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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 and 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 slow-growing, high-yield organisms with lower maintenance and much lower rates of oxidation of sulfide and thiosulfate. Moreover, the latter survived starvation much better than *Thioalkalimicrobium*. Different growth characteristics and salt resistance appear to determine the outcome of the enrichment cultures from different soda lakes: *Thioalkalimicrobium* dominated in the enrichments with freshly obtained samples from diluted soda lakes at low-medium salinity, while *Thioalkalivibrio* was the predominant organism in enrichments from aged samples and at hypersaline conditions. In mixed thiosulfate-limited chemostat cultures at low salinity, *Thioalkalimicrobium* strains ($\mu_{max} = 0.33\ h^{-1}$) out-competed *Thioalkalivibrio* strains ($\mu_{max} = 0.15\ h^{-1}$) at $D > 0.02\ h^{-1}$. The overall results suggest that *Thioalkalimicrobium* and *Thioalkalivibrio* represent two different ecological strategies.

Keywords Competition · Halolkaliphilic · Soda lakes · Sulfur-oxidizing bacteria · *Thioalkalimicrobium* · *Thioalkalivibrio*

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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) have 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 concentrations. 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 a 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, 2001c). 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 in both pure and mixed continuous cultures indicate that the genus *Thioalkalimicrobium* represents an ecological strategy of the *R*-type, while the genus *Thioalkalivibrio* fits a *K*-type.

Materials and methods

Samples and strains

Samples of the surface sediments and water from 20 northeast 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, and 360. The 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 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 22 g l^{-1} Na_2CO_3; 8 g l^{-1} $NaHCO_3$; 6 g l^{-1} NaCl; 1 g l^{-1} K_2HPO_4; 0.5 g l^{-1} KNO_3; 0.1–0.2 g l^{-1} $MgCl_2 \cdot 6H_2O$; 1 ml l^{-1} trace elements (Pfennig and Lippert 1966); 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 a rotary shaker at 200 rpm and 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 $NaHCO_3$, and 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 fermenters with a 1-l working volume and fitted with pH and oxygen controls (Applikon, Schiedam, 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 1 g l^{-1} KH_2PO_4, 0.2 g l^{-1}/ $MgCl_2 \cdot 6H_2O$, 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; and 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*, such as 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, and became transparent and yellow-pigmented after prolonged incubation. This allows us to examine the competition results in enrichment cultures, when sub-maximum 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, strains were chosen that could easily be 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 Spring Instruments, Ohio). 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 auto-oxidation. 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 for 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 $NaHCO_3$; and 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 $q_{max}(O_2)$ and apparent K_s were measured with an oxygen electrode as described above using washed cells of the different bacteria grown in either 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, and 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 washout of the biomass and accumulation of thiosulfate started.

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 iodometric 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. 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 concentrations. 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 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

