krulwich, 1988). On the other hand, the role of cardiolipin in the energy transducing membranes is well known. Cardiolipin is a major membrane lipid that binds the c-type cytochromes (Choi and Swanson, 1995; Ostrander et al., 2001; Ryotmaa and Kinnunen, 1994) and cytochrome *c* oxidases (Robinson, 1993) maintaining their optimal activity. The significance of the high cytochromes content in the alkaliphilic membranes is well documented (Hicks and Krulwich, 1995). Moreover, the strains of *Thioalkalivibrio versutus* showed a high cytochrome *c* oxidase activity in their membranes (Sorokin et al., 2001).

The lipophilic pH-indicator HDHC was chosen to estimate the membrane surface potential (Ψ_0) in metabolically active cells for several reasons. The small size and the lipophilic nature of the probe facilitate an easy binding at the membrane level without disturbing the structure and charge of the lipid bilayer. The probe bound tightly and permanently to the cells without diminishing their activity or integrity. The measurement of the probe fluorescence was highly specific since the working concentration of the dye was around 18 nmoles/ assay. Last but not least, the procedure of using the fluorescent probe is relatively simple and accurate. Only few data concerning the use of HDHC in estimation of Ψ_0 are available in the literature. Most of them have reported the measurement of Ψ_0 in the non-living systems such as (proteo)liposomes. In these situations, a clear dependency of the Ψ_0 with the nature of phospholipids and the presence of salts has been found (Fromherz and Masters, 1974; Fernandez, 1981; Pal et al., 1983, 1985; Kraayenhof et al., 1996). On the other hand, only living models lacking a true cell wall were tested. The measurement of Ψ_0 with the help of a coumarin probe in spiroplasmas and mycoplasmas gave values of -180 mV (in the presence of 115 mM NaCl + 13 mM KCl and a much lower value in the presence of 50-100 mM $CaCl_2$ + 13 mM KCl) and -68 mV respectively (in the presence of 15 mM K^+) (Schummer and Schiefer, 1984, 1988). The Ψ_0 estimated in the intact vesicular stomatitis virus by the same method was -49.6 mV (Pal et al., 1983). In the fragments of the purple membranes isolated from *Halobacterium salinarum*, using a resonance Raman dye, Ehrenberg and Berezin (1984) have found a surface potential of -34.5 mV. Our results obtained with intact Gram-negative cells but in the presence of relatively high concentrations of NaCl fit well in the range of Ψ_0 known for prokaryotes.

The presence of cations in high concentration affects strongly the membrane surface potential producing a “screening” effect. The use of at least 0.5 M Na^+ in our experiments was required by the obligate halophilic nature of the model organisms *Thioalkalimicrobium* and *Thioalkalivibrio*. These haloalkaliphilic organisms are adapted to overcome the permanent danger of the loss of their membrane potential. In such conditions we have observed significant differences in the estimated Ψ_0 in the extremely salt-tolerant, alkaliphilic strain ALJ 15 as compared with the low-salt tolerant, alkaliphilic strain $AL3^T$ and the neutrophilic, nonhalophilic *E. coli* as a control. We are aware that the increasing salt concentration may have lowered the surface potential in the control and the real value could be different. Our aim was to compare the surface potential in the two haloalkaliphiles and to link these findings with the lipid composition of their membranes. As suggested above the content of the anionic cardiolipin could be very high in the *Thioalkalivibrio* membranes. Integrating all these results we can conclude that the membrane charge in strain ALJ 15 is overall more negative than in the strain AL 3^T. This property can be related to the extreme salt tolerance of the strain ALJ 15. The strong negative charge of the membrane would help this organism to prevent the screening effect due to the high concentration of Na^+. The salt adaptation in *Thioalkalimicrobium aerophilum* strain AL 3^T and in *Thioalkalivibrio versutus* strain ALJ 15 requires the synthesis of organic osmolytes that contribute to the osmotic balance. The results in strain ALJ 15 are in close agreement with the taxonomic relatedness of *Thioalkalivibrio* and *Ectothiorhodospira*, a group in which glycine betaine is one of the main compatible solutes (Galinski, 1995). Glycine betaine is synthesized *de novo* in strain ALJ 15 but it can also be accumulated from the surrounding environment (see **Chapter 4**).

In conclusion, the salt (and pH) adaptations are reflected in the biochemical composition of alkaliphilic sulfur-oxidizing bacteria from soda lakes. In addition to constitutively high concentration of unsaturated fatty acids and of squalene, several osmoregulated processes take place. The synthesis of cyclopropane fatty acids and of diglycerides at the membrane level is accompanied by the increase of compatible solute concentration in the cytoplasm. It is therefore possible that the modification of the internal osmotic pressure is also reflected in the changes of the membrane lipid composition.

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7

General discussion

Abstract

The aim of the present thesis was to characterize the physiology of the chemolithoautotrophic sulfur-oxidizing bacteria (SOB) isolated from soda lakes in view of their possible utilization in the biotechnology of sulfur removal at haloalkaline conditions. For this purpose, special attention was paid to the growth physiology under substrate excess or limitation, to the competitive interactions and to the biochemical and metabolic adaptation to extreme conditions of pH and Na^+ concentration. Two different ecological strategies were found in the alkaliphilic SOB from soda lakes: the low-yielding and fast growing r-strategists (*Thioalkalimicrobium* sp.) and high-yielding and slow-growing K-strategists (*Thioalkalivibrio* sp.). Sulfur-oxidizing capacity, growth yields and energetic efficiency of the haloalkaliphilic SOB from soda lakes were found to be similar to those of their neutrophilic counterparts. The ability of haloalkaliphilic SOB from soda lakes to cope with the doubly extreme conditions is partially explained by the availability of a suitable energy source (sulfur), which can support the production of compatible solutes and of a cell membrane with special features. In this thesis we have proven that the bacterial growth in sodium carbonate/bicarbonate environments is more efficient than in sodium chloride medium because of a lower osmotic pressure. A detailed picture of the genus *Thioalkalivibrio* was obtained after in-depth study of several representative species and strains. A great genetic and phenotypic diversity was found in this group. Finally, the extremophilic nature of these chemolithotrophic SOB offers interesting opportunities for their utilization in the biotechnology of sulfur removal, as alternative to the classic catalyst technology.

This thesis focused on the physiological aspects of biological sulfur oxidation in the (extreme) alkaline environment. The diagram sketching the aim of the present thesis is shown in **Fig. 1.**

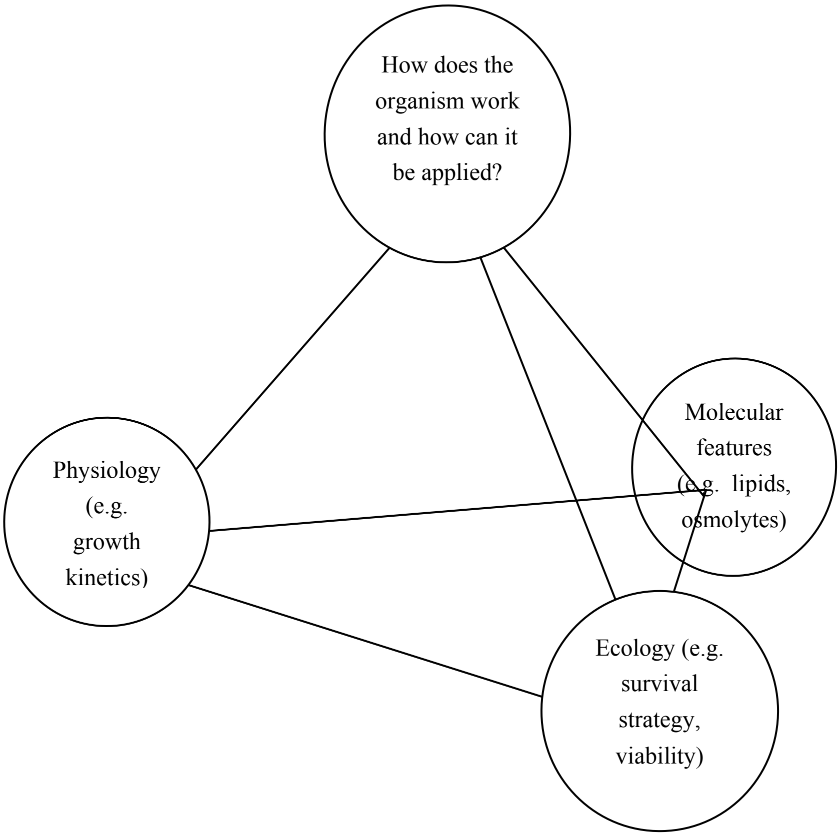

Figure 1 The integration of physiological, biochemical and ecological properties of sulfur-oxidizing alkaliphiles from soda lakes.

