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ORIGINAL PAPER

Influence of salts and pH on growth and activity of a novel facultatively alkaliphilic, extremely salt-tolerant, obligately chemolithoautotrophic sufur-oxidizing Gammaproteobacterium *Thioalkalibacter halophilus* gen. nov., sp. nov. from South-Western Siberian soda lakes

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Abstract A chemolithoautotrophic sulfur-oxidizing bacterium (SOB) strain ALCO 1 capable of growing at both near-neutral and extremely alkaline pH was isolated from hypersaline soda lakes in S-W Siberia (Altai, Russia). Strain ALCO 1 represents a novel separate branch within the halothiobacilli in the *Gammaproteobacteria*, which, so far, contained only neutro-halophilic SOB. On the basis of its unique phenotypic properties and distant phylogeny, strain ALCO 1 is proposed as a new genus and species *Thioalkalibacter halophilus* gen. nov. sp. nov. ALCO 1 was able to grow within a broad range of salinity (0.5–3.5 M of total sodium) with an optimum at around 1 M Na^+, and pH (7.2–10.2, pH_{opt} at around 8.5). Na^+ was required for sulfur-dependent respiration in ALCO 1. The neutral (NaCl)-grown chemostat culture had a much lower maximum growth rate (μ_{max}), respiratory activity and total cytochrome *c* content than its alkaline-grown counterpart. The specific concentration of osmolytes (ectoine and glycine-betaine) produced at neutral pH and 3 M NaCl was roughly two times higher than at pH 10 in soda. Altogether, strain ALCO 1 represents an interesting chemolithoautotrophic model organism for comparative investigations of bacterial adaptations to high salinity and pH.

Keywords Haloalkaliphilic · *Halothiobacillus* · Soda lakes · Sulfur-oxidizing bacteria · *Thioalkalibacter halophilus*

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Introduction

Until recently, lithoautotrophic sulfur-oxidizing bacteria (SOB) were studied mostly in neutral and acidic environments such as fresh and marine waters, wastewater treatment plants (Robertson and Kuenen 1992a, b; Kuenen et al. 1992; Kelly and Wood 2000) and acidic mine drainage (Hallberg and Johnson 2001). During the last decade, oxidation of inorganic sulfur has also been reported for chemolithoautotrophic bacteria in the saline alkaline environments such as soda lakes. To date, three gammaproteobacterial SOB genera (*Thioalkalimicrobium*, *Thioalkalivibrio* and *Thioalkalispira*), comprising 13 species, are known to grow in extremely alkaline and saline media. Their distribution, taxonomy, morphology and physiology have been recently reviewed (Sorokin and

Kuenen 2005; Sorokin et al. 2006a). All species belonging to the chemolithoautrotrophic haloalkaliphilic SOB have so far been isolated from soda lakes and are obligate halophiles, either moderate, extreme halophiles or extreme halotolerants. The majority of these haloalkaliphilic SOB were also obligate alkaliphiles (with optimum pH >9.5) and only single species was a facultative alkaliphile growing well between pH 7.5 and 9.8, with a broad optimum for growth (pH 8–9) (eg. *Thioalkalivibrio halophilus* strain HL 17, Banciu et al. 2004c). The chemolithoautrotrophic haloalkaliphilic SOB are good candidates for the biotechnological removal of sulfide from industrial waste streams (Buisman et al. 2000; Janssen et al. 2001; Banciu et al. 2004a; Bosch et al. 2007). Apart from their biotechnological use, chemolithoautotrophic haloalkaliphilic SOB also represent attractive model organisms for fundamental studies on the halo-alkaliphilic adaptations. In this regard, the facultative alkaliphiles and extreme halotolerant strains are an ideal experimental model to assess the specific physiological and molecular changes over a broad range of pH and salt.

Despite the multiple extreme conditions (pH, alkalinity–salinity, increased irradiation and, sometimes, temperature) in the saline and alkaline lakes, the (micro) diversity of SOB is remarkably high (Foti et al. 2006; Sorokin et al. 2006b). In the present paper we describe the isolation of a facultatively alkaliphilic and halophilic chemolithoautotrophic sulfur-oxidizing bacterium (strain ALCO 1) from hypersaline soda lakes in S-W Siberia. On physiological grounds, this strain resembles the previously described *Thioalkalivibrio halophilus* strain HL 17 (Banciu et al. 2004c), but it is phylogenetically distinct from all other known haloalkaliphilic SOB genera. The aim of our study was to characterize the new isolate based on its morphology, phylogeny and biochemical features, with particular attention paid to the influence of pH and salts on its growth physiology and kinetics of sulfur oxidation.

Materials and methods

Enrichment procedure and isolation of strain ALCO 1

Strain ALCO 1 was enriched and isolated in pure culture on the soda medium described previously (Sorokin et al. 2006a, b, see below), modified by increasing NaCl concentration. In total, the medium contained 1 M NaCl and 1 M Na as soda (total $Na^+ = 2$ M), pH 10. Thiosulfate (20 mM) served as energy source and ammonium (4 mM) as nitrogen source. The culture medium was inoculated with a mixture of sediments from hypersaline soda lakes in South–Western Siberia (Altai, Russia). The description of the sediments is given elsewhere (Foti et al. 2007). Development of the culture was followed by the monitoring of thiosulfate consumption. A pure culture of the SOB dominating in the enrichment was obtained from a single colony, developing on solid alkaline thiosulfate agar after preliminary purification in serial dilutions. The purity of the isolate was checked by microscopy,by substituting thiosulfate with yeast extract (1 g/L; absence of growth) and sequencing its 16 S rRNA gene. Detection of Gram-negative type of the cell wall was performed by rapid KOH string test (Gregersen 1978). The isolate was designated ALCO 1.

Growth conditions

Batch cultivation of ALCO 1

The strain was maintained and routinely grown on the soda medium, containing 2 M Na^+ (g/L): Na_2CO_3, 95; $NaHCO_3$, 15; NaCl, 16; K_2HPO_4, 1. The medium is strongly buffered at pH 10.05. After sterilization, the base medium was supplemented with 4 mM NH_4Cl, 1 ml/L of trace metal solution (Pfennig and Lippert 1966), 1 mM $MgCl_2$ and 20 mM filter-sterilized sodium thiosulfate. The neutral pH medium contained (g/L): NaCl, 120; NH_4Cl, 0.5; K_2PO4, 1.5. The pH was adjusted to 7.5. After sterilization, it was supplemented with the same additions as the alkaline medium except that 50 mM filter-sterilized $NaHCO_3$ was added as carbon source and alkaline buffer to neutralize the sulfuric acid produced from thiosulfate. Growth was conducted in 1 L serum bottles with 100 mL medium closed with gray rubber septa on a rotary shaker at 100 rpm and 30°C. For determination of the pH profile, a set of buffers containing 2 M total Na^+ (as NaCl or/and Na-soda) was used with initial pH values from 6 to 11 according to Sorokin and Kuenen 2005. The averages between initial and final values of pH were used as a data points for graphical representation.

Salt profiles were measured at pH 7.5 and 10 with varying concentrations of NaCl and soda, respectively. The ability to grow heterotrophically was tested by replacing thiosulfate with yeast extract (0.5 g/L), glucose (5 mM), or a mixture of yeast extract and acetate (5 mM) after sterile washing of the inoculum from residual thiosulfate. The ability to grow anaerobically with nitrate (20 mM) and thiosulfate (20 mM) was tested in the 2 M soda medium made anaerobic with five cycles of evacuation/flushing with argon in 60 mL serum bottles with butyl rubber stoppers. Growth with H_2 (50% gas phase) and CO (5% gas phase) as electron donor was examined at pH 10 in 100 mL serum bottles with 20 mL medium.

