Extremophiles (2004) 8:185–192
DOI 10.1007/s00792-004-0376-5

ORIGINAL PAPER

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

Received: 17 July 2003 / Accepted: 25 December 2003 / Published online: 26 February 2004

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 pH 0.6, 2 M 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 was in the micromolar range ($K_s = 6 \pm 3$ μM). The maintenance requirement ($m_s = 8 \pm 5$ 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.

Keywords Continuous cultivation · Gibbs free energy dissipation · Haloalkaliphilic · Polysulfide oxidation · Salt adaptation · Thioalkalivibrio

Communicated by W.D. Grant

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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* and *Thioalkalivibrio* species were the first characterized aerobic, chemolithoautotrophic, haloalkaliphilic bacteria that grow at pH 9–10 and at salt concentrations ranging from 0.3 M 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, 2001b). 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: (1) low salt-tolerant alkaliphilic *Thioalkalimicrobium* species (growing in up to 1.3 M Na^+), (2) extremely

salt-tolerant (growing in between 0.5 M and 4 M Na^+), and (3) 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, 2001b).

Although a large amount of data have 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-type 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 what 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 *T. versutus* strain ALJ 15 is discussed.

Materials 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 to 10.6, and at temperatures up to 50°C.

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°C. Thiosulfate and polysulfide were sterilized separately as 2 M or 800 mM solution, respectively, and added to the alkaline medium at approximately 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 it 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 five 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 (Steudel et al. 1986; Teder 1967). The mixtures were autoclaved at 120°C for 20 min 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).

Respiration measurements

Cells were collected from the effluent on an ice bath for a maximum period of 24 h. Withdrawal of large volumes of sample directly from the chemostat would have disturbed the steady state. No

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 zero-valent 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

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 resuspended 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 Spring Instruments, Yellow Spring, Ohio) 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 three to five independent measurements with standard deviations less than 10%.

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 three 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 K^+ and Cl^- was performed by instrumental neutron activation analysis at the 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, San Jose, Calif.), a 3-μm Grom-sil Amino-1PR column (Grom, Rottenburg-Hailfingen, Germany), and a Shodex refractive index detector (model RI17, Showa Denko KK, Tokyo). 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.

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 a 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 one 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 *T. 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:

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

$$\Delta G^0\ (308\ K, pH\ 10) = -1711\ kJ/reaction \quad (1)$$

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 *T. 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,

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 concentrations and at pH 10

Salinity (M Na^+)	Growth substrate	μ_{max} (h^{-1})	Y_{max}[a] (g dry weight/ mol substrate)	Y_{max}[a] (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	6.0
2	S_8^{2-}	0.21	8.5	5.0
4	$S_2O_3^{2-}$	0.11	6.1	4.0

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

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^+.

The 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 *T. 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 *T. 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 *T. versutus* strain ALJ 15, glycine betaine represents 5–9% of total dry weight at 2 M 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. 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 K^+ and 0.1% of the dry weight for Cl^-. The error limits were between 5–20% for K^+ and 1.7–3.4% for Cl^-.

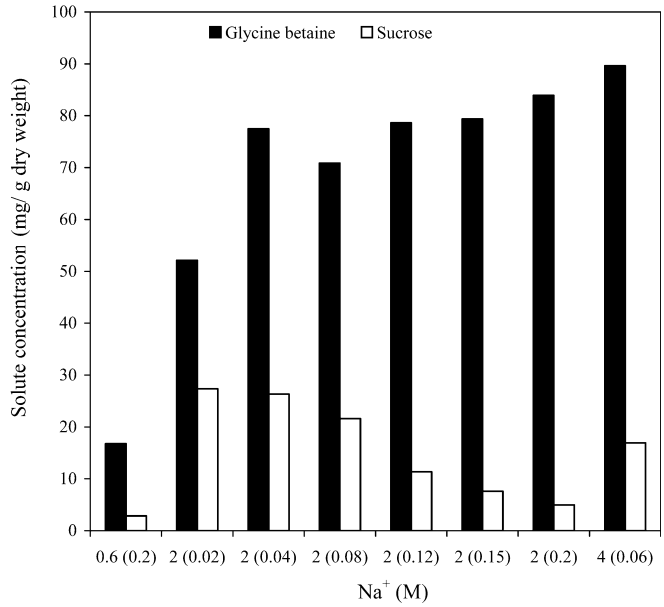

Fig. 1 Compatible solutes composition of *Thioalkalivibrio versutus* strain ALJ 15 grown in continuous culture in 0.6, 2 M and 4 M Na^+, pH 10. The numbers in the *parentheses* represent the dilution rates (D, h^{-1})