The present research used a multiple approach (physiology, biochemistry, molecular biology and ecology) in order to answer the question “How does the alkaliphilic sulfur-oxidizing organism perform in its environment?”. This fundamental knowledge is essential for a succesful application of (mixtures of) these organisms in waste water and industrial gases recycling. The physiological data including the growth kinetics and the rates of sulfur oxidation under various conditions are necessary in predicting the efficiency of sulfur-compound removal and of the elemental sulfur production. Such data, complemented by the understanding of biochemical and biophysical features of the organisms, are also important to understand how well will they be able to cope with the changing conditions.

The ecological properties of the alkaliphilic SOB from soda lakes would be useful in predicting the competitive interactions and the selection in a mixed culture, under stable or changing conditions. Knowing that, engineers can select the organism that performs best under certain (stable) conditions. On the other hand, under fluctuating conditions, the most versatile organism can be selected.

Sulfur oxidation at high pH and high Na^+ concentration

The starting idea underlying the concept of this thesis was to absorb hydrogen sulfide gas in alkaline solution before its biological conversion to insoluble elemental sulfur.

$$H_2S_{(g)} + OH^- \Rightarrow HS^-_{(aq)} + H_2O$$

The aqueous sulfide (HS^-) is chemically stable at pH > 8 and it can be used by chemolithotrophic sulfur-oxidizing bacteria in this state. In the achievement of stable alkalinity in the reaction system one must take into account the buffering capacity and the chemical stability/accessibility of sulfide. From this point of view, the Na_2CO_3/$NaHCO_3$ solution is preferable over NaCl/NaOH solution. Moreover, the carbonate solutions are more selective for H_2S absorption from CO_2-containing gases. The removal of the sulfide can best be accomplished by the partial chemical or biological oxidation to elemental sulfur (S^0) which precipitates and can easily be separated from the bulk medium (Thiopaq® technology, Janssen et al., 2001) (**Chapter 1**). The detailed investigation of bacteria capable to oxidize sulfide to sulfur at alkaline pH and in the presence of high concentration of Na^+ was therefore justified.

Significant difference in the rates of oxidation of various sulfur compounds (see **Table 1**) and production of different intermediates during thiosulfate oxidation suggest different pathways of sulfur metabolism in *Thioalkalimicrobium* and *Thioalkalivibrio*. The former never produced elemental sulfur during oxidation of thiosulfate, unless severe oxygen limitation was applied, while most of the *Thioalkalivibrio* strains formed extracellular or intracellular sulfur from thiosulfate during growth in liquid culture and in colonies. Moreover, *Thioalkalimicrobium* was virtually unable to oxidize external elemental sulfur in contrast to *Thioalkalivibrio,* which converted it to sulfate. Another important difference between these 2 groups is sulfite metabolism. Sulfite is considered as a key intermediate in sulfur oxidation pathways of many sulfur bacteria (Kappler and Dahl, 2001). The *Thioalkalimicrobium* strains released up to 3 mM sulfite into the medium during batch growth with thiosulfate (Sorokin et al., 2000), but it was never detected in the cultures of *Thioalkalivibrio*. Furthermore, neither of the *Thioalkalimicrobium* strains studied were capable of oxidizing external sulfite even at micromolar concentrations, while some of the *Thioalkalivibrio* isolates did. This correlated with the presence of sulfite dehydrogenase activity (AMP-independent type): it was uniformly detected in *Thioalkalivibrio* but not in *Thioalkalimicrobium* (Sorokin et al., 2001). Among the other activities, sulfide dehydrogenase (cytochrome *c*-dependent), SQR (sulfide-quinone reductase) and thiosulfate reductase activities have been detected in both groups. Cytochrome *c*-dependent sulfide dehydrogenase was purified from the *Thioalkalimicrobium aerophilum* AL 3 (Sorokin et al., 1998). In contrast to the known flavocytochrome *c*-containing enzymes as found in chemotrophic and phototrophic sulfur bacteria (Visser et al., 1997), the enzyme from alkaliphilic sulfur bacteria only contained cytochrome *c* as a cofactor. The presence of a second, quinone-dependent, sulfide dehydrogenase in alkaliphiles implicates that sulfide oxidation in sulfur bacteria might have different mechanisms in a single organism. Overall, the data available so far suggest that in *Thioalkalimicrobium* the oxidation pathway of reduced sulfur is probably similar to the one proposed for the facultatively autotrophic *Paracoccus* species, e.g. complete oxidation of the sulfane atom by a multienzyme complex without releasing free intermediates (Friedrich et al., 2001). In contrast, *Thioalkalivibrio* seems to employ different mechanism with the formation/consumption of free sulfur intermediates, such as elemental sulfur and sulfite, as is common for many other obligately neutrophilic sulfur-oxidizing bacteria (Kelly et al., 1997).

The studies in the present thesis confirmed and completed the previous findings on sulfur oxidation mechanisms in the two groups of alkaliphilic sulfur bacteria. The comparative growth kinetics at different Na^+ concentration (**Chapter 4**) showed that *Thioalkalivibrio versutus* strain ALJ 15 is capable of relatively high rates of sulfur oxidation as compared to the neutrophilic sulfur-oxidizing bacteria. The growth experiments with the strain ALJ 15 under thiosulfate, sulfide and polysulfide limitation showed that the oxidation pathway for thiosulfate and polysulfide may will be linked or common, whilst the sulfide oxidation occurs via different pathway (**Fig. 2**).

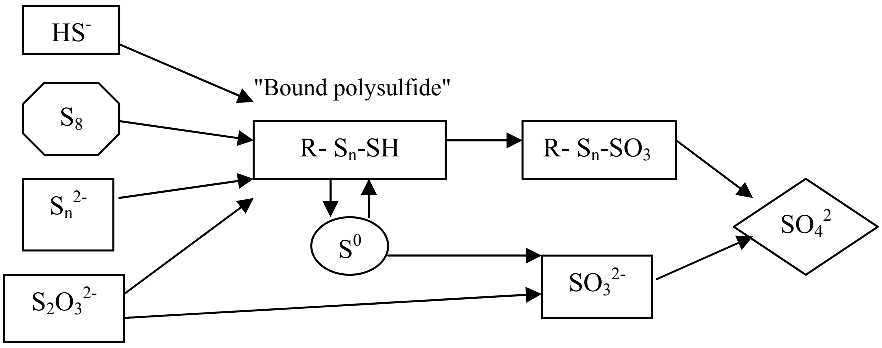

Figure 2. Possible oxidation pathways of inorganic sulfur compounds in *Thioalkalivibrio* group. Symbols: solid arrows, biological oxidation; (R-), organic residue.