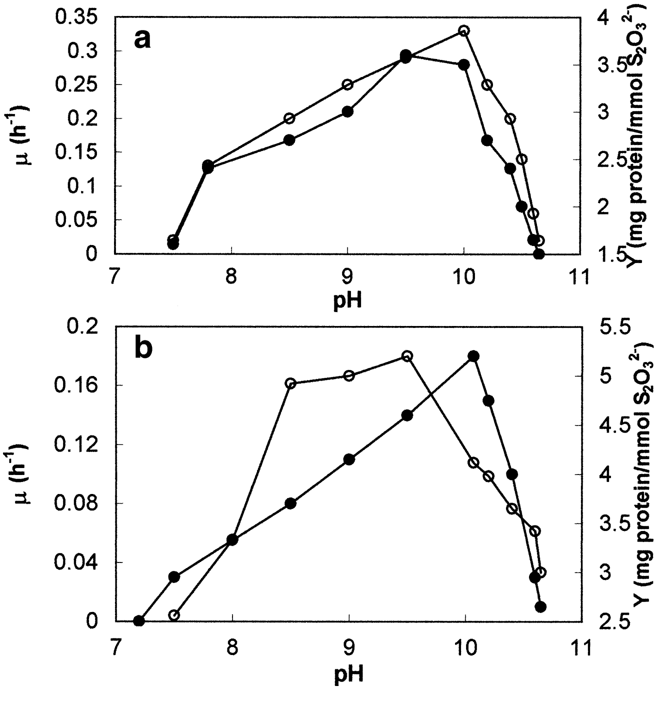

Fig. 1a, b Influence of pH on growth of moderately halophilic and alkaliphilic sulfur-oxidizing autotrophs in thiosulfate-limited and pH-controlled continuous culture at 0.5 M total Na^+. **a** *Thioalkalimicrobium aerophilum* AL 3. **b** *Thioalkalivibrio versutus* ALJ 7. *Open circles* Maximum specific growth rate (μ). *Closed circles* Maximum growth yield (*Y*) 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 $Mg(OH)CO_3$. 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 spheroplast formation and cell lysis. As indicated in Materials and methods, the μ_{max} values were determined by a washout curve. When washout 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 washout of the biomass started. The pH profiles for chemostat growth tested for another species of *Thioalkalimicrobium*, *Thioalkalimicrobium sibiricum* AL 7, was essentially the same as for *Thioalkalimicrobium 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).

Influence of salts on growth and activity of pure cultures

All of 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 belonging 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

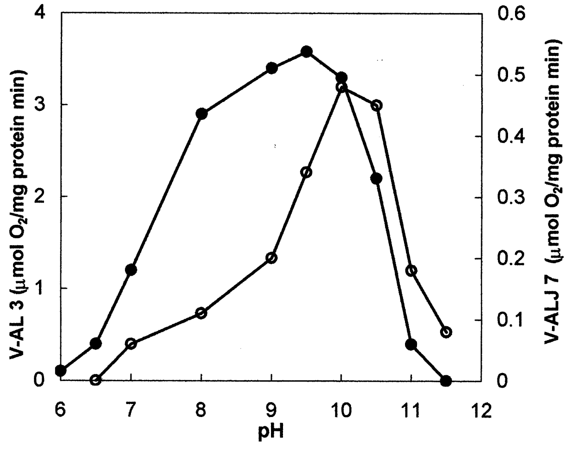

Fig. 2 Influence of pH on the activity of thiosulfate-dependent respiration by washed cells of *Thioalkalimicrobium aerophilum* AL 3 (*closed circles*) and *Thioalkalivibrio versutus* ALJ 7 (*open circles*). Buffers contained 0.6 M total Na^+ as NaCl at pH 6–8 in 0.1 M HEPES and combinations of $NaHCO_3/Na_2CO_3$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

