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REVIEW

Oxidation of Inorganic Sulfur Compounds by Obligately Organotrophic Bacteria

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Abstract—New data obtained by the author and other researchers on two different groups of obligately heterotrophic bacteria capable of inorganic sulfur oxidation are reviewed. Among culturable marine and (halo)alkaliphilic heterotrophs oxidizing sulfur compounds (thiosulfate and, much less actively, elemental sulfur and sulfide) incompletely to tetrathionate, representatives of the gammaproteobacteria, especially from the *Halomonas* group, dominate. Some denitrifying species from this group are able to carry out anaerobic oxidation of thiosulfate and sulfide using nitrogen oxides as electron acceptors. Despite the low energy output of the reaction of thiosulfate oxidation to tetrathionate, it can be utilized for ATP synthesis by some tetrathionate-producing heterotrophs; however, this potential is not always realized during their growth. Another group of marine and (halo)alkaliphilic heterotrophic bacteria capable of complete oxidation of sulfur compounds to sulfate mostly includes representatives of the alphaproteobacteria which are most closely related to nonsulfur purple bacteria. They can oxidize sulfide (polysulfide), thiosulfate, and elemental sulfur via sulfite to sulfate but neither produce nor oxidize tetrathionate. All of the investigated sulfate-forming heterotrophic bacteria belong to litho-heterotrophs, being able to gain additional energy from the oxidation of sulfur compounds during heterotrophic growth on organic substrates. Some doubtful cases of heterotrophic sulfur oxidation described in the literature are also discussed.

Key words: sulfur-oxidizing bacteria (SOB), tetrathionate-forming heterotrophs, sulfate-forming heterotrophs, marine, haloalkaliphilic, *Halomonas*, gammaproteobacteria, alphaproteobacteria.

INTRODUCTION

The sulfur cycle comprises an interrelated set of oxidation–reduction reactions of inorganic and organic the sulfur compounds (SC) with a change in the reduction state of the sulfur atom from (2–) to (6+) through several common intermediates, such as S_n^{2-} (polysulfide), $S_8(0)$, $S_2O_3^{2-}$, $S_nO_6^{2-}$ (polythionates), SO_3^{2-}. The specific well-known group of aerobic sulfur-oxidizing bacteria (SOB) is able to grow autotrophically using inorganic SC as electron donors. According to modern concepts, SOB directly originated from anaerobic photoautotrophic bacteria. Currently, several dozen representatives of this group are known, including obligately and facultatively autotrophic species. At the same time, examples of heterotrophic microorganisms for which the potential capacity for oxidation of SC has been demonstrated or suggested are also well known. Such bacteria are considered either as lithoheterotrophs, which combine organotrophy with additional use of inorganic electron donors, or as heterotrophs unable to utilize the energy of SC oxidation. Because of the absence of reliable data, the problem of heterotrophic sulfur oxidation remained vague until recently. The data on this issue obtained before 1990 were discussed in my previous review [1]. Of those data, most trustful and informative were the results of H. Jannasch's group on marine heterotrophic bacteria capable of one-electron oxidation of thiosulfate to tetrathionate (see below), the data of D.P. Kelly's team on the same process driven by heterotrophic pseudomonads [2], and physiological data from J.G. Kuenen's group on lithoheterotrophic "*Thiobacillus Q*" [3], which was later identified as a betaproteobacterium [4]. Over the decade, new data on this problem have been obtained. The aim of the present review is to analyze and generalize these data. Some of the new data have already been presented in the recent review paper on marine heterotrophic bacteria participating in the oxidation of organic and inorganic SC [5].

1. DISTRIBUTION AND ABUNDANCE OF HETEROTROPHIC SOB

Previous research of Jannasch's group demonstrated the presence of obligately organotrophic bacteria capable of the oxidation of thiosulfate to tetrathionate (hereafter, these bacteria are referred to as T-HSOB) in the redox layer of the Black Sea and Cariaco Trench [6–10]. Based on this finding, we undertook a systematic search for heterotrophic SOB in the Black Sea and some other saline environments. The samples were

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obtained during several expeditions. For enumeration of heterotrophic SOB, serial decimal dilutions were used, with the medium based on seawater, pH 7.5 (for marine heterotrophs) or with a soda mineral base, 0.5–4 M total Na^+, pH 10 (for haloalkaliphiles). In both cases, 10–15 mM thiosulfate was added, and, in some cases, 2–5 mM acetate and 50 mg/l yeast extract were added as well. The detection method was based on the change in the color of the pH indicator Bromthymol Blue (in the case of marine neutrophiles) and on measuring thiosulfate consumption followed by investigation of the dominant forms in pure cultures. It is necessary to stress that the detection of heterotrophic SOB solely on the basis of growth monitoring may lead to erroneous conclusions even if a mineral medium is used without organic additions, as was done by Podgorsek and Imhoff [11]. According to our experience, marine organotrophic bacteria unable to oxidize thiosulfate can actively grow on mineral media based on seawater (irrespective of the presence of thiosulfate) or on mineral agar media due to the presence of traces of readily utilizable organic compounds. Therefore, determination of thiosulfate consumption is necessary during the work with such "mineral" media. Moreover, in some cases discussed below, even thiosulfate disappearance may be misleading, and a final conclusion on the potential of a heterotrophic organism to oxidize SC can only be made after respiratory experiments with washed cells.