Continuous cultivation of the strain

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: the neutral (pH 7.5) NaCl based medium was titrated with 1 M Na_2CO_3, while the soda-based medium (pH 10.05) was titrated with 2 M NaOH to maintain the pH at constant value. The dissolved oxygen concentration was controlled at 20–30% air saturation by adjusting the stirring speed. The temperature was set at 30°C. Thiosulfate was sterilized separately and added to the medium at 40 mM final concentration. A steady state was assumed to be reached after at least five volumes changes, when variation in the optical density was minimized. Each day, optical density and thiosulfate concentration were monitored. The biomass collected from the effluent into the 3 L sterile bottles placed in an ice bath was concentrated by centrifugation and further used, either for activity tests or to produce cell fractions (see below).

Salt substitution experiment

To follow the growth of ALCO 1 at different ratios of Na^+/K^+ and CO_3^{2-} (or Cl^-)/SO_4^{2-} at pH 10 and 8, respectively, batch cultivation was used. As inoculum, (1/100) we used the strain grown in continuous culture at pH 7.5 and 10, respectively. Stock solutions for alkaline pH experiment were prepared as following: "2 M Na carbonate" solution (Na_2CO_3 95 g/L; $NaHCO_3$ 15 g/L; Na_2HPO_4 0.5 g/L), pH 10.15; "2 M Na sulfate" solution (Na_2SO_4 142 g/L; Na_2HPO_4 0.5 g/L), pH 8.5 and "2 M K carbonate" solution (K_2CO_3, 124 g/L; $KHCO_3$ 16 g/L; K_2HPO_4 0.5 g/L), pH 10. Stock solutions for neutral pH experiment were prepared as following: "2 M Na chloride" solution (NaCl 120 g/L; Na_2HPO_4 0.5 g/L; NH_4Cl 0.5 g/L), pH 8; "2 M K chloride" solution (KCl 74.5 g/L; K_2HPO_4 0.5 g/L; NH_4Cl 0.5 g/L), pH 8; and "2 M Na sulfate" solution as mentioned above. Stock solutions of 1 M $NaHCO_3$ or $KHCO_3$ were used for pH titration in the above media, the final concentration of HCO_3^- being 50 mM. $MgSO_4$ or $MgCl_2$ from 1 M sterile stock solutions were added. Also, 4 M NH_4Cl was prepared and sterilized separately and added to 4 mM final concentration in the alkaline mineral media. Media with varying ratio of Na^+/K^+ and CO_3^{2-} (or Cl^-)/SO_4^{2-} at pH 10 and 8, respectively, were prepared by mixing of four different base solution containing 2 M Na^+ or K^+ carbonate mixtures at pH 10 or 2 M NaCl/1 M Na_2SO_4 at pH 8. The starting substrate (sodium thiosulfate) concentration was 20 mM (= 0.02 M). The molar "1/0" or "2/0" ratios of CO_3^{2-} (or Cl^-)/SO_4^{2-} do not include the sulfate (40 mM = 0.04 M) produced by thiosulfate oxidation. Cultures were incubated as 20 mL volume in 250 mL bottles closed with rubber caps, at 30°C with slow shaking (100–150 rpm). The following parameters have been monitored over time: optical density at 590 nm; thiosulfate concentration and total protein concentration.

Activity measurements and kinetics analysis

Cells collected from the chemostat effluent and stored at 4°C were harvested by centrifugation, washed and suspended in an iso-osmotic solution of 2 M NaCl at physiological pH. For subsequent tests the concentrated cell suspension was diluted in respiration buffer to 0.05–0.1 mg protein/mL. Respiration buffers contained 50 mM KCl, 1 mM $MgCl_2$ and various concentrations of Na^+ either as NaCl or Na_2CO_3/$NaHCO_3$ and different pH values buffered with 50 mM HEPES-NaOH, Tris–HCl or soda. Respiration rates were measured at 30°C in a 5 mL glass chamber mounted on a magnetic stirrer and fitted with an oxygen electrode (Yellow Spring Instr., Ohio) connected to a chart recorder (Kipp & Zonen, model BD40). Stock solutions of sulfide (HS^-), polysulfide (S_6^{2-}) and sulfite (SO_3^{2-}) were prepared anaerobically in 0.1 M Tris–HCl, pH 10, with 5 mM EDTA to prevent auto-oxidation, and introduced into the chamber at 25–100 μM final concentration. Elemental sulfur was prepared as 17 mM stock solution in acetone.

The kinetic constants, maximum specific oxygen uptake rate ($qO_{2\ max}$) and apparent affinity constants (Ks) were determined in washed cells collected from the effluent of continuous cultures. The biomass for respiration experiments was obtained at dilution rates (D) of 0.033 h^{-1} for pH 7.5 grown culture and of 0.052 h^{-1} for pH 10.05 culture. 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 (at full scale). The K_s values were calculated based on three independent measurements by plotting the oxygen uptake rate against the substrate concentration. The maximum specific growth rate (μ_{max}) of the ALCO 1 strain grown at pH 7.5 and 10.05 was determined experimentally as the dilution rate at which washout of the biomass and accumulation of thiosulfate started.

Each determination of respiration and batch growth experiments was done in triplicate; the data represent the average values with standard deviation less than 10%.

Enzymatic and chemical analyses

Millimolar-range thiosulfate consumption in batch cultures was measured by standard iodimetric titration after

neutralization of the medium with 50% (v/v) acetic acid. Micromolar concentrations of thiosulfate and tetrathionate were assayed by the cyanolytic procedure of Kelly et al. (1969). Elemental sulfur was analyzed by cyanolysis after 12 h extraction of the cell pellet with acetone (Sörbo 1957). Cell protein was measured colorimetrically by the Lowry method using bovine serum albumin as standard after removal of interfering sulfur compounds.

The activity of sulfite dehydrogenase was measured colorimetrically using ferricyanide as an artificial electron acceptor (Kelly and Wood 1994) and of sulfide–quinone reductase (SQR), in discontinuous assay with decyl-ubiquinone (d-UQ, Sigma) as an artificial electron acceptor. For the latter assay, 4 mL of the cell membrane suspension in soda buffer, pH 9, containing 0.6 M total Na^+ with a protein content of 0.05 mg/mL was placed in 7 mL serum bottles. An ethanolic solution of d-UQ was then added at a concentration of 200 μM and after creating anaerobic conditions, 100 μM of sulfide was introduced by a syringe. The bottles were incubated up to 30 min with regular sampling of 0.2 mL directly into 1 mL of 2% Zn-acetate to analyze residual sulfide by the methylene-blue method (Trüper and Schlegel 1964). Boiled membrane preparations were used as a biological control.

The analysis of cytochromes was performed spectrophotometrically in cell-free extracts using an HP 8453 UV/Vis diode array spectrophotometer (Hewlett-Packard, Amsterdam, The Netherlands).

Hemes were extracted with acidic acetone and analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) according to a procedure described by Sone and Fujiwara (1991). RP-HPLC separation was performed using a Nucleosil 300–5 C4 column (2 × 75 mm, dp = 5 μm) and a MiliChrome A-02 chromatograph (MiliChrome, Russia).

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 Jose, CA), a 3 μm Grom-sil Amino-1PR column (Grom, Rottenburg-Haifingen, Germany) and a Shodex refractive index detector (model RI17, Showa Denko KK, Tokyo, Japan). The mobile phase consisted of 80% (v/v) acetonitrile at a flow rate of 1 mL min^{-1}. Natural abundance ^{13}C-NMR spectra of compatible solutes were recorded in the pulsed Fourier transform mode on a Bruker Avance DPX-300 spectrometer (Bruker Elektronik GmbH, Germany) operating at 75.48 MHz (^{13}C) and at 300 MHz for the proton-decoupling channel relative to sodium trimethylsilylpropionate (TMSP).

