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NOTE

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Oxidation of thiosulfate to tetrathionate by an haloarchaeon isolated from hypersaline habitat

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Abstract A novel, extremely halophilic, neutrophilic archaeon was isolated from a mixed sediment sample from different hypersaline lakes in Kulunda steppe (Altai, Russia) at 4 M NaCl with acetate and thiosulfate as substrates. The enrichment culture developed in two phases. During the first phase, a rapid growth of heterotrophic, red-colored, polymorphic rods occurred with the concomitant oxidation of thiosulfate to tetrathionate. The latter was subsequently oxidized to sulfate during a second, slower phase by extremely halophilic, chemolithoautotrophic bacteria belonging to the gamma subdivision of the Proteobacteria. The archaeal strain HG 1 was isolated from the first phase of the enrichment culture using acetate as substrate. It was able to oxidize thiosulfate to tetrathionate during heterotrophic growth with acetate—a property not yet demonstrated for any of the known haloarchaea. The presence of tetrathionate synthase, the enzyme responsible for thiosulfate oxidation, was detected in strain HG 1. The activity was associated with membranes and depended specifically on Cl^-, in contrast to the similar activity in extremely halophilic sulfur-oxidizing Gammaproteobacteria from the same enrichment, which was soluble and demanded both Na^+ and Cl^-. Strain HG 1 was identified as a member of the genus *Natronorubrum*.

Keywords Extremely halophilic · Haloarchaea · Sulfur-oxidation · Tetrathionate · Thiosulfate

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The potential for oxidation of inorganic sulfur compounds, such as sulfide and thiosulfate, is widely spread among the Bacteria. However among the Archaea, it is only confined to the thermo-acidiphilic *Crenarchaeota* (Segerer and Stetter 1999).

Many obligately heterotrophic bacteria, especially those belonging to the Gamma subdivision of the Proteobacteria, are capable of oxidizing thiosulfate, and in some cases sulfide, incompletely to tetrathionate (Sorokin 2003). Recently, these bacteria, belonging to the genus *Halomonas*, have been found abundantly among the dominant culturable aerobic heterotrophs in saline soda lakes (Sorokin 2003). The importance of this reaction for heterotrophic growth is not completely clear. In most cases it seems accidental, while only in a few bacteria the oxidation of thiosulfate to tetrathionate can be utilized as an additional energy-yielding reaction (Vedenina and Sorokin 1992; Sorokin et al. 1996).

In hypersaline habitats with neutral pH, such as salterns and inland saline lakes, extremely halophilic red Archaea represent the dominant group of aerobic heterotrophs (Oren 2002). To our knowledge, the involvement of such prokaryotes in the oxidation of sulfur compounds has never been recognized. This paper describes a first example of haloarchaea able to oxidize thiosulfate to tetrathionate.

A mixture of sediment samples from five hypersaline lakes in Kulunda steppe (Altai, Russia) with a pH range from 7.5 to 9.0 and a salt content from 300 to 380 g l^{-1} was used to enrich for halophilic heterotrophic thiosulfate-oxidizing microorganisms. The pH of the enrichment medium, which included (g l^{-1}): NaCl—240, K_2HPO_4—3, $(NH_4)_2SO_4$—0.5, was adjusted to 7.3 before sterilization. After sterilization, the medium was supplemented with 2 mM $MgCl_2$ $6H_2O$, 10 mM sodium acetate, 20 mM $Na_2S_2O_3$ $5H_2O$, 0.05 g l^{-1} yeast extract and 1 ml l^{-1} of trace elements solution (Pfennig and Lippert 1966). The same medium was also used for batch cultivation of pure cultures. The medium was solidified by adding 1.5% (w/v) agar. In case of isolation

