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ORIGINAL PAPER

**Horia Banciu · Dimitry Y. Sorokin
Erwin A. Galinski · Gerard Muyzer
Robbert Kleerebezem · J. Gijs Kuenen**

Thialkalivibrio 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, facultatively alkaliphilic, extremely salt-tolerant, sulfur-oxidizing bacterium was isolated from an alkaline hypersaline lake in the 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 *Thialkalivibrio* belonging to the γ subdivision of the *Proteobacteria* for which the name *Thialkalivibrio 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 of 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 sources and electron donors. 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.

Keywords Compatible solutes · Facultative alkaliphilic · Halophilic · Osmotic pressure · Soda · Sulfur oxidizing · *Thialkalivibrio halophilus*

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H. Banciu (✉) · D. Y. Sorokin · G. Muyzer · R. Kleerebezem
J. G. Kuenen
Department of Biotechnology, Delft University of Technology,
Julianalaan 67, 2628 BC, Delft, The Netherlands
E-mail: H.Banciu@tnw.tudelft.nl
Tel.: +31-15-2782425
Fax: +31-15-2782355

D. Y. Sorokin
Institute of Microbiology, Russian Academy of Sciences,
Prospect 60-let Octyabrya 7/2, 117811 Moscow, Russia

E. A. Galinski
Institute of Microbiology and Biotechnology,
Rheinische Friedrich-Wilhelms University,
Meckenheimer Allee, 168, 53115 Bonn, Germany

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) have 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, *Thialkalimicrobium* spp., *Thialkalivibrio* 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, 2002a, 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., submitted). 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 physicochemical 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: *Thialkalivibrio* (*Tv.*) *halophilus*.

Material and methods

Enrichment procedure and isolation of pure culture

Strain HL 17^T was isolated from the hypersaline (380 g l^{-1} total salts) and slightly alkaline (pH 9.05, total carbonate alkalinity 0.5 M) Stamp Lake in the south Kulunda Steppe (Altai, Russia). The enrichment was started by inoculating a sample in 4 M NaCl mineral medium containing 30 mM thiosulfate as the 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 the carbon source (20–50 mM).

A pure culture of an 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 240 g l^{-1} NaCl, 1.5 g l^{-1} K_2HPO_4, and 0.5 g l^{-1} NH_4Cl, with a pH of 7.5. After sterilization, 50 mM 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^{-1} 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, containing 4 M Na^+, was prepared by mixing seven volumes of 4 M soda medium (pH 10.0) with one volume of 4 M NaCl (pH 8) medium. The final concentration of sodium as carbonate/bicarbonate salt was 3.5 M, while the sodium chloride concentration was 0.5 M. The medium was strongly buffered at pH 9.6.

The 4 M soda medium contained 180 g l^{-1} Na_2CO_3, 38 g l^{-1} $NaHCO_3$, g l^{-1} $^{NaCl\ 6}$, 1 g l^{-1} K_2HPO_4, and 1 g l^{-1} KNO_3, with a pH 10. After sterilization all other salts were added as mentioned above. The electrical conductivity (mS/cm) was determined by using a pH and conductivity meter (Jenway, UK; model 4330). The osmotic pressure (osm/kg) of the growth media was determined using a vapor pressure osmometer (KNAUER, Berlin-Zehlendorf).

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 an approximately 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 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 five 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 milliliter 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 resuspended 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 Spring Instruments, Ohio, USA) connected to a chart recorder (Kipp and Zonen, model BD40). Different sulfur substrates were used at final concentrations of 34–50 μM.

The kinetic constants, maximum specific oxygen uptake rate (qO_{2max}) 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 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 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 3,000 *g*. 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). The interfering sulfur compounds were removed by two washing steps with 2 M NaCl solution (thiosulfate), followed by overnight acetone extraction (elemental sulfur). 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 $< 5\%$.

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

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 (San Mateo, Calif.), a 3-μm Grom-sil Amino-1PR column (Grom, Rottenburg-Haifingen), 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.