Analysis of fatty acid composition of the polar lipids extracted from the membranes with chloroform/acetone was performed according to Zhilina et al. (1997).

Cell-free extracts and membrane vesicles preparation

Cell-free extracts were prepared by ultrasonic disruption of the same cell suspensions used in respiratory experiments. Unbroken cells and cell debris were removed by centrifugation at 5,000×*g* for 15 min. All operations were carried out between 0 and 4°C.

For the determination of sodium influence on respiratory activity, membrane vesicles were prepared in sodium-free buffers as follows. Freshly collected cells from chemostat cultures were washed twice, first with 0.6 M NaCl then with Na-free buffer. The "Na-free buffer" consisted of 50 mM Tricine-KOH, pH 8, 0.3 M sucrose, 10% (v/v) glycerol, 0.1 M KCl, 6 mM $MgSO_4$, 1 mM DTT. PMSF (1 mM) and DNAse (0.2 mg) were added just before cell disruption. Suspended cells were disrupted in a French Press at 1.3 Kbars. After removal of cell debris by two centrifugations at 5,000×*g* for 15 minutes, the extract was centrifuged in a Beckman Optima LE-80 K ultracentrifuge for 90 min at 60,000×*g*. The supernatant containing the cytosolic fraction was stored at −80°C for further analyses, while the membrane pellet was suspended in a minimal volume of the "Na-free buffer" described above. Total protein concentration in the membrane suspension was 20 mg/mL in ALCO 1 strain grown at pH 7.5 and 16.5 mg/mL in ALCO 1 strain grown at pH 10.05. To test the respiratory activity in the membrane vesicles, buffers containing 0.6 M of total Na^+ and 0.1 M KCl were used. The buffers with pH 8.0–8.5 contained 50 mM HEPES–KOH, the buffers with pH 8.5–9.0; 50 mM Tris–HCl, and the buffers with pH 9.5–11.0; a mixture of $NaHCO_3$, Na_2CO_3 and NaCl.

SDS–PAGE

Gel electrophoresis of total proteins was performed under denaturing conditions according to Laemmli (1970) using 5–20% gradient of polyacrylamide.

Total DNA analysis

The isolation of the DNA and subsequent determination of the DNA G + C content were performed according to Marmur (1961).

Phylogenetic analysis

Genomic DNA for PCR amplification was extracted from the cells using the UltraClean Soil DNA Extraction Kit (MolBio Laboratories, USA), following the manufacturer's instructions. The nearly complete 16S rRNA gene was obtained from pure cultures using bacterial primers

GM3F and GM4R (Schäfer and Muyzer 2001). The PCR products were purified from low-melting agarose using the Wizard PCR-Prep kit (Promega, USA) according to the manufacturer's instructions. Sequencing was performed using Big Dye Terminator v.3.1 sequencing reaction kit at ABI 3730 DNA automatic sequencer (Applied Biosystems, Inc., USA). The sequences were aligned with those from GenBank using CLUSTALW. Phylogenetic trees were reconstructed with four different algorithms using the TREECONW software package (Van de Peer and De Wachter 1994). Pairwise evolutionary distances (expressed as estimated changes per 100 nucleotides) were computed using Jukes & Cantor method. A resulting phylogenetic tree was constructed by the Neighbor-joining method. Bootstrap analysis (100 replications) was used to validate the reproducibility of the branching patterns of the trees.

Results

Strain description

Cells of strain ALCO 1 are short, thick, motile rods with a single polar flagellum (Fig. 1). On a mineral alkaline solid medium, it formed reddish colonies up to 3 mm in diameter free of sulfur. The cell wall is typical for Gram-negative bacteria. The new isolate is an obligately chemolithoautotrophic aerobic sulfur-oxidizing bacteria (SOB) unable to grow heterotrophically (i.e., on yeast extract, acetate and glucose) or anaerobically with nitrate. H_2 and CO are not utilized.

Phylogenetic analysis

16S rRNA gene sequence analysis of strain ALCO 1 placed it within the *Gammaproteobacteria*, in the genus *Halothiobacillus* (Fig. 2). However, sequence similarity to the validly described species of this genus was below 95% (94% with *Htb. halophilus* cluster and 91–93% with *Htb. neapolitanus* cluster), which indicates that (1) ALCO 1 represents a new separate genus within this group and (2) the genus *Halothiobacillus* needs a revision, but this subject is beyond the scope of the present work.

Growth characteristics and optimal growth conditions

Batch and continuous cultivation of strain ALCO 1, together with the results of respiration experiments with washed cells collected from the chemostat cultures, showed that the strain is a facultative alkaliphile, growing between pH 7.5 and 10.2, with an optimum pH at around 8.5 (Fig. 3a). At the same time, ALCO 1 is an obligate halophile, requiring a minimum of 0.5 M total Na^+ in the growth medium and showing an optimum for growth at 1 M of total Na^+. The organism grew within a broad salinity range between 0.5 and 3.5 M Na^+, with no or very poor growth at 4 M Na^+ both at pH 7.5 and 10 (Fig. 3b). At near-optimal pH (8.5) and salt concentration (0.6 M Na^+), the maximum experimental yield and the observed maximum growth rate (μ_{max}) of strain ALCO 1, as measured in batch culture, were 3.5 mg protein/mmole thiosulfate and 0.09–0.1 h^{-1}, respectively. During thiosulfate oxidation, elemental sulfur formed transiently in NaCl-grown culture, at pH 7.5, but never in the soda-

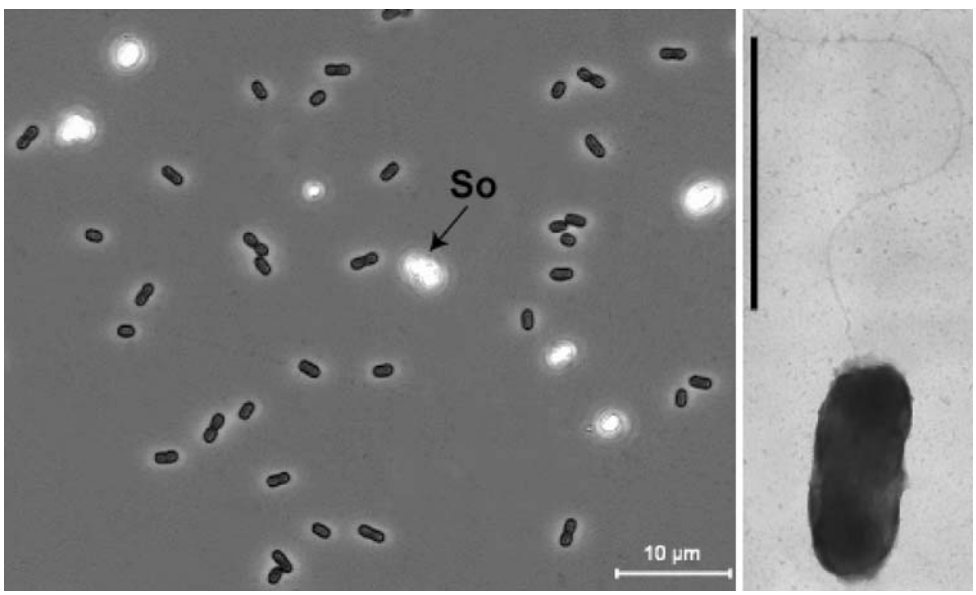

Fig. 1 Cell morphology of ALCO 1. **a** phase contrast photograph of the cells grown at 3 M NaCl and pH 7.5 with sulfur (*So*) accumulation (*light refractive globules*). **b** Transmission electron micrograph, *bar* 1 µm