We observed three types of culturable chemotrophic SOB in the redox layer of the Black Sea, including lithoautotrophs, which oxidized thiosulfate and sulfide to elemental sulfur and sulfate, and two groups of organotrophs that oxidized thiosulfate either completely to sulfate (hereafter, S-HSOB) or incompletely to tetrathionate (hereafter, T-HSOB) [12, 13]. Lithoautotrophic SOB prevailed in the lower part of the redox layer (up to 10^3/ml), being represented by small motile spirilla resembling *Thiomicrospira*. In other parts of the layer, only heterotrophic SOB could be detected on mineral medium or on medium supplemented with acetate. The group of S-HSOB, which had not been detected in the Black Sea before our work, were observed regularly in most of the samples from the redox layer, with an abundance of up to 10^4/ml. Such bacteria can develop on seawater media under oligotrophic conditions but are unable to grow autotrophically, thus belonging to the group of obligate organotrophs. 11 strains were obtained in pure cultures. The group of T-HSOB, which increased the pH during growth on medium with acetate and thiosulfate, prevailed in the upper part of the redox layer, exhibiting an abundance of up to 10^3–10^4/ml. This type constituted up to 30% of the culturable population of the aerobic acetate-utilizing heterotrophs of the redox layer. 20 strains of this type were isolated in pure culture. In addition to the deep-sea part, we also analyzed SOB population associated with the microbial mats developing on the surface of aragonite structures located on the shelf near Sevastopol. In contrast to the redox layer, only the T-HSOB group was detected here, with a high abundance of up to 10^5–10^6/g [14]. Four strains were isolated in pure culture. The density of heterotrophic SOB in the littoral sediments of the Plenty Bay (New Zealand) and Matupy Harbour (Papua–New Guinea) around sulfidic exhalations reached 10^8/ml, with domination of the T-HSOB. They constituted up to 38% of the population of culturable aerobic acetate-utilizing heterotrophs [15]. Of the 11 strains isolated, 5 were represented by S-HSOB and the rest belonged to T-HSOB. Among the latter an unusual form dominated which grew in the form of chains of cocci. A high number of heterotrophic SOB (up to 10^7/ml) was also observed in soda lakes [16].

Recently, an attempt has been made to enumerate heterotrophic SOB in a deep-sea hydrothermal area [17]. Two groups of heterotrophic SOB were detected, with an abundance of up to 10^4/ml. Unfortunately, the potential of the isolated heterotrophs to oxidize thiosulfate was not confirmed by the growth experiments with liquid cultures and by the respiratory test. This, as already mentioned above, may lead to incorrect conclusions. In particular, in my opinion, the conclusion that several *Halomonas* and *Cytophaga* isolates, whose growth was accompanied by an increase in pH, belonged to the group of S-HSOB was not justified. During prolonged incubation, tetrathionate produced from thiosulfate decomposes in neutral and alkaline conditions with the formation of thiosulfate, sulfate, and sulfur. This process can take place even under conditions of freezing, which makes it risky to store samples containing tetrathionate (R. Stuedel, personal communication). According to our experience, marine T-HSOB growing with a pH increase due to tetrathionate production never form sulfate as a product. For example, the numerous thiosulfate-oxidizing *Halomonas* strains isolated by us formed tetrathionate as the final product (see below). This suggests that at least the *Halomonas* isolate described in [17] belong to the T-HSOB.

2. CHARACTERIZATION OF THE T-HSOB GROUP ISOLATED FROM MARINE, SODA, AND HYPERSALINE HABITATS

2.1 General Characterization and Taxonomy

More than 20 strains of marine T-HSOB (mostly from the redox layer of the Black Sea), approximately the same amount of isolates from soda lakes and soda soils, and 6 isolates of extremely halophilic bacteria have been investigated (Tables 1, 2). All of the isolates were gram-negative.

Among marine isolates, motile rods and vibrios were the dominant morphotypes, except for a single coccoid strain which was described as a new genus and species, *Catenococcus thiocyclus* [18]. Rod-shaped marine isolates formed three clusters that included aerobic rods, facultatively anaerobic denitrifying curved

Table 1. Neutrophilic T-HSOB isolated from the Black Sea (ChG); Green Lake, Kermadek Archipelago, New Zealand (TG 3); and from a sulfide-oxidizing bioreactor (BG 2)

Group	Number of isolates	Depth, m	HS^-, mg/l	DNA G+C, mol %
Aerobic motile (up to 3 flagella) rods	ChG: 8 strains	120–200	0.04–2.0	7 strains: 57–59; 1 strain: 40.7
Denitrifiers; curved motile rods with a single polar flagellum	ChG: 6 strains;	120–200	0.04–2.0	
	TG3	–	0.58	
	BG2	–	–	

		DNA G+C, mol %	DNA homology with ChMG 3, %
Group of microaerophiles from the shelf of Sevastopol	ChMG 3	50.6	100
	ChMG 4	50.5	96
	ChMG 5	50.7	98
	ChMG 6	44.4	10