Ecology of alkaliphilic SOB from soda lakes

Soda lakes represent large variety of low to high salt alkaline environments offering a relatively broad spectrum of physico-chemical conditions. This is reflected by the large diversity of sulfur-oxidizing alkaliphiles in the *Thioalkalivibrio* group. The niche differentiation of *Thioalkalimicrobium* and *Thioalkalivibrio* most probably resides in their different salt tolerance and growth kinetics (**Chapter 2**).

Based on our data we suggest that *Thioalkalimicrobium* group, representing the r-strategist, predominate only during short periods of substrate excess combined with favorable conditions of low salt concentration. They are less resistant to starvation and their viability is lower than that of *Thioalkalivibrio* group. Unlike the *Thioalkalimicrobium* strains, the *Thioalkalivibrio* group has adopted the K-strategy for survival and can be found permanently in various soda lakes at relatively high density (10^3-10^6 cells/ml). Their resistance to starvation is significantly higher than that of *Thioalkalimicrobium* cells (**Chapter 2**). The intra- or extracellular production of elemental sulfur as an intermediate of sulfur compound oxidation in *Thioalkalivibrio* species may be another competitive advantage. Using molecular techniques, several authors recently reported the presence of *Thioalkalivibrio* in a variety of soda lakes (Humayoun et al., 2003; Ma et al., 2004; Rees et al., 2004; G. Muyzer, unpublished data). The development of new molecular probes to help the identification of alkaliphilic SOB in natural samples is currently in progress in our laboratory.

The genetic and phenotypic heterogeneity of the *Thioalkalivibrio* group is very high. Not only are they sodium-tolerant but can also withstand a broad range of carbonate, chloride or sulfate anions concentration. A clear division could be made based on their anion tolerance: carbonate-loving alkaliphiles and chloride-loving alkaliphiles (**Chapter 3**). We propose that the carbonate-loving organisms should be classified as natronophiles to differentiate them from the true halophiles, which clearly prefer NaCl over Na_2CO_3/$NaHCO_3$. However intermediate types growing well, both in sodium carbonate and sodium chloride media, have been found. Special attention in this thesis was paid to the versatile *Thioalkalivibrio halophilus* strain HL17^T (**Chapter 5**). This bacterium was able to withstand equally well neutral and alkaline conditions, low to high NaCl and Na_2CO_3/$NaHCO_3$ concentrations. Strain HL17^T is an typical example of extreme tolerance. The results of the present thesis suggest that a continuous spectrum of organisms living in various microniches exist in the natural extreme environment.

The differences in the chemical composition of soda lakes cause different osmotic pressure that the organisms have to cope. Using strain HL17^T as a model organism capable of growing at high sodium carbonate/bicarbonate as well as at high sodium chloride we found that a lower osmotic pressure of the carbonate-based medium is followed by a lower production of compatible solutes (**Chapter 5**). This finding corroborates the substantially metabolic differences between natronophiles and halophiles.

Contributions to the knowledge of extremophiles

In order to answer the question "how do the alkaliphilic SOB work?" a multiple approach was used in this thesis. The physiology of sulfur oxidation and the aspects of competitive interactions between the two groups of alkaliphilic SOB were discussed above. The biochemical and biophysical data concerning the salt and pH adaptation were presented in **Chapter 6**. The composition of the cell membranes in alkaliphilic SOB from soda lakes is dominated by unsaturated fatty acids. It was expected that the content of anionic lipids would be very high, especially in the extremely salt-tolerant strains. This assumption was confirmed by the highly negative values found in the membrane surface potential of *Thioalkalimicrobium* and *Thioalkalivibrio*. The presence of significant amounts of squalene, an apolar lipids acting like a barrier or as modulator of membrane fluidity, may also explain the extremely salt-tolerance of some alkaliphilic SOB belonging to *Thioalkalivibrio* group. The squalene may also play a role in the alkaliphilicity (Clejean et al., 1986).

Many extremely salt-tolerant, extremely natronophilic and halophilic strains of *Thioalkalivibrio* group synthesized a membrane-bound yellow pigment not found in the low-salt tolerant *Thioalkalivibrio* or *Thioalkalimicrobium* strains. This pigment is a 23 C-polyene compound with a structure unknown for any bacterial pigments (S. Takaichi, personal communication). Although its complete formula and function is not yet completely understood, it is clearly essential for the functioning of these bacteria at extremely high salt/pH conditions.

The high osmotic pressure is counterbalanced in the salt-loving alkaliphiles by the production of compatible solutes: ectoine, glutamate (*Thioalkalimicrobium*), glycine-betaine and sucrose (*Thioalkalivibrio*) (**Chapter 6**).

The highlights of the present thesis are: the evidence for a different survival strategy in the metabolic group of SOB from soda lakes (Chapter 2); the differences between halophilic and a concept of natronophily are discussed in Chapter 3; the continuous culture approach was used to characterize the growth physiology of an extremely salt-tolerant, alkaliphilic strain, at high salt concentration and pH 10 (Chapter 4); a newly versatile double extremophile is described in Chapter 5; some of the molecular bases for the salt and pH adaptation in the alkaliphilic SOB from soda lakes are revealed in the Chapter 6. The updated information on the alkaliphilic SOB from soda lakes is summarized in **Table 1**.

Table 1. Summary of the main properties of the two groups of alkaliphilic sulfur-oxidizing bacteria from soda lakes.

Parameter or property	***Thioalkalimicrobium*** **spp.**	***Thioalkalivibrio*** **spp.**
Isolation	Alkaline and low to moderate saline soda lakes	Alkaline and saline soda lakes with high variations of [Na^+]
Genotypic and metabolic diversity	Homogenous (3 species, 43 strains)	Heterogenous (7 species, 74 strains)
G+C mol% in DNA	47.3-51.2	61-66
Cell morphology	Rods to spirilla with 1-3 polar flagella	Mostly vibrios or short spirilla with a single polar flagellum
pH limits (optimum)	7.5-10.6 (10.0)	7.5-10.65 (10-10.2)
Upper temperature limit, ^{o}C	39	50
Salt tolerance	0.2-1.5 M Na^+ (low salt tolerance)	0.6-5 M Na^+ (moderate and extremely salt tolerance, true halophiles)
Growth rate	High	Low-moderate
Biomass yield	Low	High
Thiosulfate and sulfide oxidation rates	High	Moderate
Elemental sulfur oxidation rate	Low or absent	Moderate
Sulfur intermediates	Sulfite	Polysulfide, sulfur
Intracellular sulfur	Absent	Present in some strains
Sulfur production (at O_2 excess)	Zero to low	Low to moderate
Apparent affinity constant for $S_2O_3^{2-}$ or HS^-	Micromolar concentration	Micromolar concentration
Viability and resistance to starvation	Low (weeks-months)	High (months-years)
Survival strategy	*r*-strategists	K-strategists
Dominant cytochromes	c	c and b
Cytochrome oxidases	*cbb_3*	*o (?), cbb_3, aa_3, cd_1-nitrite reductase*
Membrane lipids (Dominant fatty acids and apolar lipids)	C16:1, C18:1, C16:0	Cyc-C19, C18:1, squalene
Compatible solutes	Ectoine, glutamate	Glycine betaine, sucrose
Yellow pigments	Absent	Present in high salt-tolerants

Practical application of SOB from soda lakes

Overall, alkaliphilic SOB isolated from soda lakes proved to be highly specialized for the double extreme conditions of high salt concentration and high pH. They can be isolated from soda lake environment only. Some strains tolerate the entire possible range of pH and salt concentrations that might transiently appear in their natural location. Based on these properties, the haloalkaliphilic SOB from soda lakes can be utilized in bioreactors with stable soda alkalinity and supplied with inorganic sulfur compounds. The production of elemental sulfur from sulfide can be assured under oxygen limitation. In such conditions, the biomass production is also decreasing. One possible scenario would be the utilization of a complex mixture of *Thioalkalimicrobium* and *Thioalkalivibrio* strains. Theoretically, such mixture would behave as presented in the **Fig. 3**. The behavior of such SOB mixtures is presently under study in a collaborative project between the TU Delft, Wageningen University and industry that aims at a wider application of alkaliphilic sulfur bacteria in the recycling of sulfur from waste and gases.