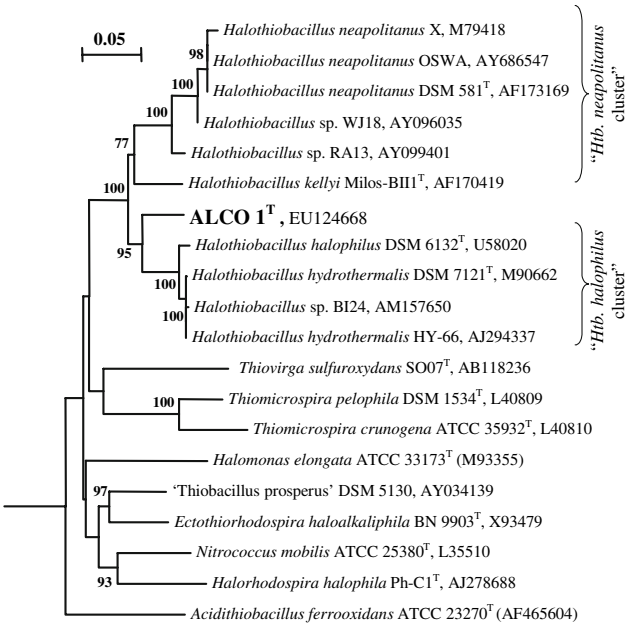

Fig. 2 Phylogenetic tree, based on the analysis of 16S rRNA gene sequences showing the position of strain ALCO1 in between the clusters *Htb. neapolitanus* and *Hbt. halophilus* in the Gammaproteobacteria

growing cultures. Tetrathionate was not detected as an intermediate of thiosulfate oxidation.

Growth kinetics in continuous culture at substrate-limited conditions

Continuous cultivation of microorganisms under substrate limitation is a good simulation of environmental conditions, providing important information on the growth characteristics and many structural features. Several kinetic parameters have been measured in strain ALCO 1 during parallel continuous cultivation under substrate limitation at neutral conditions (NaCl, pH 7.5) and alkaline conditions (soda, pH 10.05).

We chose to grow ALCO 1 strain at intermediate salt concentration (2 M of total Na^+) to obtain information about its behavior under halo-(alkali)philic conditions and also because cells cultivated at such salinity proved to be more stable as compared to lower salinity. The results obtained are summarized in Table 1. A major difference was noted between the maximum growth rates of the two cultures, the high-pH soda environment being obviously more favorable. The value of μ_{max} (0.22 h^{-1}) obtained for strain ALCO 1 at 2 M Na^+ in soda was comparable with the μ_{max} (0.21 h^{-1}) previously reported in another high salt-tolerant haloalkaliphilic SOB *Thioalkalivibrio versutus* ALJ 15 (Banciu et al. 2004b). However, the growth yield (*Y*) was similarly low in both cultures of strain ALCO 1, at neutral and alkaline pH (2 and 1.58 g protein/mole thiosulfate, respectively) as compared to the high-yield *T. versutus*. It is noteworthy that the yields observed in the chemostat cultures (1.58–2), being lower than those measured in batch (3.5), were caused by the lower growth rate and the conditions of growth chosen in continuous culture as explained above. Washed cells of ALCO 1 collected from the alkaline culture respired sulfide and thiosulfate by 20–40% more actively than the cells grown at neutral pH in NaCl. The apparent affinity constant (K_s) for thiosulfate and sulfide was 5 and 3 μM, respectively, indicating that ALCO 1 cells possess a very high affinity for theses substrates.

These results indicated that, in the continuous culture, under substrate limitation, ALCO 1 grew faster in soda at high pH but with less energy available for biomass production. At neutral pH in NaCl, the bacterium grew slower but with slightly higher biomass yield. As will be discussed below, this difference in growth kinetics might be related to the difference in the osmotic pressure of soda and NaCl brines. Nonetheless, the pH factor should also be taken into account.

Oxidation of inorganic sulfur compounds

The trends in the pH and salt effect observed for growth of ALCO 1 were confirmed with some minor modifications by the respiration experiments in the washed cells and membrane vesicles. The cells grown in batch culture at pH 7.5 showed an optimum pH for substrate oxidation (pH 8)

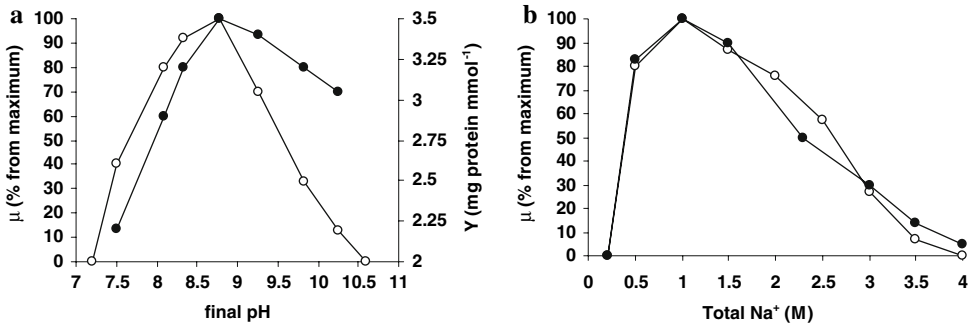

Fig. 3 Effect of pH at near-optimal salt concentration (0.6 M Na^+) (**a**) and salt (**b**) on growth yield (*Y*) and growth rate (μ) in ALCO 1 grown in batch culture. The observed maximum growth rate was 0.092 h^{-1}. Symbols in (**a**): *open* μ; *closed Y*. Symbols in (**b**): *open* culture grown in soda at pH 10; *closed* culture grown at pH 7.5 in NaCl

Table 1 Parameters of growth kinetics in facultatively alkaliphilic, halophilic strain ALCO 1 grown in continuous culture under substrate (thiosulfate) limitation at 2 M total Na^+, pH 7.5 and 10.05, respectively, at 30°C

Parameter	Growth pH	
	2 M NaCl, pH 7.5	2 M Na^+ (soda), pH 10.05
Maximum growth rate (μ_{max}, h^{-1})	0.05	0.22
Yield (g protein/mole thiosulfate)	2	1.58
Protein concentration (mg/L)	84[a]	72.8[b]
Maximum oxygen uptake rate with thiosulfate (qO_{2max}^{thio}, nmoles O_2 min^{-1} mg^{-1})	900	1,600
Maximum oxygen uptake rate with sulfide ($qO_{2max}^{sulfide}$, nmoles O_2 min^{-1} mg^{-1})	990	1,200
Maximum oxygen uptake rate with polysulfide ($qO_{2max}^{polysulfide}$, nmoles O_2 min^{-1} mg^{-1})	870	950
Oxygen uptake rate with elemental sulfur (qO_2^{So}, nmoles O_2 min^{-1} mg^{-1})	105	35
Oxygen uptake rate with sulfite ($qO_2^{Sulfite}$, nmoles O_2 min^{-1} mg^{-1})	30	160
Optimum pH for sulfur substrates respiration	9.5–10 for all substrates	10 for all substrates
Apparent affinity constants (*K*s, μM) for:		
Thiosulfate	ND[c]	5
Sulfide	ND	3
Sulfur formation	Yes but little, extracellular	No
Cell morphology	Short rods	Slightly longer rods than neutral-grown cells

Steady-state kinetic data were obtained at $D = 0.033$ h^{-1} (for culture grown at pH 7.5) and $D = 0.052$ h^{-1} (for culture grown at pH 10.05)

[a] $Na_2S_2O_3$ concentration in the influent was 42 mM (→84 mM sulfate in culture)

[b] $Na_2S_2O_3$ concentration in the influent was 46 mM (→92 mM sulfate in culture)

[c] Not determined

lower than the cells cultivated at alkaline pH, whose optimum pH for respiration was at around 10 (Fig. 4a). This shift of optimum pH for respiration might be explained by the growth history but it was not observed in the cells grown under substrate limitation in chemostat. Surprisingly, ALCO 1 cells grown in continuous culture at pH 7.5 or 10.05 exhibited the same optimum pH for respiration at around 10 (Fig. 4 b). The same was true for other tested sulfur compounds, such as sulfide, polysulfide and sulfite. Washed cells of ALCO 1 grown at neutral pH were capable of oxidizing elemental sulfur at much higher rate than the alkaline-grown cells (see Table 1) despite the fact that neutral pH cultures accumulated elemental sulfur. High activity of SQR (sulfide–quinone reductase) was measured in the membranes of ALCO 1 cells grown in soda [280 nmol HS^-/(mg protein min)], while the activity of cytochrome *c*-dependent sulfide dehydrogenase was negligible [<10 nmol/(mg protein min)].