Table 2. Haloalkaliphilic T-HSOB isolates from soda lakes

Region	Number of isolates	Group*	Strain designation	DNA G+C, mol %
1. Moderate halophiles				
Southeastern Siberia	6	I	AG	66–67
	2	II		
Transbaikal region (Chita oblast)	6	I	AGB	1 strain: 54.4
	2	II		7 strains: 66–67
Kenya	1	I	AGJ	65–66
	1	II		
	5	III		
2. Selenite-reducing moderate halophiles				
Mongolia, Kenya, Mono Lake	7	II	Se, Mono	–
3. Strains able to grow at up to 4 M total Na^+				
Mongolia, Mono Lake, Lake Magadi	2	III	AGMg	–
	1	I	AGM	54
	1	I	AGMa	–

* Group I, motile thin rods; group II, motile coccobacilli; group III, nonmotile coccobacilli with barrel-shaped cells.

rods, and microaerophilic vibrios isolated from the Sevastopol shelf area (Table 1). DNA–DNA hybridization analysis indicated presence of two genomic clusters corresponding to the clusters of aerobes and denitrifiers (Fig. 1). Phylogenetic analysis of several representatives revealed that these bacteria belong to the gammaproteobacteria. In particular, the aerobic strain ChG 7-3 belongs to the genus *Halomonas*, with closest relation to *H. meridiana*; strain ChG 7-2 was identified as a representative of the genus *Pseudoalteromonas*; and the isolate from the Black Sea shelf area is close to the genus *Psychrobacter* (Fig. 2). Phylogenetic analysis of four isolates from the cluster of denitrifying T-HSOB revealed their affiliation with the "superspecies" *Pseudomonas stutzeri* [19]. *C. thiocyclus* from the near-shore hydrothermal area in the Pacific Ocean differed from the rest of T-HSOB isolates morphologically and by its ability for sugar fermentation [18]. According to the recent phylogenetic analysis, this unusual organism is a member of the family *Vibrionaceae* within the gammaproteobacteria (Fig. 2).

Among the pure cultures of T-HSOB described in two recent publications [11, 17], were the same members of the gammaproteobacteria that we found in the Black Sea, including *Pseudoalteromonas*, *Halomonas*, and *Pseudomonas stutzeri.*

The group of haloalkaliphilic strains of T-HSOB that we isolated from soda habitats on highly alkaline medium (pH 10, 0.6–4 M total Na^+) containing acetate and thiosulfate included three morphological and genetic clusters (Table 2, Fig. 3) [16, 20, and unpublished results]. Most of the representatives of this group

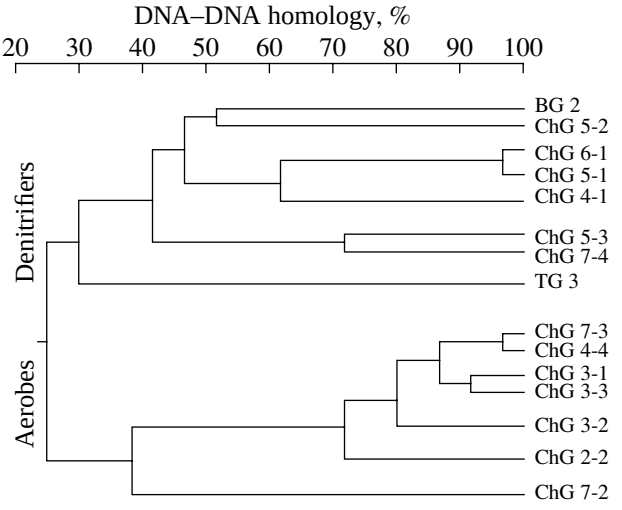


Fig. 1. Dendrogram of DNA homology within the group of marine T-HSOB isolates.

are active denitrifiers, but none was able to ferment sugars. Despite their substantial phenotypic and genetic diversity, phylogenetic analysis of the representatives of the group placed all of them into the *Halomonas* group within the gamma-3 proteobacteria (Fig. 2), where they form a compact cluster with the haloalkaliphilic denitrifying species *Halomonas disederata* [21]. Two strains from the cluster of motile rods (AG 2 and AGJ 2) phylogenetically are most closely related (B. Jones, personal communication) to another haloalkaliphilic species from the Kenyan soda lake, *Halomonas magadii* [22]. Clearly, our isolates might represent several new species of *Halomonas.* However, their taxonomic description at this moment is not feasible because of the shaky status of the whole *Halomonas* group, which is evidently much more diverse than a single genus should be and, therefore, needs reorganization [23]. Nevertheless, it is possible to state that the ability to oxidize inorganic SC incompletely to tetrathionate is widespread among the moderately halophilic and haloalkaliphilic organotrophic bacteria of the genus *Halomonas*, which is one of the dominant groups of aerobic eubacteria in saline habitats [24].