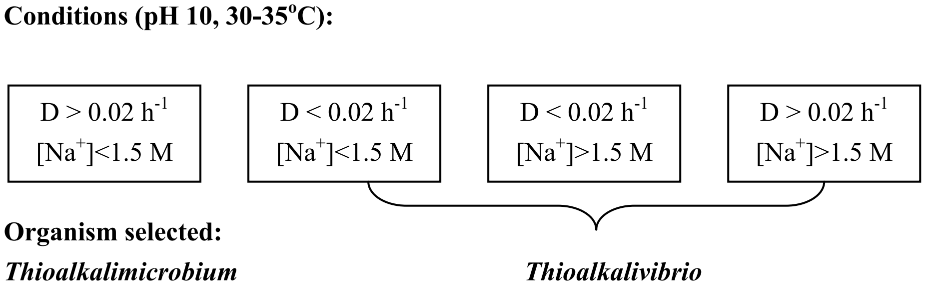

Figure 3. Selection of alkaliphilic SOB from soda lakes if used in bioreactors during the process of sulfur removal.

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Summary

The inorganic sulfur oxidation by obligate haloalkaliphilic chemolithoautotrophs was only recently discovered and investigated. These autotrophic sulfur oxidizing bacteria (SOB), capable of oxidation of inorganic sulfur compounds at moderate to high salt concentration and at high pH, can be divided into three genera belonging to the γ subdivision of the Proteobacteria: *Thioalkalimicrobium*, *Thioalkalivibrio* and *Thioalkalispira*. Their taxonomy, metabolic diversity and the potential application in biological removal of toxic sulfur compounds of haloalkaliphilic SOB were reviewed in **Chapter 1** (Introduction) of this thesis.

The scope of this thesis was to characterize the physiology and growth kinetics of the newly isolated haloalkaliphilic chemolithotrophic SOB with respect to their potential use in the biotechnology of H_2S removal under haloalkaline conditions. A second aim of this thesis was to obtain more information about the specific adaptation to high salt and alkaline conditions from the population level down to the biomolecular level.

The growth physiology of representatives of the genus *Thioalkalimicrobium* and *Thioalkalivibrio* is compared in **Chapter 2**. The competitive interaction between these two groups of organisms and their survival strategy with direct implications in their ecology is also described. The aim was to study what are the rationales for the environmental occurrence of one or another group in the soda lakes. The *Thioalkalimicrobium* strains demonstrated relatively high specific growth rates, low growth yield, high maintenance and extremely high rates of thiosulfate and sulfide oxidation. In contrast, the *Thioalkalivibrio* strains, in general, were slowly growing and high-yield organisms with lower maintenance and much lower rates of oxidation of sulfide and thiosulfate. Moreover, the latter survived starvation much better than the *Thioalkalimicrobium*. In mixed thiosulfate-limited chemostat cultures at low salinity a *Thioalkalimicrobium* strain outcompeted a *Thioalkalivibrio* strain at $D>0.02\ h^{-1}$. The overall results suggest that *Thioalkalimicrobium* and *Thioalkalivibrio* represent two different ecological strategies.

The **Chapter 3** describes the sodium salt requirement for the growth and sulfur-oxidizing potential at alkaline conditions in *Thioalkalivibrio versutus* strains isolated from soda lakes. A clear distinction was made between the NaCl- and Na_2CO_3/$NaHCO_3$-requiring and tolerant strains. The different sodium salt requirement in the strains belonging to the same species allows us to conclude that there is a clear physiological difference between the alkaliphilic, high–salt organisms: "natronophiles" which require and tolerate high $Na_2CO_3/NaHCO_3$ but not NaCl and the "halophiles" requiring and tolerating high concentrations of NaCl for their growth.

The comparative growth kinetics at different Na^+ concentration (**Chapter 4**) showed that *Thioalkalivibrio versutus* strain ALJ 15 is capable of relatively high rates of sulfur oxidation as compared to the neutrophilic sulfur-oxidizing bacteria. The growth experiments with strain ALJ 15 under thiosulfate, sulfide and polysulfide limitation showed that the oxidation pathway for thiosulfate and polysulfide may be linked or common, whilst the sulfide oxidation occurs via different pathway.

The growth physiology of an extremely salt-tolerant and facultative alkaliphile, *Thioalkalivibrio halophilus* sp. nov., is presented in **Chapter 5**. This organism tolerated high concentrations of sodium carbonate and sodium chloride. It was also capable of growing well at pH 7.5 and 9.8. The hypothesis that two aqueous solutions with same Na^+ concentration but containing different anionic species (HCO_3^- /CO_3^{2-} and Cl^-), resembling two types of saline environments, would have different osmotic pressure was verified theoretically and experimentally. This difference clearly has a direct consequence for the production of organic compatible solutes in the same organism.

The research concerning the salt-dependent compatible solutes production and membrane lipid composition of haloalkaliphilic sulfur-oxidizing bacteria is the subject of **Chapter 6** of this thesis. Significant biochemical differences were revealed in the two groups of haloalkaliphilic SOB. The *Thioalkalimicrobium* species primarily use ectoine as the compatible solute, whereas the *Thioalkalivibrio* species primarily use glycine betain and low concentrations of sucrose. The estimation of the membrane surface potential using a fluorescent lipophilic probe supported the hypothesis of a very negative surface potential in the membranes of *Thioalkalivibrio* species.

In the last chapter (**Chapter 7**) the results are integrated and general conclusions are presented, pointing to the uniqueness and importance of the chemolithoautotrophic haloalkaliphilic SOB for fundamental as well as for applied research.

Overall, alkaliphilic SOB isolated from soda lakes proved to be highly specialized for the doubly extreme conditions of high salt concentration and high pH. They can be isolated from soda lake environments only. Some strains tolerate the entire possible range of pH and salt concentrations that might transiently appear in their natural habitat. Based on these properties, the haloalkaliphilic SOB from soda lakes can be usefully exploited in bioreactors with stable soda alkalinity and supplied with inorganic sulfur compounds for sulfur removal from waste streams.

Samenvatting

De oxidatie van anorganisch zwavel door obligate haloalkalifiele chemolithoautotrofe bacteriën is pas kortgeleden ontdekt en onderzocht. Deze autotrofe zwavel oxiderende bacteriën (ZOB) zijn in staat tot oxidatie van anorganische zwavelverbindingen bij middelmatige tot hoge zoutconcentraties en bij een hoge pH, en kunnen worden onderverdeeld in drie genera behorende tot de γ-subdivisie van de proteobacteriën: *Thioalkalimicrobium*, *Thioalkalivibrio*, and *Thioalkalispira*. Een overzicht betreffende de taxonomie, de metabole diversiteit, en de potentiële biotechnologische toepassing van haloalkalifiele ZOB is gegeven in **Hoofdstuk 1** (Inleiding) van dit proefschrift.

Het onderwerp van dit proefschrift is de karakterisering van de fysiologie en groeikarakteristieken van recentelijk geïsoleerde haloalkalifiele chemolithotrofe ZOB in het kader van de potentiële biotechnologische toepassing voor verwijdering van waterstof sulfide onder haloalkalische omstandigheden. Een tweede doelstelling betrof het verschaffen van inzicht in de specifieke adaptatie, van populatie tot moleculair niveau, aan de hoge zoutconcentraties en de hoge pH-waarden waarbij deze bacteriën groeien.