Free sulfite (SO_3^{2-}) oxidation is an unusual feature among the haloalkaliphilic SOB (Sorokin et al. 2001). Washed cells of strain ALCO 1 were capable of sulfite oxidation, the cells grown in soda being much more active than the NaCl-grown cells. A similar pattern was observed when we tested the sulfite dehydrogenase activity. We found that washed cells of ALCO 1 grown in batch, at pH 7.5, had a lower activity [160–200 nmol/(mg protein min)] than their alkaline-grown counterparts [300–360 nmol/(mg protein min). Sulfite dehydrogenase activity

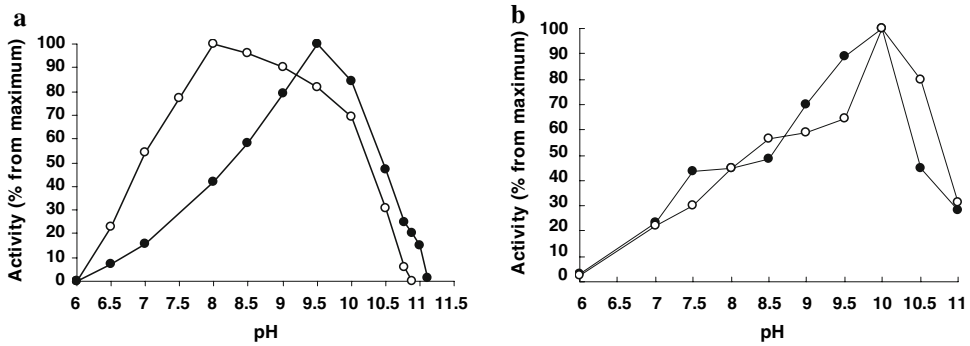

Fig. 4 Effect of pH on the activity of thiosulfate respiration in washed cells of ALCO 1 grown in batch (**a**) or continuous (**b**) culture at 2 M total Na^+. *Open symbols*, cells grown in NaCl at pH 7.5; *closed symbols*, cells grown in soda at pH 10–10.05

was AMP-independent and required the presence of $NaHCO_3$ (optimum at 0.3–0.4 M Na^+, and pH 8.8 for neutral-grown cells, and pH 10, for alkaline-grown cells).

The effect of sodium concentration on sulfur respiration of washed cells collected from the chemostat was also tested at the pH of growth (7.5 and 10, respectively). We found that a concentration of 1.5 M of total Na^+ (as mixture of NaCl and soda) was optimal for thiosulfate and sulfide oxidation in ALCO 1 cells. This value of salinity was somewhat higher than that found (1 M Na^+) for the batch-grown cells. The cells grown at pH 7.5, however, were capable of respiring thiosulfate down to 0.3 M total Na^+ (as NaCl), where it retained 60% of maximum activity, in contrast to alkaline-grown cells which could not utilize inorganic sulfur substrate below 0.5 M Na^+ (soda) (data not shown).

We also checked the influence of total sodium concentration on sulfur respiration in membrane vesicles prepared from washed cells of ALCO 1. Fresh membrane vesicles were capable of oxidizing reduced TMPD, a fact indicating that the locus cytochrome *c*-cytochrome *c* oxidase was active. The membrane vesicles of ALCO 1 were inactive with thiosulfate or sulfite as electron donors under any condition tested, suggesting that the utilization of these substrates was not limited to membranes only. In contrast, the membranes were capable of sulfide and polysulfide oxidation. At 0.6 M of total Na^+, the optimal pH for sulfide oxidation was 9 and 9.5 in membrane vesicles isolated from neutral- and alkaline-grown cells, respectively. The maximum specific oxygen uptake rates with sulfide (100 μM), measured at optimal conditions (pH 9 and 0.6 M Na^+) were 62 and 368 nmoles O_2/(mg protein min) in membrane vesicles originating from neutral and alkaline-grown cells, respectively. The maximum specific oxygen uptake rates with polysulfide (50 μM) at optimum pH and 0.6 M Na^+ was 138 nmoles O_2/(mg protein min) in membranes from the cells grown at pH 10.05. Sulfide oxidation in membranes at pH 9.0 was Na-dependent, starting at a minimum of 10 mM of Na^+ and reaching 50% of maximum activity at about 0.3 M Na^+ (Fig. 5)

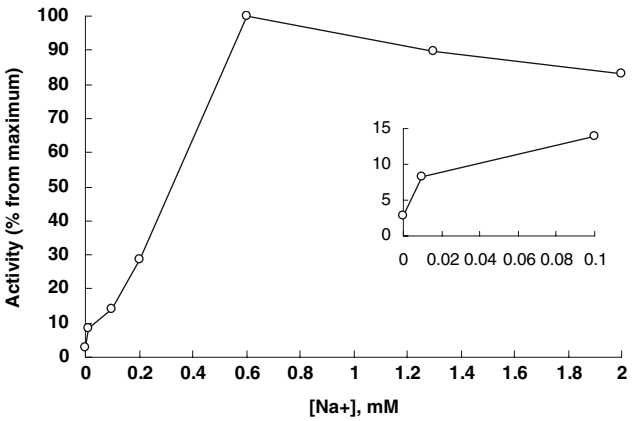

Fig. 5 Sodium effect on sulfide respiration in membranes from the cells grown in continuous culture at 2 M Na^+, pH 10.05 and 30°C ($D = 0.052\ h^{-1}$). Membranes (0.1 mg of protein/mL) were incubated in a buffer containing 50 mM Tris–HCl, 0.1 M KCl, pH 9.0, at 30°C and substrate (HS^-) concentration was 100 μM. The maximum specific activity was 368 nmoles O_2/min^{-1} mg of $protein^{-1}$. The first three points of the plot are enlarged in the *inset*

Effects of ionic composition on growth in batch culture

To find out whether ALCO 1 has an absolute growth requirement for sodium (as main cation) and for chloride or carbonate (as main anions at near-neutral or alkaline pH), the strain was grown in batch media at excess substrate, with decreasing ratios of Cl^- or CO_3^{2-} to SO_4^{2-} and Na^+ to K^+ (at pH 8 and 10). We used cells grown under thiosulfate limitation at pH 7.5 or 10 as inocula for these short-term growth tests. The results of this experiment are shown in Fig. 6.