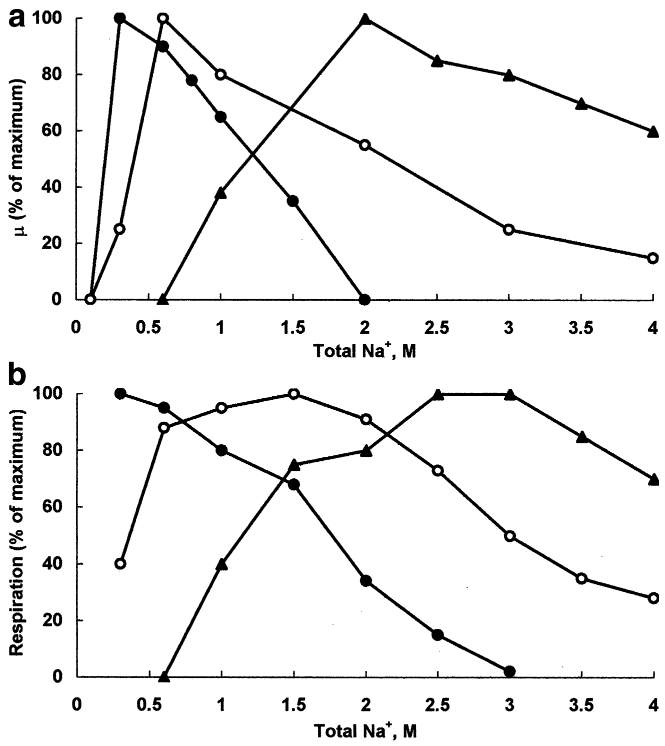

Fig. 3 Influence of sodium carbonate/bicarbonate on growth with thiosulfate (**a**) and on thiosulfate-dependent respiration activity of washed cells (**b**) at pH 10 in three different types of autotrophic sulfur alkaliphiles. *Closed circles Thioalkalimicrobium* and moderately salt-tolerant *Thioalkalivibrio* strains. *Open circles* extremely salt-tolerant *Thioalkalivibrio* strains. *Triangles* Halophilic *Thioalkalivibrio* strains

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

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 Materials and 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 *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 cannot 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 a 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.

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

For a further analysis of competitive behavior under energy limitation, two representatives of *Thioalkalimicrobium* and *Thioalkalivibrio* were used. Co-culturing 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 slow-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

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^+

Parameter	Dimension	Method	*Thioalkalimicrobium*		*Thioalkalivibrio*	
			Batch	Chemostat	Batch	Chemostat
Y	mg protein $(mmol\ S_2O_3^{2-})^{-1}$	Biomass measurements	2.3 (20)	Maximum: 3.5(2)	6.0 (15)	5.5 (5)
$K_s(S_2O_3^{2-}; HS^-)$	μM	BOM at pH 10	$3.5 \pm 1.5(5)$		$2.8 \pm 0.8(6)$	
m_s	$\mu mol\ S_2O_3^{2-}$ $(mg\ protein\ h)^{-1}$	*q*–*D* plot	n.d.	6 (1)	n.d.	2 (1)
$q_{max}(S)$		Experimental	n.d.	$94 \pm 2(1)$	n.d.	$25 \pm 1(5)$
$q_{max}(O_2)$ (thiosulfate)	$\mu mol\ O_2$ $(mg\ protein)\ h^{-1}$	BOM	225 (20)	240 (2)	33 (15)	30 (5)
μ_{max}	h^{-1}	Experimental (washout kinetics)	0.23 (20)	0.33 (2)	0.10 (15)	0.12 (5)

[a] Number in parentheses = number of strains tested. *n.d.* Not determined; *BOM* respirometry in the biological oxygen monitor; *Y* growth yield; K_s: affinity constant; m_s maintenance coefficient; $q_{max}(S)$ maximum substrate-utilizing potential; $q_{max}(O_2)$ maximum respiration activity with thiosulfate; μ_{max} maximum growth rate

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}) was strain ALJ 7 able to out-compete strain AL 3 (Fig. 4a). The course of the competition was complex. At first, within 2 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 $D \geq 0.05$ h^{-1} the result of the competition was opposite, with the total domination of strain AL 3 at both pH 10.05 and pH 10.5. Moreover, the course of the competition was straightforward, with rapid elimination of the loser (Fig. 4b).

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. The bacteria were pre-grown in batch culture with thiosulfate at pH 10 and 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. 5a). The actual cell death kinetics was much slower and was substantially different in the two strains. The viable population of strain AL 3 decreased by a factor of 10 within the first week and then rapidly dropped by several orders of magnitude. In contrast, the viability of strain ALJ 7 decreased by only one order of magnitude within 20 days of starvation (Fig. 5b). Overall, this experiment demonstrated a much higher resistance of the *Thioalkalivibrio* group to starvation in comparison with the *Thioalkalimicrobium* group.