Een vergelijking tussen de groeifysiologie van vertegenwoordigers van *Thioalkalimicrobium* en *Thioalkalivibrio* is beschreven in **Hoofdstuk 2**. De competitieve interactie tussen beide groepen organismen en de verschillende overlevingsstrategieën worden beschreven, evenals de ecologische implicaties. Het doel van dit onderzoek was om te achterhalen waardoor beide groepen coëxisteren in zogenaamde soda-meren. De *Thioalkalimicrobium* stammen worden gekarakteriseerd door relatief hoge specifieke groeisnelheden, lage groeiopbrengst, hoge onderhoudscoëfficiënt, en extreem hoge specifieke respiratiesnelheden met thiosulfaat en sulfide. *Thioalkalivibrio* soorten echter, hebben een lage maximale specifieke groeisnelheid, een hoge groei opbrengst, en een lagere onderhoudscoëfficiënt en aanzienlijk lagere specifieke respiratiesnelheden. *Thioalkalivibrio* soorten zijn bovendien beter bestand tegen substraatloze perioden dan *Thioalkalimicrobium* soorten. In een met thiosulfaat gevoede chemostaat reactor bij een laag zoutgehalte en een hoge verdunningssnelheid ($D>0.02$ uur^{-1}) bleek *Thioalkalimicrobium* in staat om *Thioalkalivibrio* te overgroeien. De resultaten suggereren dat *Thioalkalimicrobium* en *Thioalkalivibrio* groepen verschillende strategieën toepassen om zich in het ecosysteem te kunnen handhaven.

Hoofdstuk 3 beschrijft de natriumzout behoefte en zwaveloxiderende capaciteit van uit soda-meren geïsoleerde *Thioalkalivibrio versutus* stammen. Een duidelijk onderscheid kon worden aangebracht tussen stammen die hoge concentraties (natrium)-chloridezouten of -carbonaatzouten nodig hebben voor groei. De verschillen in de natriumzout-behoefte tussen diverse stammen binnen een soort geeft aan dat er duidelijke fysiologische verschillen zijn tussen "natronofielen" – die optimaal groeien bij hoge concentraties natrium bicarbonaat – en "halofielen" –die hoge concentraties natrium chloride behoeven. Metingen aan de groeikinetiek van *Thioalkalivibrio* versutus stam ALJ 15 bij verschillende zoutconcentraties (**Hoofdstuk 4**) hebben aangetoond dat de specifieke respiratiesnelheden relatief hoog zijn in vergelijking met neutrofiele zwavel oxiderende bacteriën. Groei-experimenten met stam ALJ 15 met thiosulfate, sulfide en polysulfide als elektronen donor hebben aangetoond dat thiosulfaat en polisulfide worden afgebroken via vergelijkbare afbraakroutes, terwijl sulfide oxidatie via een andere route plaatsvindt.

De groeifysiologie van de extreem zout tolerante en facultatief alkalifiele soort *Thioalkalivibrio halophilus* sp. nov. wordt beschreven in **Hoofdstuk 5**. Dit organisme tolereert zowel hoge concentraties natrium carbonaat als natrium chloride. Het was bovendien in staat tot efficiënte groei bij pH-waarden tussen 7.5 en 9.8. De hypothese dat bij vergelijkbare natriumconcentraties een verschillende saliniteit en osmotische druk worden verkregen als gevolg van het type anion (bicarbonaat en chloride) is zowel theoretisch als experimenteel onderzocht. Het anion afhankelijke verschil tussen carbonaat en chloride zouten heeft duidelijke implicaties voor de cellulaire productie van organische zoutvervangende componenten.

Onderzoek naar de zoutconcentratie afhankelijke productie van zoutvervangende componenten en de samenstelling van het celmembraan van haloalkalifiele zwavel oxiderende bacteriën is het onderwerp van **Hoofdstuk 6** van dit proefschrift. Significante biochemische verschillen konden worden aangetoond tussen de twee voornoemde groepen haloalkalifiele ZOB. Daar waar *Thioalkalimicrobium* soorten voornamelijk ectoine toepassen als zoutvervangende component, maken *Thioalkalivibrio* soorten gebruik van glycine betaine en in mindere mate sucrose. Metingen aan de potentiaal van het oppervlak van het bacteriële membraan met behulp van een fluorescente lipofiele sonde bevestigt de hypothese van een sterk negatieve oppervlaktepotentiaal in de membranen van *Thioalkalivibrio* soorten.

In het laatste hoofdstuk (**Hoofdstuk 7**) worden de resultaten uit de voorgaande hoofdstukken geïntegreerd en worden algemene conclusies gepresenteerd. Hiermee worden de unieke karakteristieken duidelijk van haloalkalifiele ZOB, alsmede het belang van zowel fundamenteel als toegepast onderzoek naar dit type organisme.

In het algemeen kan worden vastgesteld dat haloalkalifiele ZOB geïsoleerd uit soda-meren uitstekend toegerust en gespecialiseerd zijn voor de tweevoudig extreme omstandigheden waar ze aan worden blootgesteld: hoge zoutconcentraties en hoge pH-waarden. Dit type organismen wordt alleen in soda-meren aangetroffen. Een aantal stammen hebben een hoge tolerantie voor verschillende pH-waarden en zoutconcentraties die tijdelijk kunnen voorkomen in soda-meren. Op basis van deze unieke eigenschappen kunnen haloalkalifiele ZOB interessant zijn voor toepassing bij biotechnologieën gericht op verwijdering van gereduceerde zwavelverbindingen uit afvalstromen.

Translation in Dutch by **Robbert Kleerebezem** and **Jeanine Geelhoed**. Thank you.

Curriculum Vitae

Horia Leonard Banciu was born on 8th of August 1974, at Sântana, Arad county, Romania. In 1992 he graduated from the Chemistry-Biology section of the "Elena Ghiba-Birta" Theoretical School, Arad. In the same year he left the hometown Arad for Cluj-Napoca to follow the university studies in Biology. In 1997 he obtained the BSc. Degree in Biology with the highest record in his class, at the Department of Biology, Faculty of Biology and Geology, "Babeş-Bolyai" University of Cluj-Napoca. His graduation thesis dealt with the levan synthesis by the *Bacillus* strains isolated from soil. In this work Prof. Dr. Mihail Dragan-Bularda supervised him. In 1998 he obtained the MSc. Degree in Cell and Molecular Biology at the same Department and University. His master degree dissertation dealt with the use of fluorescent probes for the bioenergetic studies in an alkaliphilic *Bacillus*. In this research he was supervised by Prof. Dr. Corneliu Tarba.

Since 1997 he has been working as teaching assistant in Biophysics and since 1998 he enrolled as PhD. Student at the Department of Biology, "Babeş-Bolyai" University of Cluj-Napoca, Romania. His doctoral supervisor in Romania is Prof. Dr. Corneliu Tarba.

In 1999 he had the fortune to work for five months as research assistant at the Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York City, U.S.A. His supervisors were Prof. Dr. Terry Ann Krulwich, Dr. David B. Hicks and Dr. Arthur A. Guffanti. Horia was involved in a project dealing with the molecular studies on facultatively alkaliphilic *Bacillus pseudofirmus* OF4. Two years later, in 2001 he was granted with an Erasmus/Socrates fellowship and he studied for six months at the Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, The Netherlands. His luck did not stop here and he began to work as PhD. student at Department of Biotechnology. His doctoral supervisors are Prof. Dr. J. Gijs Kuenen and Dr. Dimitry Y. Sorokin. The product of this collaboration that has lasted two years is the present PhD. thesis.

List of publications and presented work

Journal articles

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Takaichi, S., Maoka, T., Akimoto, N., Sorokin, D.Y., **Banciu, H.,** Kuenen, J.G. Two novel yellow pigments natronochrome and chloronatronochrome from the natrono(alkali)philic sulfur-oxidizing bacterium *Thialkalivibrio versutus* ALJ 15. Submitted to *Tetrahedron Lett*.