The tolerance for sulfate was higher in the cells of ALCO 1 cultivated at alkaline pH (where the main anion is carbonate) than in those grown at neutral pH (with chloride as the main anion). Interestingly, at 2 M total Na^+ and pH 10, carbonate could be almost entirely replaced by sulfate without loss of growth ability (Fig. 6a), while the demand for Cl^- is much less obvious at high pH. At pH 8, the strain was capable of growing reasonably well with the increasing concentration of sulfate ions, that replaced chloride on the basis of charge (1 SO_4^{2-}/2 Cl^-), up to a molar ratio of 0.66 M Cl^-/0.66 M SO_4^{2-} (Fig. 6b). However, high obligate dependence on Cl^- at neutral pH was still evident from this experiment.

The yields were $Y = 2.09$ mg of protein/mmole thiosulfate for Cl^-/SO_4^{2-} at a ratio of 2/0 ("low" sulfate concentration) and $Y = 1.96$–1.98 for carbonate/SO_4^{2-} at ratios of 0.83/0.17 and 0.66/0.33 ("high" sulfate concentration). Increasing the sulfate concentration (vs. Cl^-) resulted in a decrease of Y in neutral batch culture, but an increase of Y in soda culture (where Y varied between 1.37 and 1.98). This indicates that sulfate is more favorable for growth at high pH.

At neutral pH, ALCO 1 grew extremely poor when Na^+/K^+ ratio was 1.66 M/0.33 M and no growth was observed at a lower Na^+/K^+ ratio (data not shown). At pH 10 the bacterium was a little more tolerant to the substitution of Na^+ with K^+, still retaining the growth capacity at a molar Na^+/K^+ ratio of 1.33/0.66 (Fig. 6c).

Compatible solutes, membrane lipids, total proteins, hemes and cytochromes

Analysis of organic compatible solutes in the cells grown at 3 M total Na^+, either as NaCl or as soda, demonstrated the presence of ectoine and glycine–betaine in different

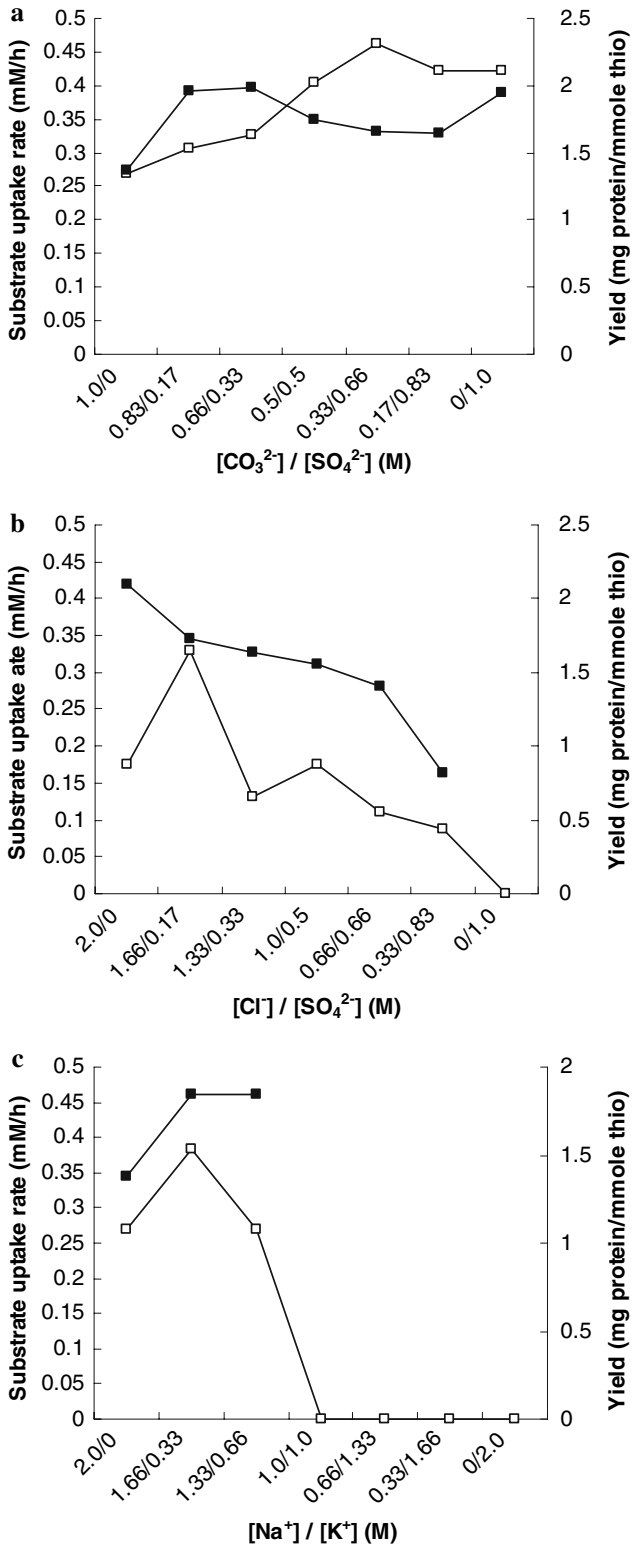

Fig. 6 Effect of replacement of CO_3^{2-} by SO_4^{2-} (**a**) at pH 10, Cl^- by SO_4^{2-} (**b**) at pH 8, and Na^+ by K^+ (**c**) at pH 10, on substrate uptake rate (*open symbols*) and growth yield (*closed symbols*) in batch culture of strain ALCO 1 grown at 2 M of total cations and 2 M of total (monovalent) anions. As an inoculum, cells of ALCO 1 strain grown in continuous culture at pH 7.5 ($D = 0.033\ h^{-1}$) and 10.05 ($D = 0.052\ h^{-1}$), respectively, were used

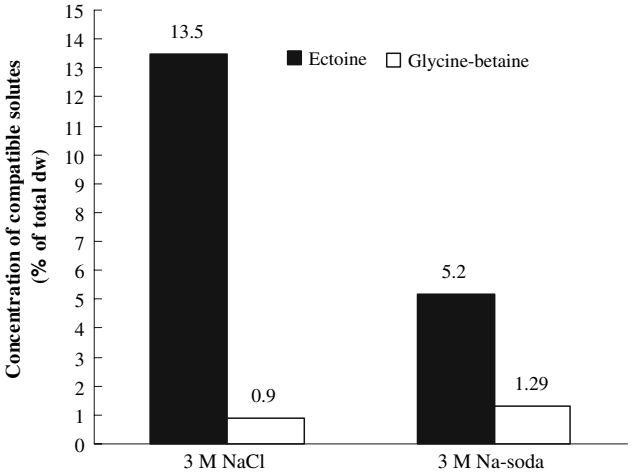

Fig. 7 Compatible solutes in the cells of ALCO 1 grown at 3 M total Na^+ and at different pH

proportions (Fig. 7). In total, the cells grown in 3 M NaCl at pH 7.5 accumulated two times more solutes than those grown in soda (14.4 vs. 6.5% of dry biomass). This confirmed our previous conclusion on a dramatically different response of haloalkaliphilic bacteria to NaCl (strong, fully dissociating electrolyte) versus soda (weak, partially dissociating electrolyte) as observed in a study of the facultatively alkaliphilic *Thioalkalivibrio halophilus* (Banciu et al. 2004c). Furthermore, cells grown in NaCl accumulated a higher proportion of ectoine as compared to the cells, grown in soda. At present we do not have any reasonable explanations for this fact.

The analysis of fatty acid composition in the membrane polar lipids, surprisingly, demonstrated almost identical profiles for the cells grown in soda at pH 10 and in NaCl at pH 8 except the presence of *19cyc* species in the former (Table 2). In our previous experience, halophilic neutrophilic SOB species growing in NaCl clearly differed from the natronophilic SOB growing in soda by the dominance of saturated and branched fatty acid species in their polar lipids. In cells of ALCO 1, however, the dominant species was unsaturated C18:1w7 (60–61% from the total) and the content of the second dominant saturated C16:0 was three times lower (21–24%), followed by C14:0 (5%). Such a composition is, in fact, very similar to the fatty acid profile of natronophilic *Thioalkalivibrio versutus* ALJ 15 (Banciu et al. 2005). This indicates that ALCO 1 is rather a natronophile than a halophile, but a possible adjustment of the membrane lipid composition from halophilic to natronophilic conditions (or vice versa) was clearly not detectable or might have escaped detection.