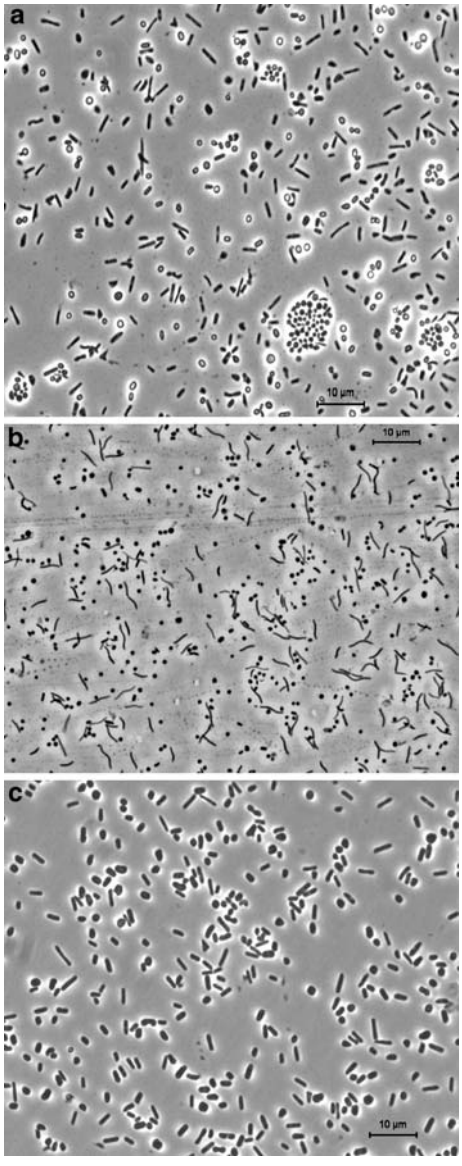

Fig. 1 Phase contrast microphotographs of an enrichment (**a, b**) and pure (**c**) cultures from hypersaline lake sediments at 4 M NaCl with acetate and thiosulfate as substrates. **a** First, heterotrophic phase of the enrichment, producing tetrathionate from thiosulfate; **b** second, autotrophic phase of the enrichment, oxidizing tetrathionate to sulfate; **c** pure culture, strain HG 1, isolated with acetate from the first heterotrophic stage of enrichment

and cultivation of extremely halophilic lithoautotrophic sulfur-oxidizing bacteria (SOB) out of the enrichment, the medium lacked organic additives and was supplemented with 50 mM $NaHCO_3$. Tetrathionate ($K_2S_4O_6$, Fluka) was filter-sterilized as a 1 M solution immediately before use. The cultures were grown in closed bottles with liquid to air ratio of 1:10 on a rotary shaker at 150 rpm at 30°C. Growth was followed by turbidity measurements at 590 nm. Thiosulfate was determined by standard iodimetric titration; tetrathionate and trithionate were determined by the cyanolytic procedure as described by Kelly et al. (1969). 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 Instruments, OH, USA) using cells from the late exponential phase, that were washed and resuspended in mineral medium. Cell-free extract was prepared by sonication of washed cells; the extract was subsequently separated into a membrane and a soluble fraction by ultracentrifugation at 150,000 *g* for 2 h. The activity of tetrathionate synthase was routinely measured spectrophotometrically with ferricyanide ($[K_3(FeCN)_6]$, 1 mM) as the electron acceptor and thiosulfate (1 mM) as the electron donor. Protein concentrations were determined with the Lowry method (Lowry et al. 1951). Pigments were extracted from wet biomass with 7:3 methanol/acetone mixture and absorption spectra were recorded with a UV–Vis diode-array HP 8453 spectrophotometer (Hewlett Packard, Amsterdam, The Netherlands). *16S rDNA* gene sequencing and phylogenetic analysis were performed as described previously (Sorokin et al. 2003).

When the 4 M NaCl medium containing acetate and thiosulfate was inoculated with a mixture of sediments from five hypersaline lakes, a rapid development of red colored, pleomorphic rods and cocci was observed accompanied by the oxidation of thiosulfate and an increase in pH (Fig. 1a). The latter indicated the formation of polythionates as products of thiosulfate oxidation rather than the formation of sulfate (which was confirmed by chemical analysis), according to reaction 1. During the second phase of the enrichment, tetrathionate started to disappear and the pH dropped, indicating the development of lithotrophic SOB oxidizing tetrathionate to sulfate (reaction 2). At this stage, thin motile spirilla and thin, long, flexible rods appeared in the community (Fig. 1b). In total, complete utilization of the two substrates resulted in biomass growth of at least three different prokaryotic morphotypes and the oxidation of thiosulfate to sulfate (reaction 3).