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Appendix

Methods used in the study of alkaliphilic sulfur-oxidizing bacteria from soda lakes

Separation of elemental sulfur from the cell biomass in a solution containing high concentration of salts

Principle

This method aims for the separation of the elemental sulfur from the biomass suspended in a solution containing 0.5 to 4 M Na^+ The density of this medium is relatively high and the sedimentation of the cells is preceded by that of the elemental sulfur particles.

Procedure

- The culture sample is collected and placed on the table for 5-10 min. The bulk of insoluble elemental sulfur settles during this period.
- The supernatant is transferred into a new Eppendorf tube. The elemental sulfur present is pelleted by short centrifugation at low speed (1200-2400xg, 0.5-1 min). This procedure can be repeated twice.
- The elemental sulfur separated in this way is suitable for quantitative analysis (Sörbo method, see below).
- The biomass suspension is treated as following:
  - (i) if Na^+ concentration does not exceed 3 M, the suspension can be centrifuged at high speed (15 minutes at 15,000xg);
  - (ii) if Na^+ concentration exceeds 3 M, the suspension must be diluted with a solution of NaCl of lower molarity (0.6-2 M). At 4 M Na^+, the biomass cannot be pelleted because of the high density of the medium.
- The next step following the biomass pelleting is the washing with an isotonic solution of NaCl. The washing procedure is repeated at least twice.

A diagram showing the main steps of sample preparation is presented in **Fig.1**.

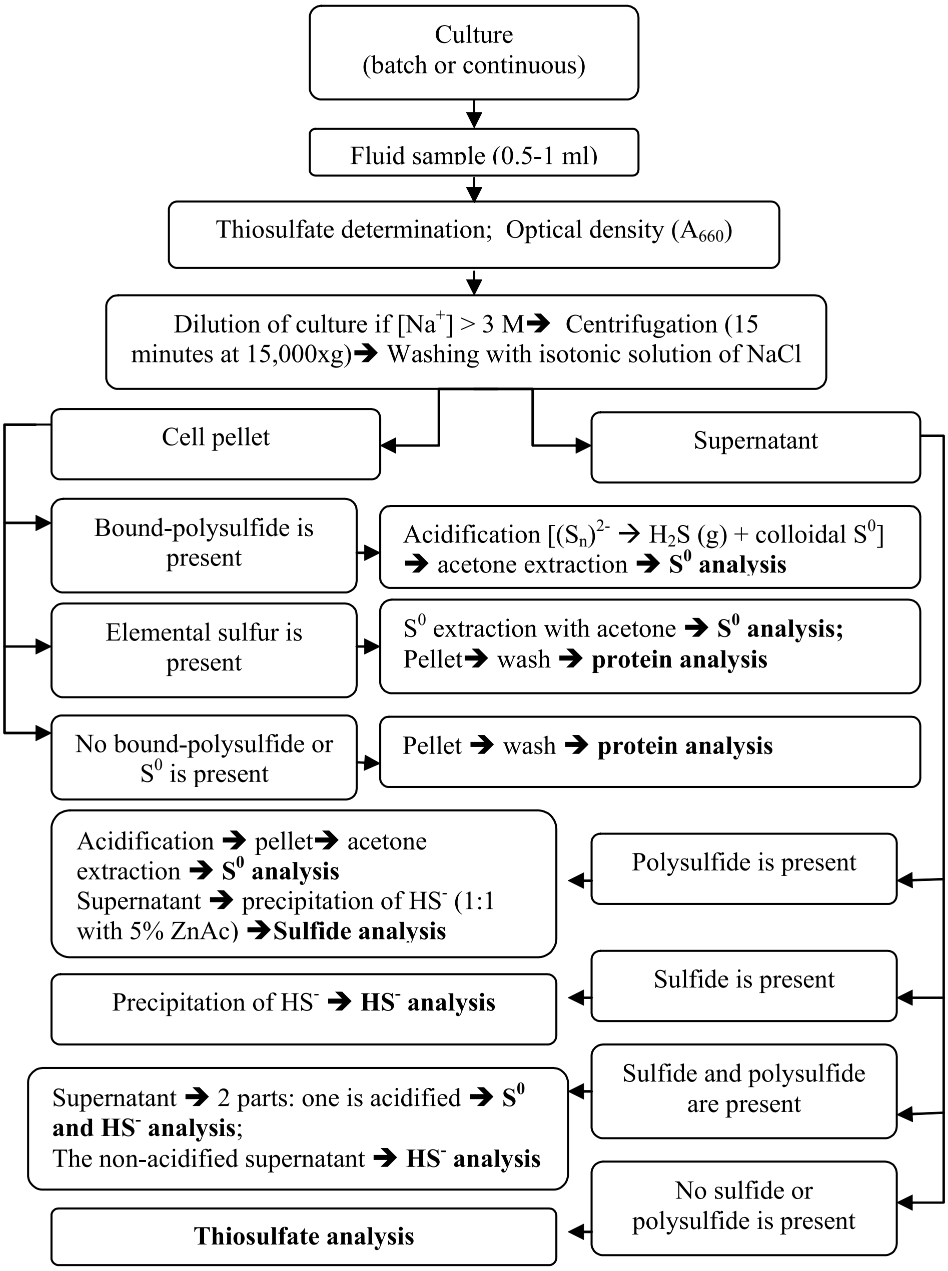

Fig. 1 The sampling diagram of pure cultures of sulfur-oxidizing bacteria from soda lakes.

Sörbo method for determination of elemental sulfur

Principle

This method is used for the determination of elemental sulfur cultures and cell suspensions. This assay is based on the conversion of sulfur to thiocyanate. In a next step, thiocyanate forms a complex with Fe^{3+}, which has an absorbance maximum at 460 nm. Also, the thiosulfate can be determined. In this case thiosulfate is cyanolyzed to thiocyanate and sulfite.

Reaction mechanisms of sulfur determination:

$S^0 + CN^- \rightarrow SCN^-$

$SCN^- + Fe^{3+} \rightarrow \text{at low pH} \rightarrow Fe(SCN)_3$

Other reaction mechanisms:

$S_2O_3 + CN^- \rightarrow \text{low temperature (T)}, Cu^{2+} \rightarrow SCN^- + SO_3^{2-}$

$S_4O_6 + 3CN^- + H_2O \rightarrow \text{low T} \rightarrow SCN^- + S_2O_3^{2-} + SO_4^{2-} + 2HCN$

$S_4O_6 + CN^- \rightarrow \text{low T}, Cu^{2+} \rightarrow 2\ SCN^- + 2\ SO_3^{2-}$

$S_3O_6 + 3CN^- + H_2O \rightarrow Cu^{2+}, 100°C \rightarrow SCN^- + SO_3^{2-} + SO_4^{2-} + 2HCN$

Reagents

- Acetone
- KCN solution (815 mg/100 ml) (stored at 4°C);
- $Fe(NO_3)_3$ solution 12.5% (w/v) in 20% HNO_3 (6.15 ml of 65% solution per 100 ml) (stored at room temperature);
- Stock solution of elemental sulfur in acetone (1 mM).

Procedure

Sampling and sulfur extraction

- The culture samples (0.5-1.0 ml) are diluted 1:1 with 0.6 M of NaCl solution (for cultures with 0.6-3 M of Na^+) and centrifuged (15-20 min. at 15,000xg).
- Cell pellets are washed in 0.6 or 2.0 M NaCl (depending on the growth history).

- 1.0 ml of acetone is added to the suspension, mixed vigorously and incubated at room temperature overnight. Samples can be stored in acetone at room temperature for several weeks. The extraction period can be shortened by incubationg the sample at 35^{O}C for 2-4 hours with frequent mixing.
- If the production of bound polysulfide is expected, an acidification step is necessary. This consists in addition of few drops of a concentrated solution of HCl to the cell suspension. The suspension is mixed vigorously and incubated at room temperature for 10 minutes. The suspension is centrifuged and the pellet is washed twice with NaCl solution. From this pellet both elemental sulfur and the sulfur resulted from the polysulfide decomposition can be extracted.
- After extraction, the suspension mixture is vortexed vigorously and centrifuged at high speed for a short time (5 min. at 15,000xg). The supernatant is used for the Sörbo method and the pellet can be used for protein determination after it has been washed with 0.6 M NaCl.