The RP-HPLC analysis of the hemes that are noncovalently membrane-bound in the strain ALCO 1 has revealed the presence of types B, D and trace amounts of heme O. The latter was detectable only in the membranes isolated

Table 2 Fatty acid composition of polar lipids in strain ALCO 1 grown in batch at pH 10 (in soda) and at pH 8 (in NaCl) at 2 M total Na^+, 30°C

Fatty acid	ALCO 1 (soda)	ALCO 1 (NaCl)
12:0	2.09	2.16
12:0 3OH	0.39	0
14:0	**5.21**	**5.42**
15:0	0.15	1.80
16:1w7	1.14	1.93
16:1w5	0.25	0
16:0	**21.23**	**24.29**
17:0	0.13	1.30
18:1w9	0.58	0.37
18:1w7	**61.33**	**60.56**
18:1w5	0.55	0
18:0	1.53	1.67
11 Me18:1	1.14	1.10
19 cyclo	**3.15**	0

Dominant fatty acids are in bold type

from ALCO 1 cells grown at pH 7.5. A detailed analysis of heme types in several strains of sulfur-oxidizing strains belonging to *Thioalkalimicrobium* and *Thioalkalivibrio* genera has shown that the hemes profile of ALCO 1 is similar to that of *Thioalkalimicrobium* sp. (data summarized in Table 3).

The analysis of cytochromes in the cell-free extracts of strain ALCO 1 grown in batch culture at 2 M of total Na^+, pH 7.5 and 10, respectively, has revealed the abundance of a membrane-bound cytochrome c_{554} (data not shown). The specific content of the cytochrome c_{554} (calculated on the basis of millimolar extinction coefficient, $E = 20$) was two times higher in soda-grown cells as compared to the NaCl-grown cells (2.7 vs. 1.5 nmoles/mg protein). Such a trend of increase in the total cytochromes at high pH seems to be common for facultatively alkaliphilic *Bacillus* strains (Yumoto 1992). Apart from c_{554}, difference spectra also indicated the presence of cytochrome b_{558} as a minor component and the presence of CO-reacting covalently bound hemes C and A. The latter might point on the presence of a cytochrome *c* oxidase type caa_3. Similar to the cytochrome *c*, the specific activity of cytochrome oxidase (measured with TMPD as substrate) was two times higher in the soda-grown cells compared to NaCl-grown cells [1,040 vs. 490 nmoles/(mg protein min)].

The one-dimensional gel electrophoresis of total protein in the strain ALCO 1 did not reveal any difference in this aspect. The more appropriate 2-D SDS–PAGE should be used for catching the possible changes that may appear in the expression of some functional proteins whose concentrations are very low.

Discussion

A new chemolithoautrotrophic, extremely salt-tolerant and facultatively alkaliphilic SOB strain ALCO 1 represents a new taxon within the *Gammaproteobacteria*, which, to date, accommodates all known groups of extreme (halo)alkaliphilic sulfur bacteria, both chemo- and phototrophic groups. Both phylogeny and physiology clearly differentiate the new bacterium from its closest relatives among the genus *Halothiobacillus*. Moreover, when comparing the studied strain with its chemolitoautotrophic haloalkaliphilic SOB counterparts, we observed a mixture of physiological and biochemical properties (Table 3).

ALCO 1 can grow and remain active within a broad range of salinity and pH, exhibiting facultatively alkaliphilic and halophilic nature but clearly preferred soda environments. ALCO 1 is also a facultative natronophile since it was capable of growing within both wide ranges of NaCl concentration and Na_2CO_3/$NaHCO_3$ (Na-carbonate or soda). In these aspects, strain ALCO 1 resembles *T. halophilus* HL 17.

Considering its growth and respiration kinetics, ALCO 1 differs from all other known chemolitotrophic haloalkaliphilic or neutrophilic halophilic SOB. A first important aspect to be stressed is the relatively low growth yield both at near-neutral and alkaline pH.

The yield of the soda-grown (pH 10.0) cells is 20% lower than that of NaCl-grown (pH 7.5) cells, in spite of the fact that the NaCl grown cells have to synthesize significantly more compatible solutes. This lower yield and a higher μ_{max} in soda than in NaCl (Table 1) may indicate that the strain adopts a different strategy in the energy conservation. This phenomenon of an apparent trade-off between faster μ_{max} and higher (ATP) yield is commonly observed, both in pure cultures at different growth rates (De Vries et al. 1970) as well as in physiologically similar organisms with a R-strategy as opposed to a K-strategy of survival (Sorokin and Kuenen 2005). It is assumed that in order to grow faster, the rate of ATP production must be optimized at the expense of maximal energy conservation. In addition, in case of growth at high pH and salt, the high optimum pH may require considerably more maintenance. These observations, together with the finding of higher contents and specific activity of cytochrome *c* in soda-grown cells than those in NaCl-grown cells, show that the efficiency of energy conservation in *halo*-philes (NaCl-loving organisms) and *natrono*-philes (trona- or soda-loving organisms) is clearly different at the same concentration of total Na^+. The main compatible solute (ectoine) in cells of ALCO 1 is also found in alkaliphilic SOB belonging to genus *Thioalkalimicrobium* sp. and is widespread among heterotrophic, aerobic, neutrophilic halotolerant bacteria, such as *Halomonas* spp. with a very

Table 3 Characteristics of strain ALCO 1 and other chemolithoautotrophic halo(alkali)philic SOB

Property	*Thioalkalimicrobium* spp.[a,b]	*Thioalkalivibrio* spp.[a,b,c]	*Thioalkalivibrio halophilus* strain HL 17[d]	Strain ALCO 1[e]	*Halothiobacillus halophilus*[f,g]
Maximum growth rate (h^{-1})	0.33 (at pH 10)	0.2 (at pH 10)	0.11 (at pH 10)[h]	0.22 (at pH 10) 0.055 (at pH 7.5)	0.072 (at pH 7)
Range of protein yield (g protein/mole thiosulfate)	1.5–3.5	4–7	4–6[h]	3.5 (at 0.6 M Na^+) 1.58–2 (at 2 M Na^+)	6.4
Maximum oxygen uptake rate for thiosulfate	Very high	Low to moderate	Low	Very high	High
pH limits (optimum)	7.5–10.6 (10)	7.5–10.65 (10–10.2)	6.5–9.8 (8–9)	7.5–10.05 (8–9)	6–8.4 (7.0–7.3)
Na^+ limits (optimum, M)	0.3–1.5 (0.6–1)	0.5–4.3 (0.6–2)	0.2–5 (2)	0.3–3.8 (1.5)	Up to 4 M (0.8–1)
Optimum temperature (°C)	30	30–35	30	30	30–32
RuBisCO activity	+, Type I	+, Type I	+, Type I	?[i]	+, Type I[h]
SO_3^{2-} oxidation and SO_3^{2-} dehydrogenase activity	−	+	−	+	−
Dominating cytochromes	c	c	c	c	ND [j]
Heme types in membrane [h]	B, D	B, D and O	ND	B, D	ND
Compatible solutes	Ectoine	Glycine betaine (+low amounts of sucrose)	Glycine betaine	Ectoine (+low amounts of glycine betaine)	ND
DNA G + C mol%	47.3–51.2	61–66	65.1	54.6	64.2
Physiology	Obligate alkaliphiles and low halotolerants	Obligate alkaliphiles, extreme salt-tolerants and extreme natronophiles	Facultative alkaliphile, extreme halotolerant and natronotolerant	Facultative alkaliphile, extreme halotolerant and natronotolerant	Neutrophile and extreme halotolerant