Our recent analysis of the hypersaline chloride–sulfate lakes in the Kulunda steppe (Altai, Russia) and in northeastern Mongolia (total salt content, 120–360 g/l; pH 7.5–8.5) demonstrated the presence of a unique population of extremely halophilic T-HSOB. Spiral-shaped organotrophic eubacteria able to oxidize thiosulfate and sulfide to tetrathionate dominated in aerobic and denitrifying enrichment cultures on medium containing 4–5 M NaCl and supplemented with acetate and thiosulfate. Overall, six strains were obtained in pure cultures, representing two distantly related genetic clusters. In contrast to the *Halomonas* T-HSOB, these bacteria fail to grow at low salinity (growth range is 2–5 M NaCl). Phylogenetic analysis put them in the "*Pseudomonas halophila*" cluster within the gammaproteobacteria, despite the striking differences in the phenotypes, which made us doubt the authenticity of the type strain of "*Pseudomonas halophila*". Indeed, our careful investigation of the properties of "*P. halophila*" revealed its identity with the extremely halophilic gammaproteobacterium originally described as "*Halovibrio variabilis*" [25]. This bacterium, at present known as "*Halomonas variabilis*," also proved to be able to oxidize thiosulfate to tetrathionate. Therefore, our extremely halophilic denitrifying T-HSOB isolates belong to the genus "*Halovibrio*," whose taxonomic status needs to be restored.

Apart from the eubacterial T-HSOB, in some enrichment cultures from hypersaline lakes we observed unusual associations that included red organotrophic haloarchaea and autotrophic spirilla-shaped eubacteria. During the first stage, haloarchaea rapidly developed, using acetate as the carbon source and oxidizing thiosulfate to tetrathionate. After most of thiosulfate was converted, the tetrathionate produced was oxidized further to sulfate by extremely halophilic thioautotrophic spirilla. The haloarchaeal member of these associations was obtained in pure culture, and its ability to oxidize thiosulfate to tetrathionate was proved in growth and respiratory experiments. The oxidation of thiosulfate mostly occurred in the end of the exponential and beginning of the stationary growth phase. The phylogenetic position of the haloarchaeal T-HSOB is currently under investigation. To our knowledge, it is the first evidence of participation of haloarchaea in oxidation of SC. Overall, it can be concluded, however, that various groups of gammaproteobacteria dominate among the culturable forms of T-HSOB in saline habitats.

2.2 Influence of Thiosulfate on the Growth of T-HSOB

Most of our halophilic and haloalkaliphilic T-HSOB strains oxidized thiosulfate in parallel with growth in batch culture with acetate as the carbon and energy source; the total amount of converted thiosulfate was comparable with that converted in the cultures of autotrophic SOB or was even higher [16, 26]. Neutrophilic T-HSOB increased the pH of the medium during thiosulfate oxidation from 7 to 9 due to tetrathionate production, which constituted up to 95% of the thiosulfate oxidized. Most of the strains were also capable of the reverse reaction of tetrathionate reduction to thiosulfate under anaerobic conditions in the presence of acetate [26]. Haloalkaliphilic representatives growing at pH 10 converted to tetrathionate no more than 60% of the thiosulfate oxidized; the rest could be recovered as trithionate, the product of chemical decomposition of tetrathionate in alkaline medium [27].

The capacity of T-HSOB to utilize the energy of oxidation of thiosulfate to tetrathionate was of special interest to us. This reaction gives 8 times less energy than the complete oxidation to sulfate, and this makes it doubtful that T-HSOB can be lithoheterotrophs.