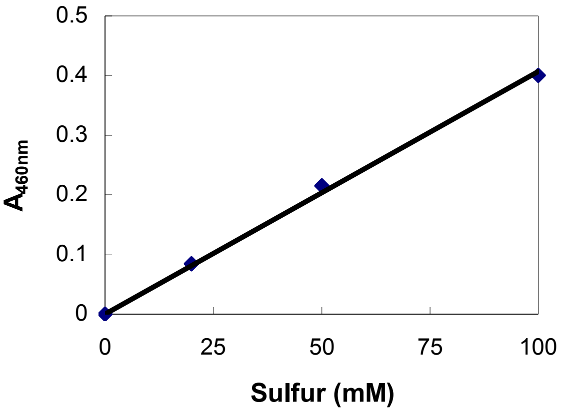

Fig. 2. Calibration curve for sulfur determination by the Sörbo method as used by author. The coefficient is 240 and the r^2 0.9979.

Sulfur determination

Maximum solubility of elemental sulfur in acetone is ≈ 17 mM. For a reliable sulfur extraction, elemental sulfur concentration in acetone should be lower than 10-12 mM. The elemental sulfur detection by Sörbo method is linear from 20-300 μM.

- The samples are diluted 10-50 times into acetone to a final concentration of ≈ 100 µM.
- 1 ml of (diluted) sample is pipetted in a test tube. 1.0 ml acetone serves as control.
- After 0.1 ml of CN^- solution is added, the reaction solution is mixed and incubated for 10 min.
- 0.1 ml $Fe(NO_3)_3$ solution is added, mixed and the OD is measured immediately at 460 nm.
- Standards are prepared in duplicates (20-300 µM sulfur in acetone)
- The absorbances of standards and samples are read at 460 nm and a calibration curve is drawn. An example of calibration curve is given in **Fig. 2**.

Reference

Sörbo B (1957) A colorimetric determination of thiosulfate. Biochim Biophys Acta 23:412-416

Kelly method for determination of thiosulfate

Principle

This method is used for the determination of thiosulfate, trithionate and tetrathionate in cultures and cell suspensions. This assay is based on the alkaline cyanolysis of sulfur from these compounds to thiocyanate. Thiocyanate then forms a complex with Fe^{3+} that has an absorbance at 460 nm. Thiosulfate, trithionate and tetrathionate react with cyanide to form thiocyanate according to the reaction equations shown below.

Reaction mechanisms:

$S_2O_3 + CN^- \rightarrow \text{low T}, Cu^{2+} \rightarrow SCN^- + SO_3^{2-}$

$S_4O_6 + 3CN^- + H_2O \rightarrow \text{low T} \rightarrow SCN^- + S_2O_3^{2-} + SO_4^{2-} + 2HCN$

$S_4O_6 + CN^- \rightarrow \text{low T}, Cu^{2+} \rightarrow 2\,SCN^- + 2\,SO_3^{2-}$

$S_3O_6 + 3CN^- + H_2O \rightarrow Cu^{2+}, 100°C \rightarrow SCN^- + SO_3^{2-} + SO_4^{2-} + 2HCN$

Materials

- 0.1 M KCN; 3.25 g KCN dissolved in 500 ml of deionized water; stored at 4°C;

- 1.5 M $Fe(NO_3)_3$ solution in 4 M perchloric acid ($HClO_4$) (stored at room temperature); 303 g of $Fe(NO_3)_3 \bullet 9H_2O$ dissolved in 172 ml of 70 % perchloric acid (11.64 M) and adjusted to 500 ml with deionized water;
- 0.1 M $CuSO_4$**;** 4 g of $CuSO_4$ dissolved in 250 ml of deionized water;
- 0.1 M NaH_2PO_4-NaOH buffer (pH 7.4)**;** 12 g of NaH_2PO_4 dissolved in 400 ml of deionized water, pH is adjusted to 7.4 with 1 M NaOH; finally the volume is brought up to 500 ml.
- 0.1 M stock solution of thiosulfate ($Na_2S_2O_3 \bullet 5H_2O$).

Procedure

Thiosulfate determination (**procedure II** after Kelly et al., 1969):

Thiosulfate detection by the Kelly method is linear from 20-250 µM. Minimal thiosulfate concentration of the samples is therefore 100-1250 µM.

- The appropriate amount of sample is pipetted into a test tube (to achieve a sample concentration of 20-250 µM in final assay volume).
- Deionized water is added to a total volume of 2.3 ml.
- 0.8 ml phosphate buffer (0.2 M, pH 7.4) is added to the reaction mixture.
- 1.0 ml of KCN solution is added, mixed and incubated for 20-30 minutes at 4°C.
- 0.3 ml of Cu^{2+} solution is added, mixed rapidly and incubated 5 min. at room temperature.
- 0.6 ml $Fe(NO_3)_3$ solution is added, mixed and OD is measured immediately at 460 nm.
- Stoichiometry: $S_4O_6^{2-} \rightarrow 2SCN^-$ and $S_2O_3^{2-} \rightarrow 1SCN^-$

An example of calibration curve is presented in **Fig. 3**.

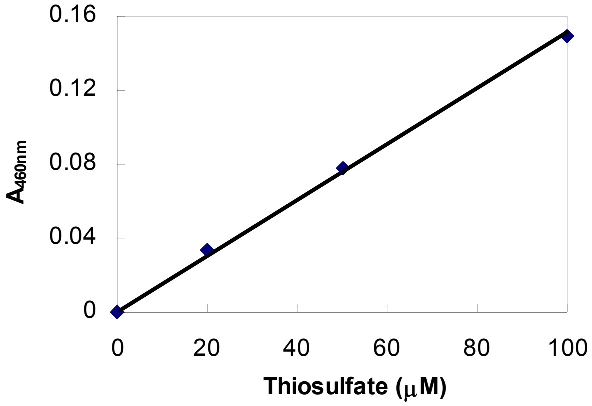

Fig. 3. Calibration curve for thiosulfate determination by Kelly method as used by author. The coefficient is 745 and the r^2 0.9980.

Reference

Kelly DP, Chambers LA, Trudinger PA (1969) Cyanolysis and spectrophotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. Anal Chem 41:898-901.

Thiosulfate titration method

Principle:

This method is used for fast and rough estimation of the (remaining) thiosulfate concentration in the medium. The method is based on the titration of thiosulfate with iodine at the presence of starch. The reaction should be performed at acid pH (4.5-6.5) to prevent formation of sulfate ($S_2O_3^{2-} + I_2 \rightarrow (OH^-) \rightarrow 2I^- + SO_4^{2-}$). Also the titration should be done rapidly since thiosulfate is decomposed at acid pH.

Reaction mechanism:

Partial:

$2S_2O_3^{2-} \rightarrow S_4O_6^{2-} + 2e^-$

$I_2 + 2e^- \rightarrow 2I^-$

Total:

$2S_2O_3^{2-} + I_2 \rightarrow (\text{acid pH}) \rightarrow S_4O_6^{2-} + 2\,I^-$

Materials

- Stock 50 mM I_2 solution is prepared from ampulles with I_2, the contents are dissolved in 0.5 l deionized water. 5 g KI are added to 1 liter of 50 mM I_2 solution. Bottles are wrapped in aluminium and stored at 4°C.
- 5 mM I_2 in 5% acetic acid: 50 ml of 50 mM I_2 are diluted to 400 ml, then 25 ml of concentrated acetic acid is added. 1 g KI is added to this solution. The volume is adjusted to 500 ml, bottles are wrapped in aluminium and stored at 4°C.
- Stock solution of 10% starch: 10 g of soluble starch are dissolved in 20 ml deionized water. The paste is diluted with 80 ml of warm deionized water.
- Starch solution (1%): 10 ml of stock starch solution are added to to 90 ml of deionized water.
- 50 % acetic acid.
- 0.1 M stock solution of thiosulfate ($Na_2S_2O_3 \bullet 5\,H_2O$); 2.48 g of $Na_2S_2O_3 \bullet 5\,H_2O$ are dissolved in 100 ml deionized water. 0.1 ml of chloroform per liter is added to prevent microbial growth.