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 (5× concentrated, 2% SDS final) 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

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

Phylogenetic analysis

Genomic DNA was obtained with the DNA Plant Extraction kit from Mo Bio Laboratories (Carlsbad, Calif.). 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 neighbor-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 *Thialkalivibrio* in the γ subdivision of the *Proteobacteria* (Fig. 1) with as closest relative to *Tv. nitratis* (96.88% similarity). The DNA–DNA hybridization was used for further discrimination of strain HL 17^T from the other species of *Thialkalivibrio*. A relatively low similarity was found between strain HL 17^T and the type strain *Tv. versutus* AL 2 (29%) and the extremely salt-tolerant strain *Tv. jannaschii* ALM 2 (34%). The mol% G + C content of DNA from strain HL 17^T was $65.1 \pm 0.5\%$. 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 *Thialkalivibrio* for which the name *Tv. halophilus* is proposed.

Growth characteristics and optimal growth conditions

Batch cultivation of *Tv. 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 (0.2–5 M Na^+). The minimal salt requirement for growth and respiration was 0.2 M Na^+. Strain HL 17^T, unlike other species of *Thialkalivibrio* 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 and subsequent cell lysis.

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 17^T 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 five times higher number of viable cells in soda culture as compared to NaCl culture (5×10^9 vs $\leq 10^9$ cells ml^{-1}).

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 in the 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 *Tv. versutus* strain ALJ 15, grown under similar conditions (Banciu et al. 2004).

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

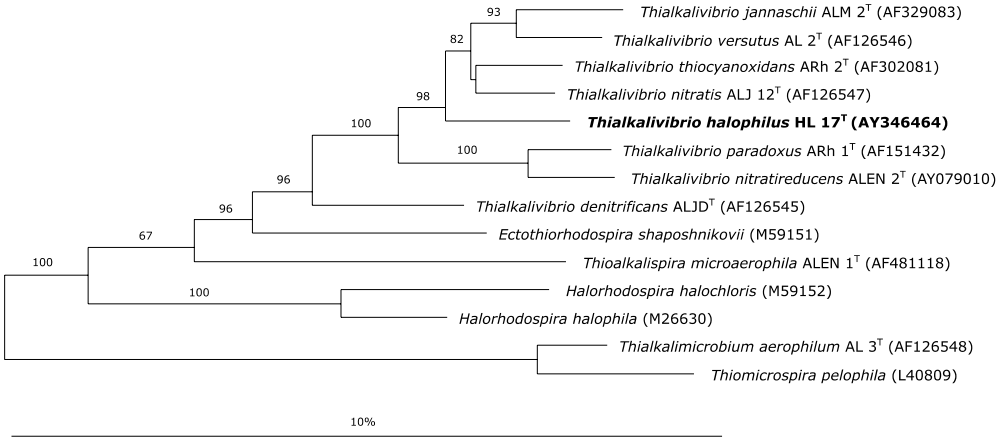

Fig. 1 Neighbor-joining tree showing the phylogenetic affiliation of *Thialkalivibrio* (*Tv.*) *halophilus* strain HL 17^T. *Paracoccus denitrificans*, *Rhodobaca bogoriensis*, and *Sulfitobacter mediterraneus*, all belonging to the α subdivision of the *Proteobacteria*, were used as an outgroup, but were pruned from the tree. The *numbers on the branches* represent results of bootstrap analysis (1,000 replications). Only values above 80% are shown

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 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 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 (Banciu et al. 2004).

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 (nine to ten times) in soda buffer compared to NaCl buffer at pH 9.6.

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 + Tris-HCl buffer, pH 9.6), while the 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 factors 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 (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