Growth-rate dependence of biomass production, maintenance requirement and ΔG_{DIS}

During growth of *T. versutus* strain ALJ 15 at different dilution rates, the biomass yield values did not change significantly. At 0.6 M and 2 M Na^+, the yields were already high at a low specific-growth rate (0.02–0.04 h^{-1}), 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. 2). At this 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 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 *T. 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.

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 *T. versutus* strain ALJ 15 gave values of 6 ± 3μM. 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 *T. versutus* strain ALJ 15 produced elemental sulfur. The sulfur production was higher at 0.6 M 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).

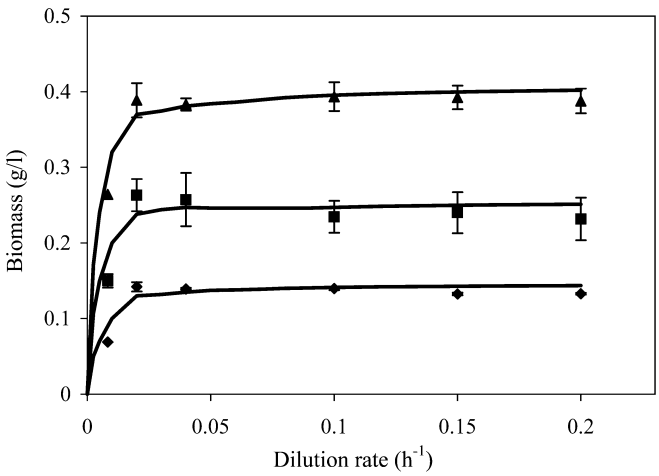

Fig. 2 Steady-state biomass concentration as a function of dilution rate in *T. versutus* ALJ 15 grown in thiosulfate-limited chemostat at 2 M Na^+, pH 10, and 35°C. ▲ dry weight, ■ protein, ♦ total organic carbon

The cultures grown at different salt concentration and substrate limitation were tested for their capacity of sulfur substrate utilization. The values of maximum specific rates of $qO_{2\ max}$ 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, 1970b). Sulfate was the final product of thiosulfate, sulfide and polysulfide oxidation.

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 *T. 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^+ ($qO_{2\ max} = 0.75$ μmol O_2/mg protein min^{-1}).

Table 3 Optimal growth conditions, 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)	Nitrogen source	Y_{max} (g dry weight/ mol substrate)	ΔG_{DIS} (kJ/g dry weight)	References
Acidiphilium acidophilum	3	30	NH_4^+	5.7	123.8	Heijnen and van Dijken (1992)
Halothiobacillus halophilus	7.1	30	NH_4^+	6.4	113.1	Wood and Kelly (1991)
Acidithiobacillus ferrooxidans	2	30	NH_4^+	6.3	105.1	Hazeu et al. (1986)
Paracoccus versutus	7.8	30	NH_4^+	8.3	83.8	Mason et al. (1987)
Halothiobacillus neapolitanus	6.8	30	NH_4^+	8.6	80.2	Mason et al. (1987)
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	Wood and Kelly (1986)
Thermothrix thiopara	6.8	65	NH_4^+	18.0	32.5	Mason et al. (1987)
Thiobacillus denitrificans	7	30	NH_4^+	20.6	21.1	Kelly (1999)

Table 4 Maximum specific rate of oxygen consumption ($qO_{2\ max}$) of *T. versutus* strain ALJ 15 grown in continuous culture under energy limitation at different salt concentrations, 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 averages ± standard deviations of three independent experiments

Salt concentration (M Na^+)	Growth substrate	$qO_{2\ max}$ (μmol 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