Note: KI is added to I_2 solution to prevent or slower the spontaneous decomposition of I_2 to I^-. High concentration of I^- (product of decomposition) inhibits this decomposition of I_2. The ratio of I_2/I^- used, should not be too high since thiosulfate is then oxidized to sulfate (due to presence of OH^-).

Procedure

Calibration of 5 mM I_2 solution:

- To 10 ml of deionized water are added 0.5 ml of 1% starch solution.
- 0.1 ml of $Na_2S_2O_3$ (0.1 M) solution is added to the above mixture.
- The mixture should be titrated with about 1 ml of I_2 solution (5 mM).
- Adjusting normality: If more I_2 solution is used (e.g. 1.1 ml instead of 1.0 ml) I_2 solution (50 mM) is added to the 5 mM I_2 solution to adjust the final concentration to 5 mM (e.g. 5 ml of 50 mM I_2).

Thiosulfate titration:

- To 10 ml of water in a small beaker is sdded a sufficient amount of acetic acid to equilibrate the alkaline sample (e.g. 0.25 ml 50% acetic acid for 0.5 ml sample of 0.6 M sodium carbonate medium).
- 200 µl of 1% starch solution is added and mixed.
- An appropiate amount of sample (e.g. 0.5 ml) is added.
- The mixture is titrated with the 5 mM I_2 (in 5% acetic acid) solution
- The necessary amount of I_2 to reach point of titration (blue color) is estimated.

Calculation: $[Thiosulfate, mM] = \frac{V(I_2) \bullet M(I_2)}{V(Sample)}$ where $V(I_2)$ is the volume of I_2 used (ml); $M(I_2)$ is the molarity of I_2 solution (5 mM); V(Sample) is the volume of sample titrated (ml).

Turbidimetric determination of sulfate ions (SO_4^{2-})

Principle

Sulfate is precipitated with $BaCl_2$ as $BaSO_4$. The precipitate is resuspened in a viscous solution containing glycerol, ethanol and NaCl. The turbidity of the suspension is measured at 420 nm.

Materials

- 100 mM Na_2SO_4 (or K_2SO_4) in MilliQ water
- "Conditioning Reagent": 150 g NaCl, 100 ml glycerol, 60 ml concentrated HCl and 200 ml 95% ethanol up to 1 l with deionized water).
- Solid crystals of $BaCl_2$.
- Sulfate stock solution (80 mM Na_2SO_4).

Procedure

- To 0.5 ml of neutralized sample, 1 ml of conditioning reagent is added, the mixture is vortexed vigorously.

- 60 mg of crushed barium chloride is dissolved in 0.5 ml deionized water and added; the turbid solution is vortexed for 30 s. This step is critical because only a thorough mixing assures reliable results.
- The OD of the mixture is immediately measured at 420 nm against a blank consisting of the complete reaction mixture excluding sulfate.
- The calibration curve is drawn between 0.5 and 2 mM sulfate. This is a polynomial curve with a linear part between 0.5 and 1.5 mM sulfate (**Fig. 4**).

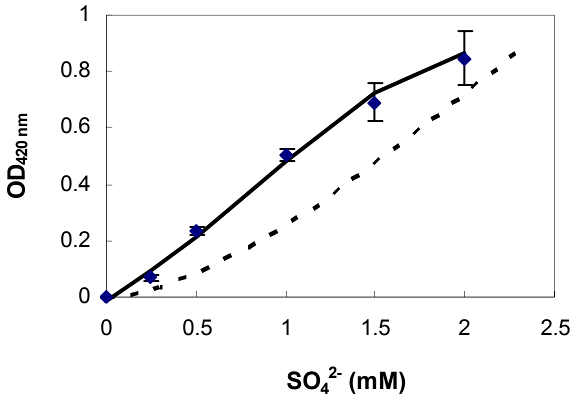

Fig. 4. Calibration curve for sulfate determination by turbidimetric method as used by author (straight line) or as given by Kolmert et al. (2001) (dotted line). The coefficient is 2.05.

Reference

Kolmert A., Wikström P., Hallberg K.B. (2000) A fast and simple turbidimetric method for the determination of sulfate in sulfate-reducing bacterial cultures. J Microbiol Meth 41:179-184

Methylene Blue method (determination of sulfide and polysulfide)

Principle

This method is used for the determination of sulfide and polysulfide in cultures and cell suspensions. This assay is based on the precipitation of sulfide (or polysulfide-sulfane atoms) with ZnAc and subsequent conversion of dimethyl-p-phenylenediamine sulfate with hydrogen sulfide and ferric iron to methylene blue in acidic medium.

Materials:

- 5 % (w/v) ZnAc (stored at room temperature); 50 g of ZnAc dissolved in 800 ml of distilled water. To this solution, 0.2 ml of concentrated acetic acid is added and the final volume is adjusted to 1000 ml.
- Reagent A: 2 g dimethyl-p-phenylenediamine (oxalate salt) dissolved in 200 ml of deionized water, 200 ml of concentrated H_2SO_4 are added and the volume is brought to 1 liter; stored in a brown bottle.
- Reagent B: 10 g $Fe(NH_4)(SO_4)_2 \bullet 12\ H_2O$ dissolved in 50 ml deionized water, 2 ml of concentrated H_2SO_4 is added and the volume is adjusted to 100 ml with water.
- Stock 0.1 M Na_2S in 1 mM EDTA, prepared anaerobically: 2.4 g of $Na_2S \bullet 9H_2O$ are dissolved in 100 ml of boiled and argon flushed 1 mM Na_2EDTA (pH 10). From this stock solution a concentration series is prepared in 1-2.5% ZnAc solution (final concentration in assay (5 ml) should be 2-35 µM.

Procedure:

Fixation of sulfide:

Sulfide detection by the Methylene Blue method is linear from 2-35 µM (concentration in the final assay volume).

- To 0.5 ml culture fluid is added 0.5 ml ZnAc (5%);
- The solution is mixed and incubated for 30 minutes at room temperature (until precipitation of ZnS is completed).

- If thiosulfate is present, the ZnS pellets should be washed with deionized water and resuspended in the same volume of water. These fixed samples can be stored a long time.

Sulfide determination:

- The ZnS suspension is homogenized and pipetted to the test tube in order to reach a concentration of 2-35 μM in the assay volume.
- The volume is adjusted to 4.5 ml with deionized water.
- Simultaneously, 0.5 ml reagent A and 30 μl reagent B are added to test tube. The test tube is closed with a rubber stopper, mixed thoroughly and incubated for 15-30 min at room temperature.
- Before reading, the precipitate is removed by centrifugation. (2 min at 15,000xg)
- The absorbance is read at 660 nm.

Note:

The presence of elemental sulfur can result in a delay of the coloring and in an underestimation of about 15%. Therefore the elemental (colloidal or crystalline) sulfur should be removed from the samples (e.g. by centrifugation)

Reference

Trüper HG, Schlegel HG (1964) Sulphur metabolism in *Thiorhodaceae*. 1: Quantitative measurements on growing cells of *Chromatium okenii*. Antonie van Leeuwenhoek 30:225-238