ND not determined

[a] Sorokin et al. 2001;

[b] Sorokin et al. 2006a;

[c] Banciu et al. 2004b;

[d] Banciu et al. 2004c;

[e] This paper;

[f] Wood and Kelly (1991);

[g] Kelly and Wood (2000);

[h] Our unpublished results;

[i] Molecular detection failed so far (our unpublished results);

[j] c-type cytochromes are dominant in *Halothiobacillus neapolitanus* (Ambler et al. 1985);

broad salinity range for growth. Many truly halophilic bacteria accommodate glycine betaine as organic compatible solutes (eg., *Ectothiorhodospira* sp., *Halorhodospira* sp.), an osmolyte also found in the alkaliphilic, halophilic or extremely halotolerant SOB of the genera *Thioalkalivibrio* and *Thiohalomonas* (Banciu et al. 2005; Galinski 1995; Oren 2002; Sorokin et al. 2007).

Figure 8 attempts to represent a salt/pH ecological niche characteristic for strain ALCO 1 in comparison with several halo(alkali)philic SOB. Every apparently minor structural and physiological property of one group that could not be found in another "competing" group may provide the different organisms with an opportunity to take their advantage under specific environmental conditions. For example, the versatility (or extreme tolerance) to pH and salt variations could be highly beneficial for facultatively alkalipihilic and halophilic or halotolerant SOB (such as *T. halophilus* HL 17) in an environment where the fluctuations of pH and salinity are significant. ALCO 1 grows in a little narrower range of salinity (0.5–3.8 M of

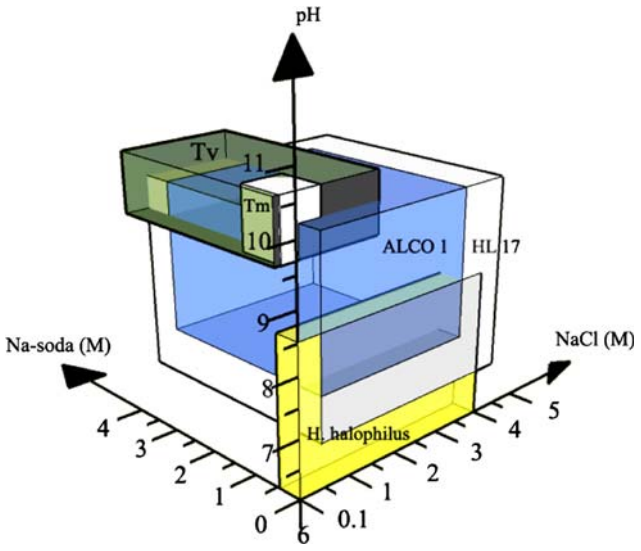

Fig. 8 Three-dimensional diagram of salt/pH response of strain ALCO 1 and other chemolithotrophic halo(alkali)philic SOB. Abbreviations used: *Tm* low salt-tolerant obligately alkaliphilic *Thioalkalimicrobium* spp., *Tv* high salt-tolerant obligately alkaliphilic *Thioalkalivibrio* spp., *HL* 17, high salt-tolerant facultatively alkaliphilic and halophilic strain *T. halophilus* HL 17, *H. halophilus* moderately halophilic neutrophilic *Halothiobacillus halophilus*

total Na^+) than HL 17 (0.2–4 M Na-carbonate and up to 5 M NaCl). Being isolated in a comparable habitat with that of strain HL 17 (saline alkaline lake) and having a similar pH and Na^+ optimum for growth and respiration, ALCO 1 could have a competitive advantage with its higher μ_{max} (0.22 h^{-1}) at natronophilic conditions (soda, pH 10) than HL 17 ($\mu_{max} = 0.11\ h^{-1}$) and its ability to utilize inorganic sulfur compounds at much higher rates. It is worth mentioning that during continuous cultivation of strain ALCO 1 at high pH, the molar ratio between carbonate and sulfate was around 1/0.092, while at neutral pH, the chloride/sulfate ratio was about 1/0.041. These ratios resemble closely the natural conditions in some soda lakes where carbonate could reach concentrations of 4 M while sulfate ions are found up to 0.2 M concentration, i.e., a ratio of 1/0.05.

Based on growth kinetics, we can speculate that ALCO 1 would adopt a similar survival strategy as *Thioalkalimirobium* (R-strategy) but over a much broader range of pH and salinity (Sorokin et al. 2003). Faster growth in an environment where the carbonate/sulfate ratio is low (i.e., at relatively high sulfate concentrations) could be another advantage for ALCO 1, especially at low values of pH (8–9). These observations of high sulfate tolerance in strain ALCO 1 are in good agreement with our similar studies made on several obligate haloalkaliphilic sulfur-oxidizing strains belonging to the genus *Thioalkalivibrio*, able to survive well at high concentration of sulfate ions (3.5 M), but only at alkaline pH (Banciu 2004). The graph demonstrates that even such a narrow ecophysiological group as chemolithoautotrophic SOB can cover nearly all imaginable, permanent or transient, conditions of salt/pH found in saline habitats. In this context, it is interesting to mention that the halo-alkaliphilic SOB are now increasingly exploited in the removal of sulfide from industrial waste gases and water in the form of insoluble elemental sulfur. Clearly, insight into the performance of these organisms under a spectrum of slightly different practical conditions is crucial to control and direct the selection and performance of these organisms in the industrial mixed culture installations.

Overall, the new SOB described here is clearly different from all the known species with the same physiology. Based on phylogenetic position and distinct phenotypic properties, we proposed to assign the new bacterium to a new genus and species *Thioalkalibacter halophilus* gen. nov. sp. nov.

Description of *Thioalkalibacter* gen. nov

(*thi.o. al.ka.li.bac.'ter.* Gr. n. *thios* sulfur; M.L. n. *alkali* soda ash; Gr. n.; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Thioalkalibacter* rod-shaped bacterium that love alkaline conditions and utilizes sulfur compounds).

Rod-shaped Gram-negative bacterium, utilizes reduced sulfur compounds as electron donors and oxygen as electron acceptor. Moderately halophilic and alkaliphilic. Member of the *Gammaproteobacteria*. Habitat: hypersaline alkaline lakes. The type species is *Thioalkalibacter halophilus*.

Description of Thioalkalibacter halophilus sp. nov

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

Cells are motile fat rods, 0.8–1 × 1.5–3 μm, with a single polar flagellum and a Gram-negative type of cell wall. Obligately chemolithoautotrophic and aerobic. Utilizes sulfide and thiosulfate and elemental sulfur electron donor with sulfate as the final oxidation product. Thiocyanate, H_2 and CO are not utilized. Moderately halophilic and extremely salt-tolerant with a salinity range for growth from 0.3 to 3.5 M total Na^+, and an optimum at 1.0 M. Facultatively alkaliphilic with a pH range for growth between 7.5 and 10.2 with an optimum between around 8.5. Obligately depends on high concentrations of Na^+ (all pH range) and Cl^- (at near-neutral pH). The dominant cellular fatty acids are 18:1w7 and 16:0. The G + C content of the DNA is 54.6 ± 0.5 mol% (T_m). Isolated from hypersaline alkaline lakes in South-Western Siberia (Russia). The type strain is ALCO 1^T (DSMZ 19224 = UNIQEM U263; 16S rRNA gene sequence accession number is EU124668).

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