$$2S_2O_3^{2-} + 0.5O_2 + H_2O \rightarrow S_4O_6^{2-} + 2OH^- \quad (1)$$

$$S_4O_6^{2-} + 3.5O_2 + 3H_2O \rightarrow 4SO_4^{2-} + 6H^+ \quad (2)$$

$$2S_2O_3^{2-} + 4O_2 + 2H_2O \rightarrow 4SO_4^{2-} + 4H^+ \quad (3)$$

From the second enrichment phase, two extremely halophilic, obligately chemolithoautotrophic SOB strains, HL 18 and HL 19, were isolated in pure culture using thiosulfate and tetrathionate as substrates, respectively. They belonged to two new lineages in the Gamma subdivision of the Proteobacteria (unpublished results). Both of the strains oxidized thiosulfate in two stages via tetrathionate to sulfate. But their growth rates were relatively low (around 0.05 h^{-1}), which might be a reason, why the heterotrophic component, growing much more rapidly in presence of acetate, took over during the first, heterotrophic phase of the succession. Given its ability to oxidize thiosulfate to tetrathionate, it obviously outcompeted the autotrophs on the basis of fast biomass production. When acetate, however, was con-

sumed, the heterotrophic component stopped growing and the autotrophic SOB started to use what was left over, e.g. inorganic product tetrathionate.

Using acetate as substrate, the enrichment was subcultured from the first stage and then was either serially diluted to extinction on liquid medium or plated onto solid medium. This resulted in the isolation of several strains with identical partial 16S rDNA sequences, from which only strain HG 1 was selected for further analysis.

Strain HG 1 is a polymorphic rod, 0.5–1 × 1.5–5 μm, transforming into irregular coccoid cells during the stationary growth phase (Fig. 1c). The biomass of fully grown cultures was red. The pigment extracted from the cell pellets with a mixture of methanol/acetone had absorption maxima at 470, 497 (main peak) and 530 nm, which correspond well to the common peaks of the haloarchaeal bacterioruberins (Oren 2002). The cells were extremely halophilic, with a NaCl range for growth between 2.5 and 5.0 M and an optimum at 3.5 M and neutrophilic, with a pH optimum at pH 7.5–8.0. It did not grow at pH above 8.5 and below 6.5. Demand for Mg^{2+} was very low, 1 mM being sufficient for maximal growth rate. At 4 M NaCl, strain HG 1 grew optimally

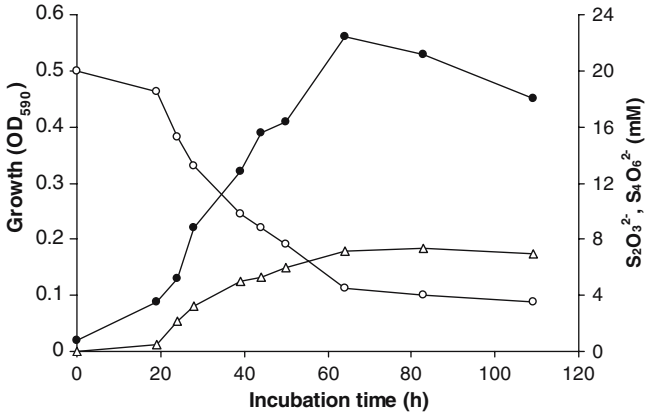

Fig. 2 Growth and oxidation of thiosulfate in batch culture of strain HG 1 at 4 M NaCl in the presence of 20 mM acetate, pH 7.2–7.5. *Closed circles* biomass growth, *open circles* thiosulfate, *opened triangles* tetrathionate

at 30–32°C and up to 46°C (lowest temperature limit was not examined). It was deposited in the DSMZ culture collection under the number DSM 17079.

Strain HG 1 was obligately heterotrophic, being unable to grow without organic compounds on purely mineral medium with thiosulfate. However, on the mixotrophic medium with acetate and thiosulfate, the latter was converted to tetrathionate more or less parallel to the biomass growth (Fig. 2). As a result, in the low-buffered medium the pH increased up to 9 and growth was inhibited. In this case the produced tetrathionate was partially chemically hydrolized to trithionate. However, if the pH of the medium was kept below 8.0 by periodic titration with HCl or by using a phosphate buffer at pH 7.2, the amount of tetrathionate produced accounted for up to 95% of the oxidized thiosulfate. The presence of 2–20 mM thiosulfate in the medium containing 10 mM acetate and buffered at pH 7.2 did not influence the final growth yield of strain HG 1 indicating furtive character of this reaction. But the final evidence on the potential to extract metabolic energy from the thiosulfate oxidation can only be drawn from acetate-limited continuous cultivation experiments (Sorokin 2003).