Discussion

Among the non-halophilic 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,

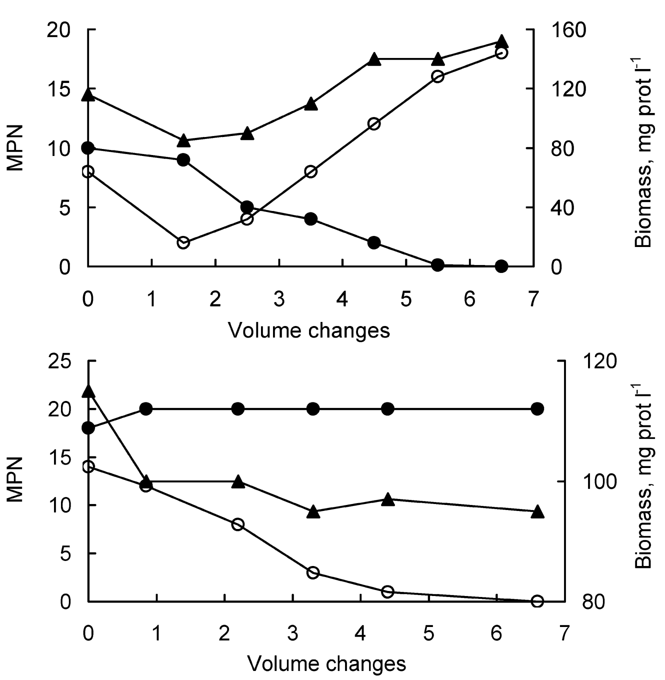

Fig. 4a, b Competition between two different strains of obligately autotrophic, alkaliphilic sulfur bacteria in thiosulfate-limited (40 mM) continuous culture at pH 10.05 and salt content 0.5 M total Na^+. **a** $D = 0.02$ h^{-1}. **b** $D = 0.05$ h^{-1}. MPN: viable counts on liquid medium with 80 mM thiosulfate at pH 10, $\times 10^8$ cells ml^{-1}. *Open circles Thioalkalivibrio versutus* ALJ 7. *Closed circles Thioalkalimicrobium aerophilum* AL 3. *Triangles* Biomass

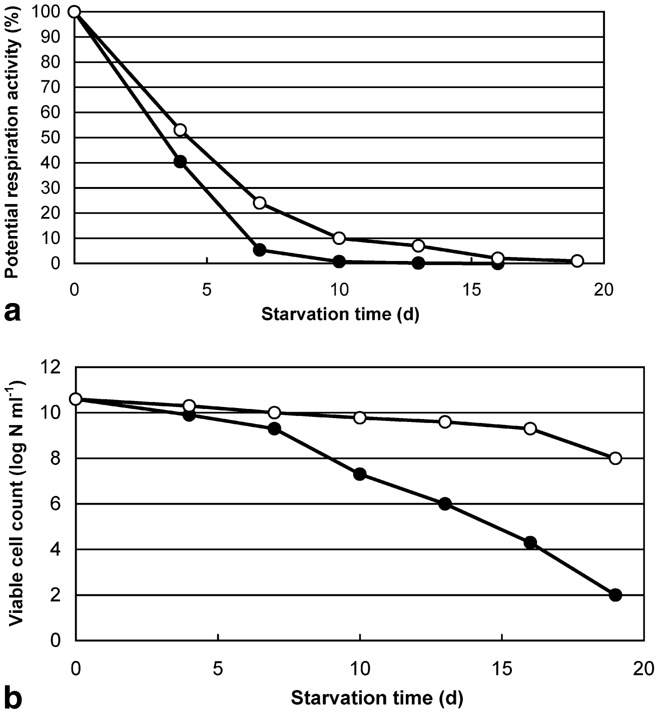

Fig. 5 Comparison of the respiratory inactivation (**a**) and the death rates (**b**) in alkaliphilic sulfur bacteria *Thioalkalimicrobium aerophilum* AL 3 (*closed circles*) and *Thioalkalivibrio versutus* ALJ 7 (*open circles*) at pH 10 and 30 °C during starvation. Batch cultures were pre-grown on mineral medium at pH 10 and 0.6 M total Na^+ until all thiosulfate was oxidized

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, in particular, the influence of pH and salts. Meanwhile, such data and their accuracy are extremely important for general biology because they extend our basic knowledge and understanding about the borders of life.