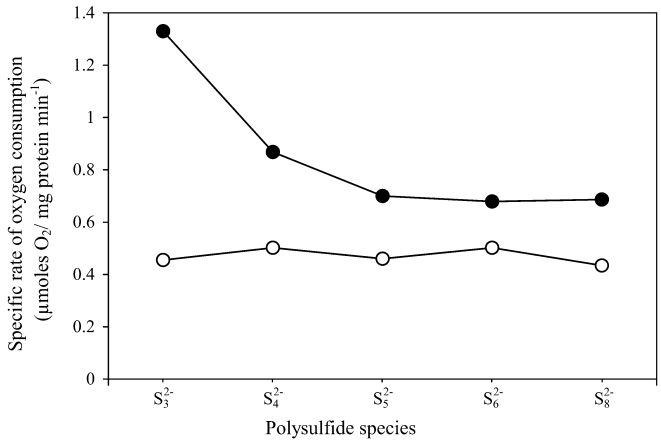

Fig. 3 Oxygen consumption rates as a function of polysulfide length (S_n^{2-}) of washed cells of *T. versutus* strain ALJ 15 pregrown under polysulfide (S_8^{2-})- limiting conditions at 2 M Na^+, pH 10, and $D = 0.04\ h^{-1}$. Polysulfide concentration was 50 μM total sulfur. • first stage of oxidation, ○ second stage of oxidation

The oxidation of polysulfides of different length (S_n^{2-}) was studied with washed cells of *T. 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. 3). From this point of view, trisulfide was the fastest oxidizable polysulfide substrate.

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 concentrations. 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 M 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^+.

$$4S_2O_3^{2-} + 20H_2O \rightarrow 8SO_4^{2-} + 40H^+ + 32e^- \quad 8e^-/\text{mol[S]}$$
$$S_8^{2-} + 32H_2O \rightarrow 8SO_4^{2-} + 64H^+ + 50e^- \quad 6.25e^-/\text{mol[S]} \qquad (2)$$

Thus, the use of either thiosulfate or polysulfide yielded a 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 to reach near-maximum biomass yield at

Table 5 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)	References
T. tepidarius	$S_2O_3^{2-}$	0.4	10.7	nd[a]	Wood and Kelly (1986)
H. halophilus	$S_2O_3^{2-}$	0.072	6.4	0.86–1	Wood and Kelly (1991)
Thiobacillus W5	HS^-	0.2	6.4	nd	Visser (1997)
Thiobacillus W1	HS^-	0.18	5.5	nd	Stefess (1993)
Thiobacillus o	$S_2O_3^{2-}$	0.3	4.2	nd	Stefess (1993)
Thiomicrospira thyasirae	$S_2O_3^{2-}$	0.038	2.5	nd	Wood and Kelly (1989)
H. kellyi	$S_2O_3^{2-}$	0.45	nd	0.45	Sievert et al. (2000)
H. hydrothermalis	$S_2O_3^{2-}$	0.6	2.5	0.43	Wood and Kelly (1988)

[a]*nd* No data

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).

In the energetic balance of the halophiles, one must take into account the production of compatible solutes. 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 K^+ 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 Na^+ 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 *T. versutus* strain ALJ 15 imply 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 the 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.

From the results obtained with polysulfides of different degrees of polymerization, it is clear that the sulfane atoms from polysulfide chains are oxidized first. It is possible that different forms of sulfur atoms from polysulfide molecules 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) are selected by haloalkaliphilic sulfur-oxidizing chemolithoautotrophs, such as *T. versutus* strain ALJ 15. This could 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).

Acknowledgements This work was financially supported by the Dutch Technology Foundation (STW) projects DST.4653 and WCB.5939.

References

Baumgarte S (2003) Microbial diversity of soda lake habitats. PhD thesis, Carolo-Wilhelmina University, Braunschweig, p 197

Chen KY, Gupta SK (1973) Formation of polysulfides in aqueous solution. Environ Lett 4:187–200

Duckworth AW, Grant WD, Jones BE, van Steenbergen R (1996) Phylogenetic diversity of soda lakes alkaliphiles. FEMS Microbiol Ecol 19:181–191

Galinski EA (1995) Osmoadaptation in bacteria. Adv Microb Physiol 37:272–328

Galinski EA, Herzog RM (1990) The role of trehalose as a substitute for nitrogen-containing compatible solutes (*Ectothiorhodospira halochloris*). Arch Microbiol 153:607–613