Strain HG 1 possessed constitutive thiosulfate-oxidizing activity, but growth in the presence of thiosulfate enhanced the activity up to five to seven times, from 3–4 to 15–20 nmol O_2 (mg protein min)$^{-1}$. In contrast to most other known tetrathionate-producing heterotrophs (Sorokin 2003), the new isolate was unable to oxidize sulfide. The pH optimum for thiosulfate oxidation by whole cells was 6.0. The activity of the enzyme tetrathionate synthase (thiosulfate-acceptor oxidoreductase), responsible for the production of tetrathionate from thiosulfate, was measurable either with ferricyanide or oxidized cytochrome c_{550} as the electron acceptors in the cell-free extract obtained from the HG 1 cells grown in the presence of thiosulfate. With ferrycianide, the activity was maximal at pH 6.0 in 3 M NaCl, with 50% activity at 1 M. The activity depended on Cl^- ions, being equally high at 3 M NaCl and KCl, but decreased sharply in Na_2SO_4 or K_2SO_4 solutions. A salt stimulating effect was found in the following order:

Table 1 Comparative properties of tetrathionate-synthase activity in (halo)alkaliphilic microorganisms

Property	Haloarchaea str. HG 1	Extremely halophilic sulfur-oxidizing gamma-proteobacterium str. HL 4[a]	Haloalkaliphilic *Halomonas* sp. str. AGJ 1-3[b]
Localization	Membrane-bound	Soluble (periplasmic)	Soluble (periplasmic)
Na^+ -dependence	–	+	+
Cl^- -dependence	+	–	–
Optimum salt concentration	3.0 M (NaCl, KCl)	2.0 M (NaCl, Na_2SO_4)	0.3 M (Na carbonate, NaCl)
Optimum pH	6.0–7.0	5.0–6.0	6.0; 8.0
Electron acceptor	Ferricyanide, cyt. *c*	Ferricyanide, cyt. *c*	Ferricyanide, cyt. *c*
Maximal activity (μmol) (min mg protein)$^{-1}$	0.42	5.2	4.0

[a]Strain HL 4 is an obligate chemolithoautotroph isolated from the hypersaline lake in N-E Mongolia (unpublished results)
[b]*Halomonas* sp. AGJ 1-3 was isolated from the Kenyan soda lake Naivasha (Sorokin and Mityushina 1998)

NaCl = KCl > LiCl > NaBr. After fractionation by ultracentrifugation, more than 85% of the tetrathionate synthase activity was found in the membrane fraction, which, apparently, differentiated it from the soluble periplasmic enzymes of this type known so far. Comparison of the enzyme from HG 1 with the same activity in the extremely halophilic lithoautotrophic SOB isolated from the same environment and with the heterotrophic *Halomonas* sp. from soda lake environment is given in Table 1. Apart from the localization, the enzyme from HG 1 was clearly much less active and has different ion specificity. It would therefore be interesting to purify the tetrathionate synthase from the (halo)alkaliphiles with different metabolism for comparative study, especially since only a few data are available on this class of sulfur-oxidizing enzymes.

From the irregular morphology, the red pigment spectrum similar to bacterioruberine and the extreme halophilic nature, it was already clear that the new isolate might belong to the haloarchaea. This was confirmed by *16S rRNA* gene sequencing and phylogenetic analysis, demonstrating its affiliation with the genus *Natronorubrum* (the gene bank accession number for the *16S rRNA* gene sequence of strain HG 1 is AY862140). This is surprising, since strain HG 1 is clearly neutrophilic in contrast to the two recognized species of the genus *Natronorubrum*, although there are some examples of such mixing of neutrophilic and alkaliphilic haloarchaea, e.g., in the genus *Halorubrum* (Feng et al. 2005). According to the difference in its *16S rRNA* gene sequence (around 97% similarity) with the other two species, the new isolate from Altai hypersaline lakes might formally be considered as a new species in the genus *Natronorubrum*, but some additional phenotypic and genetic comparison with the other members of the genus is necessary to confirm.

Furthermore, it would have been interesting to check whether the potential to oxidize thiosulfate is also present in the other representatives of haloarchaea. Our preliminary testing of the type strains of the genera *Natronococcus*,*Natronomonas* and *Natronobacterium* and of *Haloterrigena turkmenica*, however, gave negative resuts.

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