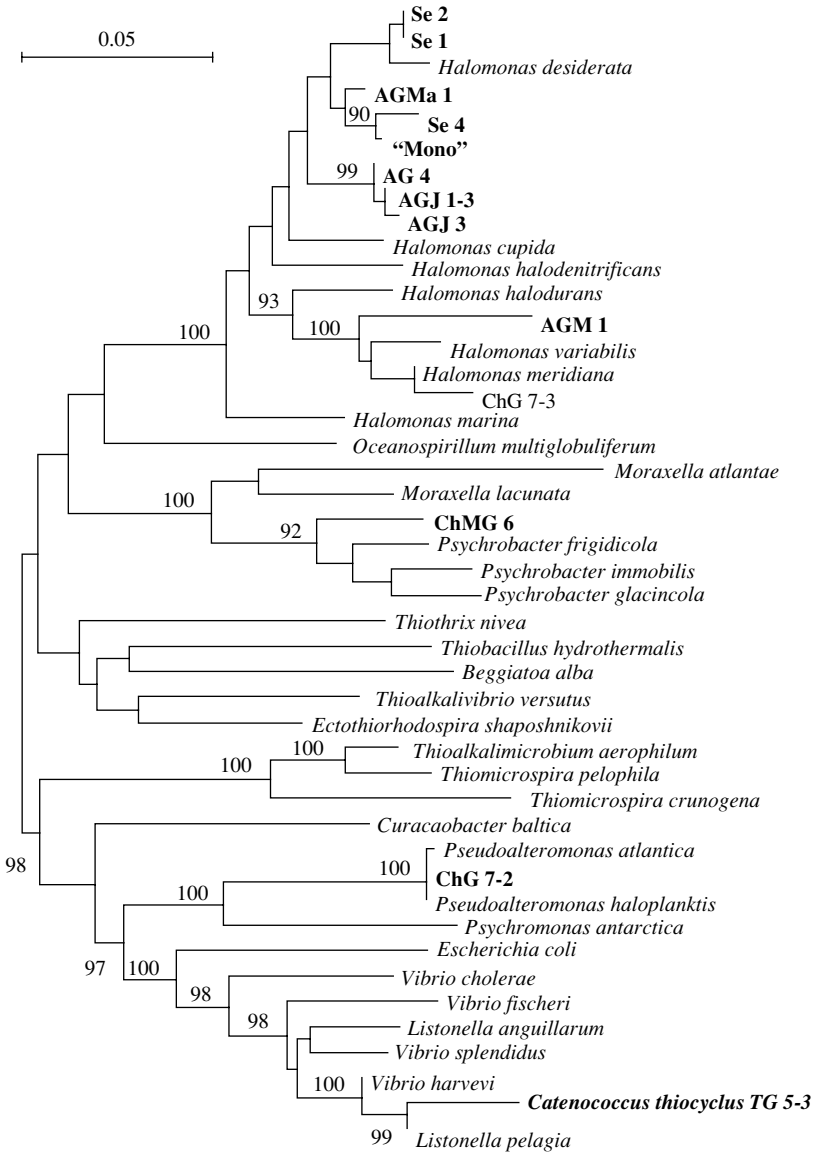


Fig. 2. Phylogenetic tree showing the position of marine and haloalkaliphilic T-HSOB within the gammaproteobacteria. Reference species are represented by the type strains. Scale bar corresponds to 5 substitutions per 100 nucleotides. Numbers indicate bootstrap values (only values above 90% are presented). The sequence data have not been published. The phylogenetic analysis was performed by T. Tourova.

Indeed, our experiments with batch cultures of marine and haloalkaliphilic T-HSOB together with data published on a Black Sea isolate [8] and some other T-HSOB strains [2] demonstrated the furtive nature of thiosulfate oxidation in these bacteria. In marine strains, thiosulfate oxidation increased the growth rate, but this increase was caused by an increase in the suboptimal pH due to tetrathionate production and did not result in an increase of the final biomass yield [27]. To eliminate the pH effect and to create organic substrate limitation, several T-HSOB strains were cultivated in continuous culture with pH controlled at a level of 7.5 for marine neutrophiles and 10 for haloalkaliphiles. Cultivation was performed with acetate limitation (10 mM influent concentration) at $D = 0.05–0.1\ h^{-1}$ and gradual increase of thiosulfate loading. Under such conditions, no growth stimulation by 2–20 mM thiosulfate was detected in the cultures of marine (strain ChG 3-3) and haloalkaliphilic (AGJ 1-3) *Halomonas* strains despite complete oxidation of thiosulfate to tetrathionate. In contrast, a reliable increase in the biomass yield in the presence of increasing thiosulfate concentration was observed for a culture of the marine neutrophilic T-HSOB *Catenococcus thiocyclus* [29]. Moreover, the effect observed dramatically exceeded the theoretical values of the energy available from the one-electron oxidation of thiosulfate to tetrathionate, being closer to the effect expected from complete oxi-

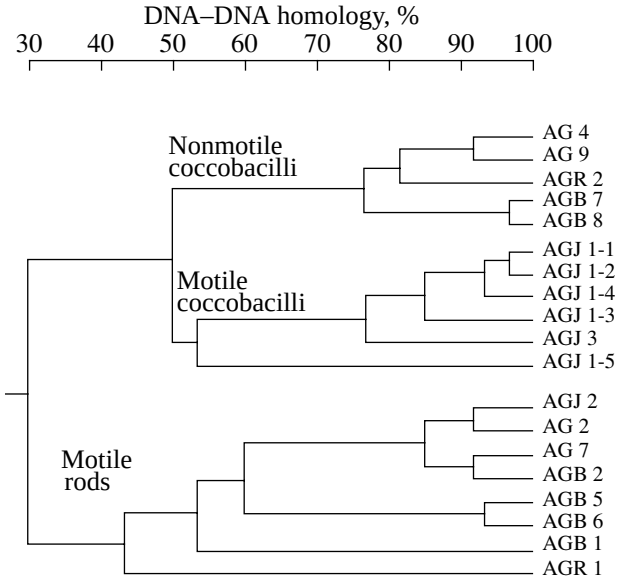


Fig. 3. Dendrogram of DNA homology within the group of T-HSOB from alkaline habitats.

dation to sulfate, which yields 8 times more energy. We can only speculate that the influence of thiosulfate on the metabolism of this organotrophic marine bacterium is more complex than merely direct supply of additional metabolic energy provided by the oxidation of thiosulfate to tetrathionate.

Apart from the interaction with the metabolism of organic substrates, thiosulfate oxidation was found to stimulate the anaplerotic CO_2 assimilation in T-HSOB [30]. The endogenous level of CO_2 assimilation in 12 investigated isolates of marine T-HSOB was similar to that observed for ordinary acetate-utilizing organotrophs from the same habitats used as controls (0.3–4.8 (mol mg protein^{-1} h^{-1}). The addition of thiosulfate increased this level by 20 to 300% in different strains depending on the activity of tetrathionate production. Control strains unable to oxidize thiosulfate did not experience such an effect.