Giggenbach WF (1972) Optical spectra and equilibrium distribution of polysulfide ions in aqueous solution at 20°. Inorg Chem 11:1201–1207

Giggenbach WF (1974) Equilibria involving polysulfide ions in aqueous sulfide solutions up to 240°. Inorg Chem 13:1724–1730

Ginzburg B, Dor I, Chalifa I, Hadas O, Lev O (1999) Formation of dimethyloligosulfides in Lake Kinneret: biogenic formation of inorganic oligosulfide intermediates under oxic conditions. Environ Sci Technol 33:571–579

Gorlenko VM, Namsaraev BB, Kulyrova AV, Zavarzina DG, Zhilina TN (1999) The activity of sulfate-reducing bacteria in bottom sediments of soda lakes of the southeastern Transbaikal region. Microbiology (Moscow) 68:664–670

Grant WD, Tindall BJ (1986) The alkaline saline environment. In: Herbert RA, Codd GA (eds) Microbes in extreme environments. Academic, London, pp 25–54

Gun J, Goifman A, Shkrob I, Kamyshny A, Ginzburg B, Hadas O, Dor I, Modestov AD, Lev O (2000) Formation of polysulfides in an oxygen-rich freshwater lake and their role in the production of volatile sulfur compounds in aquatic systems. Environ Sci Technol 34:4741–4746

Hazeu W, Bijleveld W, Grotenhuis JT, Kakes E, Kuenen JG (1986) Kinetics and energetics of reduced sulfur oxidation by chemostat cultures of *Thiobacillus ferrooxidans*. Arch Microbiol 52:507–518

Hazeu W, Batenburg-van der Vegte WH, Bos P, Van der Pas RK, Kuenen JG (1988) The production and utilization of intermediary elemental sulfur during the oxidation of reduced sulfur-compounds by *Thiobacillus ferrooxidans*. Arch Microbiol 150:574–579

Heijnen JJ, van Dijken JP (1992) In search of a thermodynamic description of biomass yields for the chemotrophic growth of microorganisms. Biotechnol Bioeng 39:833–858

Humayoun SB, Bano N, Hollibaugh JT (2003) Depth distribution of microbial diversity in Mono Lake, a meromictic soda lake in California. Appl Environ Microbiol 69:1030–1042

Jones BE, Grant WD, Duckworth AW, Owenson GG (1998) Microbial diversity of soda lakes. Extremophiles 2:191–200

Kelly DP (1999) Thermodynamic aspects of energy conservation by chemolithotrophic sulfur bacteria in relation to the sulfur oxidation pathways. Arch Microbiol 171:219–229

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

Kolmert A, Wikstrom P, Hallberg KB (2000) A fast and simple turbidimetric method for the determination of sulfate in sulfate-reducing bacterial cultures. J Microbiol Methods 41:179–184

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin-phenol reagents. J Biol Chem 193:265–275

Ma Y, Zhang W, Xue Y, Zhou P, Ventosa A, Grant WD (2003) Bacterial diversity of the Inner Mongolian Baer Soda Lake as revealed by 16S rRNA gene sequence analyses. Extremophiles 8:45–51, DOI 10.1007/s00792-003-0358-z

Mason J, Kelly DP, Wood AP (1987) Chemolithotrophic and autotrophic growth of *Thermothrix thiopara* and some thiobacilli on thiosulphate and polythionates and a reassessment of the growth yields of *Thx. thiopara* in chemostat culture. J Gen Microbiol 133:1249–1256

Moriarty DJ, Nicholas DJ (1970a) Products of sulphide oxidation in extracts of *Thiobacillus concretivorus*. Biochim Biophys Acta 197:143–151

Moriarty DJ, Nicholas DJ (1970b) Electron transfer during sulphide and sulphite oxidation by *Thiobacillus concretivorus*. Biochim Biophys Acta 216:130–138

Oren A (1999) Bioenergetic aspects of halophilism. Microbiol Mol Biol Rev 63:334–348

Oren A (2002) Halophilic microorganisms and their environments. Kluwer, Dordrecht, The Netherlands