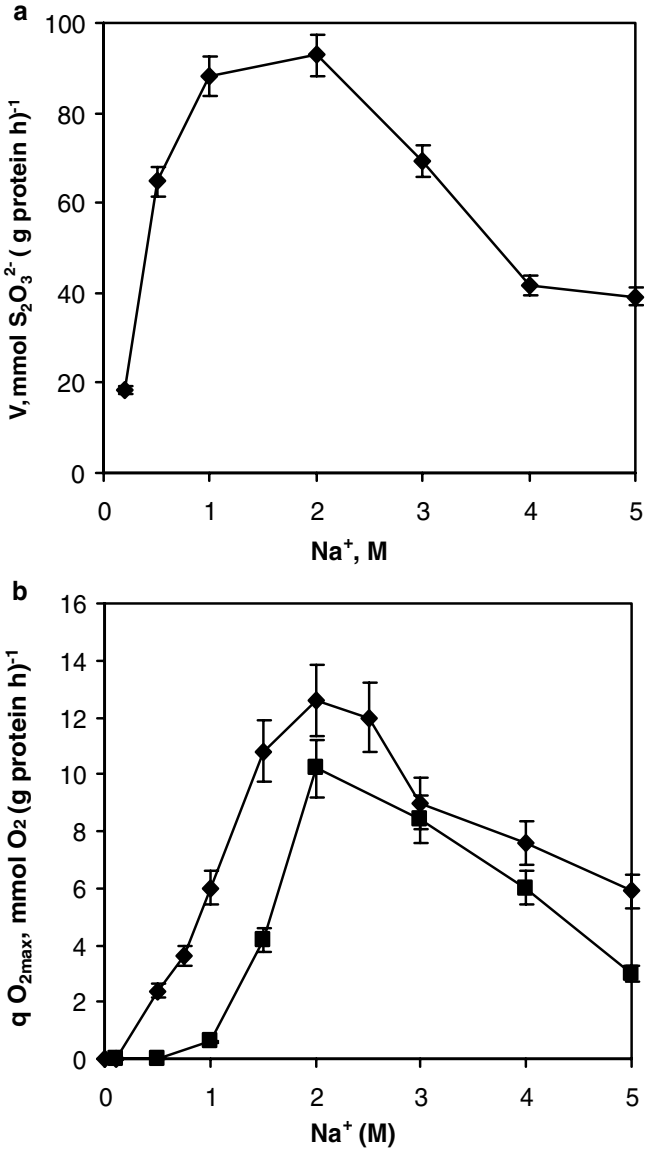

Fig. 2a, b Optimal salt concentration for growth and respiration in strain HL 17^T. **a** Salt effect on growth of strain HL 17^T in batch culture at different concentrations of NaCl, pH 7.8, and 50 mM $NaHCO_3$ as the carbon source. **b** Salt effect on thiosulfate respiration of washed cells grown 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