2.3 Thiosulfate Interaction with the Respiratory Chain and Its Influence on the ATP Synthesis in T-HSOB

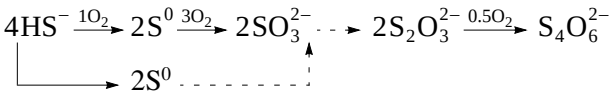
All of the marine (10) and haloalkaliphilic (4) strains of T-HSOB investigated in our laboratory lacked type *a* cytochrome oxidase in the membrane fraction; the spectral characteristics of their oxidase corresponded to the *b*(*o*) type. All the strains possessed an extremely high activity of soluble tetrathionate synthase. Thiosulfate addition to cell-free extracts resulted in rapid reduction of the pool of the periplasmic cytochrome $c_{552–553}$ (up to 70% of the complete reduction by dithionite) [26, 28]. The system of thiosulfate oxidation to tetrathionate in these bacteria seems to include (1) a thiosulfate dehydrogenase (tetrathionate synthase) sensitive to inhibition by sulfite and SH-reagents; (2) cytochrome(s) *c* of the respiratory chain; and (3) cytochrome oxidase of the *b*(*o*) type. Therefore thiosulfate can potentially influence both the energy state and the redox level of the respiratory chain in T-HSOB. The pH optimum of tetrathionate synthase is in the slightly acidic region for marine neutrophiles and in the neutral region for haloalkaliphiles. The activity was mostly constitutive, but it increased sharply upon thiosulfate addition to the growth medium. Previously, only a single attempt had been made to study this enzyme in the marine T-HSOB strain 16 B in detail [10, 31]. The authors managed to demonstrate that the enzyme includes cytochrome c_{553} and has an apparent molecular weight of 100 kDa. We also attempted purification of tetrathionate synthase from the haloalkaliphilic *Halomonas* strain AGJ 1-3. However, the process was not finished successfully because of the high instability of the enzyme during purification. We only managed to demonstrate that the enzyme was located in the periplasm and associated with the protein fraction with a high content of cytochrome c_{552} fully reducible by thiosulfate addition (our unpublished results). Of considerable interest would be a comparison of the properties of tetrathionate synthase from T-HSOB with those of the analogous enzyme in autotrophic SOB.

Using washed cells of marine T-HSOB, we managed to demonstrate a reliable increase in the endogenous ATP level during oxidation of thiosulfate to tetrathionate [32]. Interestingly, this effect was observed not only with *C. thiocyclus* cells but also with cells of *Halomonas* sp. ChG 7-3, while growth was stimulated by thiosulfate only in cultures of *C. thiocyclus.* Assuming that the connection with the respiratory chain and the ability to produce ATP in the presence of thiosulfate represent a potential of T-HSOB to lithoheterotrophy, it might be speculated that the actual use of the energy of thiosulfate oxidation to tetrathionate by T-HSOB could be determined by the efficiency of their organic substrate metabolism. Particularly, the efficiency of acetate utilization (the assimilation/dissimilation ratio for organic C, i.e. $Y_{acetate}$) in *C. thiocyclus* was 1.3–1.5 times lower than in the *Halomonas* strains ChG 3-3, ChG 7-3, and AGJ 1-3. It seems likely that the potential of the reaction of thiosulfate oxidation to tetrathionate can be realized as a biomass increase only by those T-HSOB which have an inefficient metabolism of organic substrates.

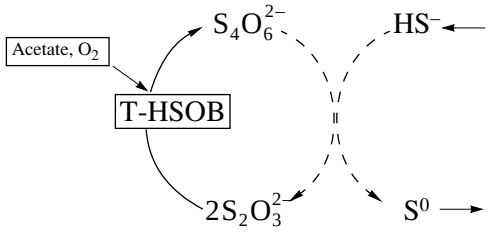
2.4 Oxidation of SC Other Than Thiosulfate by T-HSOB

The ability of organotrophs to oxidize thiosulfate to tetrathionate depends on the presence of a single specific enzyme, tetrathionate synthase. Hence, this property could be considered as nonspecific and accidental, transferable, for example, by plasmids. However, upon careful examination of our T-HSOB collection, most of the strains were found to be able to oxidize, in addition to thiosulfate, sulfide and elemental sulfur, although with a much lower activity. The final product in all

cases was tetrathionate. Batch cultures of marine *Halomonas* strains grown with acetate and crystalline sulfur produced up to 1 mM tetrathionate within 3 days. Oxidation of hydrophilic sulfur formed in a bioreactor proceeded 5 times faster in the *Halomonas* ChG 3-3 culture. An acetate-limited continuous culture of *C. thiocyclus* oxidized up to 3 mM sulfide to elemental sulfur and tetrathionate at D = 0.05–0.075 h^{-1} [29]. The rates of oxygen consumption by washed cells of T-HSOB in the presence of thiosulfate, sulfide, and elemental sulfur were within the ranges of 120–1000, 10–800, and 10–200 nmol O_2/(mg protein min), respectively. The haloalkaliphilic representatives differed from the marine strains by exhibiting elevated rates of sulfide oxidation [16, 33]. The stoichiometry of oxygen consumption with any of the three SC corresponded to their oxidation to tetrathionate, which allows the following scheme of reactions to be suggested (dashed lines indicate abiotic reactions):