Pfennig N, Lippert KD (1966) Über das Vitamin B12-Bedürfnis phototropher Schwefelbacterien. Arch Microbiol 55:245–256

Rees HC, Grant WD, Jones BE, Heaphy S (2003) Diversity of Kenyan soda lake alkaliphiles assessed by molecular methods. Extremophiles DOI 10.1007/s00792-003-0361-4

Sievert SM, Heidorn T, Kuever J (2000) *Halothiobacillus kellyi* sp. nov., a mesophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium isolated from a shallow-water hydrothermal vent in the Aegean Sea, and emended description of the genus *Halothiobacillus*. Int J Syst Evol Microbiol 50:1229–1237

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

Sorokin DY, Lysenko AM, Mityushina LL (1996) Isolation and characterization of alkaliphilic heterotrophic bacteria capable of oxidation of inorganic sulfur compounds to tetrathionate. Microbiology (Moscow) 65:370–383

Sorokin DY, Robertson LA, Kuenen JG (2000) Isolation and characterization of alkaliphilic, chemolithoautotrophic, sulphur-oxidizing bacteria. Antonie van Leeuwenhoek 77:251–262

Sorokin DY, Lysenko AM, Mityushina LL, Tourova TP, Jones BE, Rainey FA, Robertson LA, Kuenen JG (2001a) *Thioalkalimicrobium aerophilum* gen. nov., sp. nov. and *Thioalkalimicrobium sibericum* sp. nov., and *Thioalkalivibrio versutus* gen. nov., sp. nov., *Thioalkalivibrio nitratis* sp. nov., novel and *Thioalkalivibrio denitrificans* sp. nov., novel obligately alkaliphilic and obligately chemolithoautotrophic sulfur-oxidizing bacteria from soda lakes. Int J Syst Evol Microbiol 51:565–580

Sorokin DY, Tourova T, Schmid MC, Wagner M, Koops HP, Kuenen JG, Jetten M (2001b) Isolation and properties of obligately chemolithoautotrophic and extremely alkali-tolerant ammonia-oxidizing bacteria from Mongolian soda lakes. Arch Microbiol 176:170–177

Sorokin DY, Banciu H, van Loosdrecht MCM, Kuenen JG (2003) Growth physiology and competitive interaction of obligately chemolithoautotrophic, haloalkaliphilic, sulfur-oxidizing bacteria from soda lakes. Extremophiles 7:195–203

Stefess GC (1993) Oxidation of sulphide to elemental sulphur by aerobic thiobacilli. PhD thesis, Delft University of Technology, p 128

Steudel R, Holdt G, Nagorka R (1986) On the autooxidation of aqueous sodium polysulfide. Z Naturforsch 41b:1519–1522

Teder A (1967) Spectrophotometric determination of polysulfide excess sulfur in aqueous solutions. Sven Papperstidn 70:197–200

Then J, Trüper HG (1983) Sulfide oxidation in *Ectothiorhodospira abdelmakeii*. Evidence for the catalytic role of cytochrome c-551. Arch Microbiol 135:254–258

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

Visser JM (1997) Sulfur compound oxidation and sulfur production by *Thiobacillus* sp. W5. PhD thesis, Delft University of Technology, p 107

Wood AP, Kelly DP (1986) Chemolithotrophic metabolism of the newly-isolated moderately thermophilic, obligately autotrophic *Thiobacillus tepidarius*. Arch Microbiol 144:71–77

Wood AP, Kelly DP (1988) Isolation and characterisation of *Thiobacillus aquaesulis* sp. nov., a novel facultatively autotrophic moderate thermophile. Arch Microbiol 149:339–343

Wood AP, Kelly DP (1989) Isolation and physiological characterisation of *Thiobacillus thyasiris* sp. nov., a novel marine facultative autotroph and the putative symbiont of *Thyasirae flexuosa*. Arch Microbiol 152:160–166

Wood AP, Kelly DP (1991) Isolation and characterisation of *Thiobacillus halophilus* sp. nov., a sulphur-oxidising autotrophic eubacterium from a Western Australian hypersaline lake. Arch Microbiol 156:277–280

Zavarzin GA, Zhilina TN, Kevbrin VV (1999) The alkaliphilic microbial community and its functional diversity. Microbiology (Moscow) 68:579–599