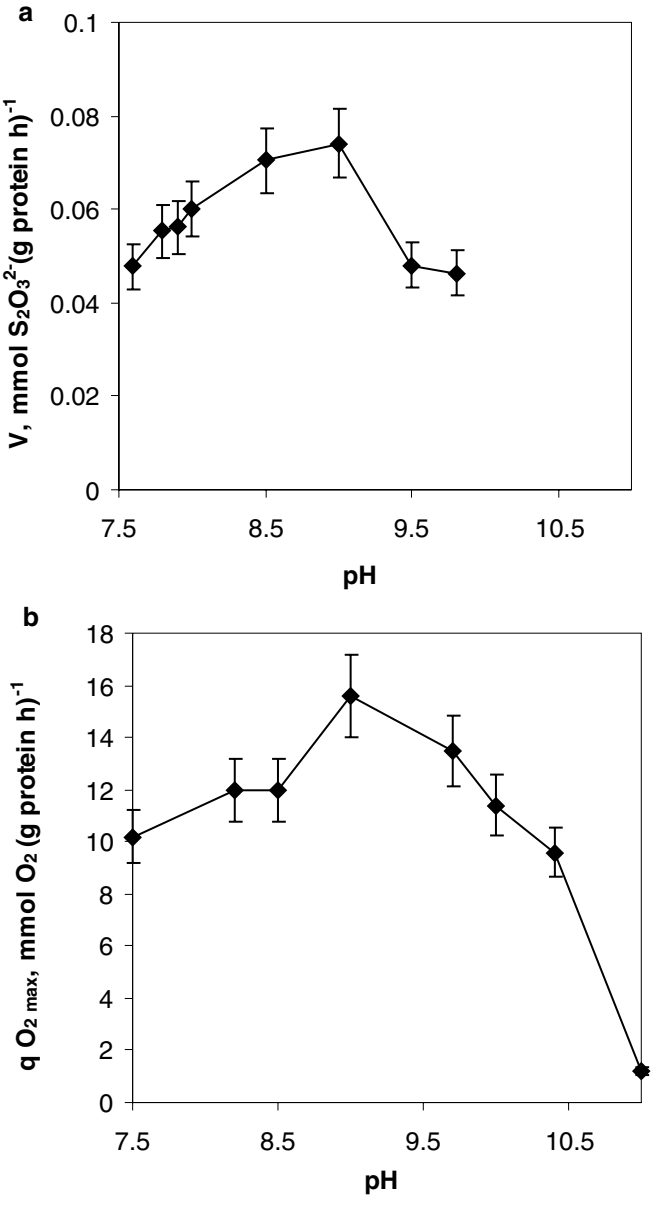

Fig. 3a, b Optimal pH for growth and respiration of strain HL 17^T. **a** pH effect on growth of strain HL 17^T in batch culture at 4 M Na^+. At low pH (6.5–8.0), 50 mM $NaHCO_3$ was added to buffer the medium containing 4 M NaCl. At alkaline pH, 0.5 M $NaHCO_3 + Na_2CO_3$ was added to 3.5 M NaCl. **b** 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 (pH 7.5–9.8) and by addition of small quantities of Na_2CO_3 (pH 10.0–11.0)

appearance, while the soda-grown cell-free extract was bright yellow. The absorption spectrum 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.

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

Table 1 Maximum specific growth rates (μ_{max}), maximum growth yields (Y_{max}) and maintenance coefficients (m_s) of *Thioalkalivibrio* (*Tv.*) 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})	2.6×10^{-3}	5.3×10^{-3}	ND[d]

[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
[d]*ND* No data

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

Substrate	qO_{2max} (mmoles O_2/g 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

(9.3 osm kg^{-1}) is much higher than in 4 M soda medium (5 osm kg^{-1}).

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 *Thialkalivibrio* species (Banciu et al. 2004) and in *Halorhodospira* sp. (Galinski and Trüper 1982), the closest relative of members of the *Thialkalivibrio* genus. The NaCl-grown biomass of strain HL 17^T contained 19.8% (w/w) glycine betaine, while the soda-grown biomass contained 12.4% glycine betaine.

Discussion

Strain HL 17^T was isolated from the hypersaline and alkaline lake that can be regarded as an intermediate between saline and soda lakes since the alkaline salts (sodium carbonate) comprised only small fraction of the total salts. These chemical characteristics seem to favor a domination of facultative haloalkaliphiles rather than neutrophilic halophiles thriving in hypersaline lakes with pH ≤ 8.5 (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 *Thialkalivibrio* species—many of which being extremely salt-tolerant organisms—accumulate the organic osmolyte glycine betaine (Banciu et al. 2004). 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