Apart from direct sulfide oxidation, which was usually about 10 times less active than thiosulfate oxidation in most of the marine T-HSOB, we discovered an interesting fact of indirect catalysis of sulfide oxidation with the participation of T-HSOB. Its essence is that tetrathionate is acting as a very efficient sulfide oxidant. During chemical reaction of tetrathionate and sulfide in the presence of oxygen, the sulfide is oxidized to elemental sulfur, reducing thereby tetrathionate to thiosulfate. The presence of the T-HSOB cells substantially accelerate sulfide oxidation. Moreover, with the cells, sulfide can be oxidized in the presence of thiosulfate instead of tetrathionate. The reason for this is that the bacteria regenerate the catalyzer (tetrathionate) by oxidizing the product of the chemical reaction of sulfide and tetrathionate (thiosulfate). Overall, the process is semicircular, and bacteria act as a pseudocatalyzer. Possessing an active tetrathionate synthase, the T-HSOB can participate in sulfide oxidation when supplied with catalytic amounts of acetate and thiosulfate [28], according to the following scheme:

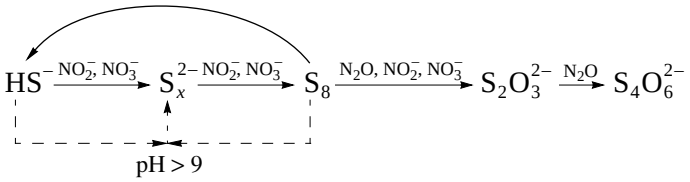


The investigation of the activity of heterotrophic SOB in the Baltic Sea sediments led to similar conclusions [11]. The principle of such a combined microbial–chemical process [34] was used in our study of the possibility of T-HSOB application for biological oxidation of sulfide. For this, the biomass of the marine strain *Halomonas* ChG 3-3 was grown in a continuous culture with 10 mM acetate + 14 mM thiosulfate at pH 8. From the fermentor, the culture was pumped into a chemical reactor that was continuously fed with a sulfide solution. Sulfide was oxidized to elemental sulfur by tetrathionate produced by the bacterium from thiosulfate during growth. Tetrathionate was reduced back to thiosulfate, which was reoxidized by the *Halomonas* cells in the chemical reactor. As a result, tetrathionate was constantly present in the chemical reactor until the sulfide loading rate exceeded a certain level. The elemental sulfur produced in the process can be separated from the culture and the latter can be returned to the reactor. Using this model, we demonstrated that T-HSOB can be used as an indirect catalyzer for the conversion of sulfide to elemental sulfur with rates comparable to the parameters of autotrophic bioreactors. The process has been patented [35].

2.5 Anaerobic Thiosulfate Oxidation by Denitrifying T-HSOB

Our marine isolates belonging to *P. stutzeri* and most of the haloalkaliphilic T-HSOB isolates, being active denitrifiers, were found to be capable of thiosulfate (*P. stutzeri*) [19] or sulfide (haloalkaliphiles) [16] oxidation during anaerobic growth with the production of tetrathionate as the final product. *P. stutzeri* strains oxidized thiosulfate most actively using nitrous oxide as electron acceptor. Nitrate was also used albeit less actively because of nitrite accumulation, which inhibited tetrathionate production. Washed cells of these bacteria could also oxidize thiosulfate with nitrite as an electron acceptor. In contrast to the marine strains, which were unable to oxidize sulfide during anaerobic growth, the haloalkaliphilic *Halomonas* isolates actively converted sulfide to thiosulfate (via polysulfide and elemental sulfur) using nitrate or nitrite as an electron acceptor. However, only the isolates from Kenyan soda lakes could anaerobically oxidize thiosulfate further on to tetrathionate, and this was possible only with nitrous oxide as the electron acceptor. Soluble polysulfide was the first product of anaerobic sulfide oxidation by the haloalkaliphilic *Halomonas* strains. It was further oxidized to elemental sulfur via cell-bound polysulfide. This reaction proceeded relatively fast, in contrast to the slow second phase of elemental sulfur oxidation to thiosulfate, which started after polysulfide consumption. Moreover, haloalkaliphilic heterotrophs were able to reduce elemental sulfur back to sulfide

during anaerobic incubation after the nitrogen oxides had been completely consumed. The anaerobic sulfide conversion by the denitrifying T-HSOB can be represented by the following overall scheme:



3. CHARACTERIZATION OF THE GROUP OF S-HSOB ISOLATED FROM MARINE AND SODA HABITATS

3.1 Diversity, Phenotypic Properties, and Phylogeny of the Pure Cultures

Heterotrophic bacteria oxidizing thiosulfate completely to sulfate and different from both autotrophic SOB and the T-HSOB discussed above proved to be one of the dominant groups of SOB in the redox layer of the Black Sea. These isolates (S-HSOB) can develop on mineral medium with thiosulfate and seawater. When the medium was prepared using distilled water, the growth ceased after 2–3 culture transfers. The inability of this group to grow autotrophically was confirmed by enzymatic analysis (absence of RuBisCo). In contrast to T-HSOB, S-HSOB acidify the medium during growth due to the formation of sulfuric acid from thiosulfate. Overall, ten pure cultures of S-HSOB have been obtained from the redox layer of the Black Sea and from the near-shore hydrothermal area in the Pacific Ocean [12, 15], and one haloalkaliphilic isolate of this type was isolated from the soda lake Gorbunka (Chita region, Siberia). The marine isolates form four genetic clusters according to DNA homology data (Table 3). Group 1 is represented by four strains with lemon-shaped cells covered with a thick capsule and forming in aged cultures brown cyst-like structures with a thick cell wall. Group 2 (three strains) is represented by thin motile rods often occurring in chains and rosettes. Group 3 includes two similar strains of motile rod-shaped bacteria forming dumbbell forms and rosettes. Group 4 includes a single strain, whose cells tend to branch during growth on rich media [36]. According to the lipid (B. Tindall) and phylogenetic (F. Rainey) analyses, these bacteria belong to the alphaproteobacteria: strains of groups 1–3 are members of the alpha-3 subgroup of the *Roseobacter* cluster, while strain ChLG 11 of group 4 is in the alpha-2 subgroup (Fig. 4). Recently, the strains of group 3 were described as a new genus and species, *Sulfitobacter pontiacus* [37]. Group 1 undoubtedly represents another new genus with 2 separate species.