The two groups of sulfur-oxidizing bacteria 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 neutrophilic and acidophilic sulfur bacteria. In particular, the soda lake alkaliphiles grow optimally at pH values at which other sulfur oxidizers cannot grow at all. The highest validated pH limit demonstrated for heterotrophic alkaliphilic *Bacillus* strains 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 a 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 carbon 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 *Thiobacillus 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 that need not only high pH but also high salt content for optimal growth, with a minimal requirement of > 0.2 M Na^+. This might imply the possible presence of specific Na^+-dependent metabolic adaptations in such bacteria that are different from the well-known mechanisms of alkaliphily in non-halophilic *Bacillus* species (Krulwich et al. 1998), which would be very worthwhile to investigate. Interestingly, we observed that the nitrifiers, in contrast to the sulfur-oxidizing bacteria, were incapable of growing at extremely high salt concentrations (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 a 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 has a direct effect on the selection of different types of sulfur-oxidizing alkaliphiles even 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 has been observed in the Mongolian and the Kenyan lakes. We postulate that the latter type might be active during the rainy seasons when the salt content of the shallow soda lakes can decrease several-fold.

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 slow-growing autotrophs may out-compete 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 slow-growing *Thioalkalivibrio* strains and the 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 of 3. Competition experiments done under thiosulfate limitation showed that at $D = 0.02$ h^{-1} the *Thioalkalivibrio* sp. out-competed *Thioalkalimicrobium*. This confirmed the prediction made by modeling 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–Cs curves would be well above

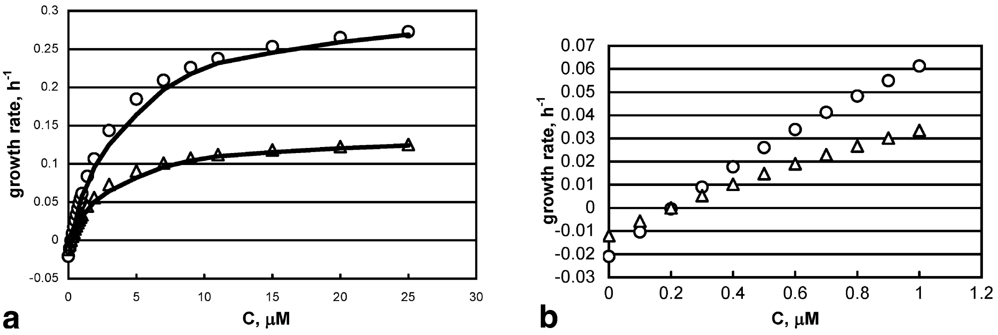

Fig. 6 Simulation of the μ–Cs curves within coarse (**a**) and fine (**b**) scales for the moderately halophilic, sulfur-oxidizing alkaliphiles *Thioalkalimicrobium aerophilum* AL 3 (*circles*) and *Thioalkalivibrio versutus* ALJ 7 (*triangles*) based on Pirt's growth equation: $\mu = [q_{\max}(S) \cdot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

$\mu = 0.02\ h^{-1}$ (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 the case of unlimited substrate supply in batch cultures. On the basis of its growth kinetic parameters (low growth efficiency at a very high substrate consumption rate), the *Thioalkalimicrobium* group can be regarded as a typical *R*-strategist, the ecotype that becomes dominant in the system during short periods of excessive substrate supply, being able to out-compete 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 a 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 slow-growing *Thioalkalivibrio* to take over. Low growth rates and maintenance and the ability to survive prolonged starvation are characteristic properties of *K*-strategists.

Considering the substantial differences 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, particularly during starvation (N. Loiko and D. Sorokin, unpublished). These studies have demonstrated that *Thioalkalivibrio versutus* formed several orders of magnitude more cyst-like resting cells in a starving population than did *Thioalkalimicrobium aerophilum*, which might be one of the reasons for the much better survival of the former. On the other hand, the cyst-like resting cells of *Thioalkalimicrobium*, although fewer in number, 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 numbers. 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 concentration – the dominant extreme factors in such an unusual environment. Moreover, despite narrow physiological specialization common for obligately autotrophic bacteria, the example of haloalkaliphilic, sulfur-oxidizing representatives demonstrates the presence of substantially different subtypes within the same physiological type of the chemolithoautotrophs.

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