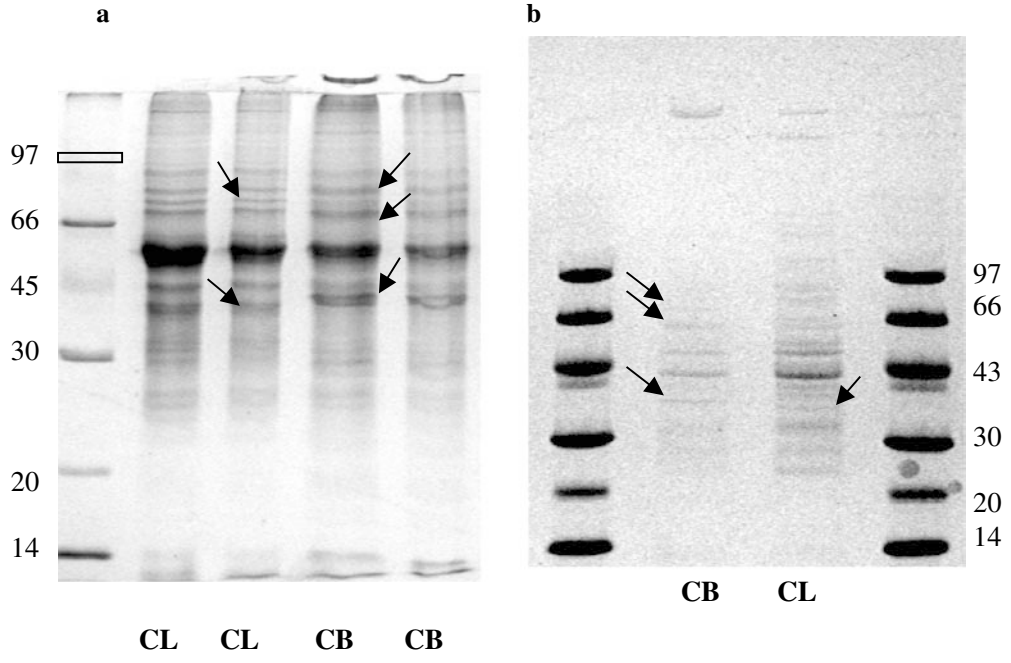

Fig. 4 Total protein analysis of crude cell extract using **a** 12% SDS-PAGE and **b** 8–25% gradient SDS gel. The *arrows* indicate the bands which appear to be overexpressed in one or another culture. *CL* Chloride-grown culture, *CB* soda (carbonate)-grown culture

the growth of strain HL 17^T, the high concentration of sodium carbonates may represent a stressful condition.

The higher pigment production in soda cultures along with changes in protein expression is another indication 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 physicochemical 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):

$$\Pi = \phi \times (vmRT) \quad (1)$$

where v is the number of ions from one molecule of salt ($v = 2$ for NaCl and $v = 3$ for Na_2CO_3); m the moles solutes per 1,000 g solvent (molality); R is the gas constant (8.3143 J mol^{-1} K^{-1}); 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°C (Table 3). The values of the vapor 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 *Thialkalivibrio* 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 *Thialkalivibrio* species at a pH below 9.5 and at more than 1 M NaCl. Together with the special physiological features, the genetic data indicate that strain HL 17^T is a new species, *Tv. halophilus*—the first halophilic facultative alkaliphilic chemolithoautotroph able to grow in NaCl and Na_2CO_3/$NaHCO_3$ brines.

Description of *Thialkalivibrio halophilus* sp. nov.

Thialkalivibrio halophilus (ha·lo'phi·lus; Greek *n. halos*, salt; Greek *adj. philus*, loving; NewLat. *adj. halophilus*,

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	References
Actual molality (m) (moles kg^{-1})	4.58	2.36	–
Solubility (moles kg^{-1})	6.15	2.75	c,d
Density (ρ) (kg l^{-1})	1.145	1.182	e
Absolute viscosity (η) (cP)	1.34	2.40	e
Ionic strength (I)[a]	4	6	–
Vapor 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) (kJ kg^{-1})[b]	26.2	9.1	–

[a]Calculated from equation: $I = 1/2\Sigma\,(c_i \cdot z_i^2)$; 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 Eq. (1)
[c]Apelblat and Korin (1998)
[d]Apelblat and Manzurola (2003)
[e]Lobo and Quaresma (1989)
[f]Our determination

salt-loving) is a Gram-negative, non-spore-forming, aerobic, obligately chemolithoautotrophic, halophilic, and facultatively alkaliphilic bacterium. Cells are 0.3–0.4×1–2 μm and made motile by a single polar flagellum. At low salinity it often generates extracellular sulfur from thiosulfate. It 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. *T. halophilus* 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. It oxidizes sulfide, thiosulfate, polysulfide, sulfur, and tetrathionate to sulfate. The G+C content in DNA is 65.1 ± 0.5 mol% (Tm). Other properties are the same as for the genus.

The type strain is HL 17^T (DSM 15791, UNIQEM 225), which is isolated from the surface sediments of alkaline hypersaline Stamp Lake. The GenBank accession number of the 16S rDNA sequence is AY346464.

Emended description of genus *Thialkalivibrio*

The genus *Thialkalivibrio* (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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