The haloalkaliphilic S-HSOB strain ALG 1 isolated from a microbial mat in a soda lake at pH 10 is a member of erythrobacteria, which include obligately aerobic bacteria synthesizing bacteriochlorophyll *a* [38]. Strain ALG 1 is a nonmotile rod producing a red–orange carotenoid. It is an obligate alkaliphile (with a pH range for growth from 8.5 to 10.5) and moderate halophile (grows between 0.3 and 2 M total Na+), obligately organotrophic and aerobic. It belongs to the alpha-3 subgroup of the alphaproteobacteria, with closest relation to nonsulfur purple bacteria of the genus *Rhodobacter* and was recently described as a new genus and species, *Roseinatronobacter thiooxidans* [39].

Thus, all our isolates of T-HSOB belong to the alphaproteobacteria and are related to nonsulfur purple bacteria and their chemotrophic (including sulfur-oxidizing) descendants. This group also includes the recently described soil S-HSOB isolate *Bosea thiooxidans* [40]. On the other hand, among freshwater T-HSOB isolates, there are members of the betaproteobacteria, particularly the lake heterotroph *Limnobacter thiooxidans* [4], "*Thiobacillus Q*" from a thiodenitrifying reactor [3, 4], and the moderately thermophilic heterotroph *Tepidomonas ignava* from a hot spring [41].

3.2 Influence of SC on Growth of S-HSOB

In contrast to T-HSOB, our S-HSOB isolates started thiosulfate oxidation only at the end of active growth in batch culture with acetate, which indicates a more tight interaction between the inorganic sulfur and organic electron donor in such organotrophs. Overall, up to 20 mM thiosulfate was oxidized during growth with 20 mM acetate. Sulfite was often detected in the medium as an intermediate of thiosulfate oxidation (up to 2.5 mM), while sulfate was the final product. Crystalline sulfur was oxidized much more slowly than thiosulfate, although substantial amounts of sulfate did accumulate in the medium after prolonged incubation (up to 36 mM after 9 days).

In batch culture, only group 1 marine strains and the haloalkaliphilic strain ALG 1 produced extra biomass yield in the presence of thiosulfate. On the other hand, all S-HSOB increased their growth efficiency in the presence of thiosulfate in acetate-limited continuous culture [39, 42]. The average value of the thiosulfate-dependent biomass increase was about 4 mg protein/mmol thiosulfate which corresponds to average yield values of autotrophic SOB [43] and, therefore, indicates efficient utilization of the energy of thiosulfate oxidation in the metabolism of

these organotrophic bacteria. A thiosulfate-dependent yield increase has also been observed in batch cultures of other recently described representatives of S-HSOB, such as *Bosea thiooxidans* [39], *Limnobacter thiooxidans* [4], "*Thiobacillus Q*" [3, 4] and *Tepidomonas ignava* [41].

3.3 Activity and Mechanism of SC Oxidation by S-HSOB

All our S-HSOB isolates oxidized thiosulfate (20–350 nmol O_2/(mg protein min), sulfide (2–220), elemental sulfur (20–150) and sulfite (20–1480) but none of them were able to oxidize or produce tetrathionate. *S. pontiacus* differed from other S-HSOB by exhibiting a very low thiosulfate-oxidizing activity and extremely active sulfite oxidation (see below). Thiosulfate oxidation was inducible and was sharply activated during growth in continuous culture. The K_s values for thiosulfate and sulfide in strain ChLG 1 were in the range of 2–5 and 3–5 μM, respectively, which is within the range known for autotrophic SOB [36, 42]. The activities of the thiosulfate-splitting enzymes rhodonese and thiosulfate reductase and AMP-independent sulfite dehydrogenase (but not of tetrathionate synthase) were found in the cell-free extracts obtained from the S-HSOB cells grown with thiosulfate. These data allowed us to suggest that the mechanism of SC oxidation in our S-HSOB isolates could be similar to one of the pathways common among autotrophic SOB, with sulfite as a key intermediate:

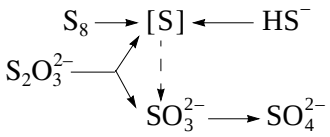


Table 3. S-HSOB isolated from the Black Sea

Group	Strain	DNA G+C, mol %	DNA homology (%) with		
			ChLG 1		
1	ChLG 1	68.9	100		
	ChLG 2	69.0	88		
	ChLG 15	69.2	93		
	ChLG 16	67.5	44		
				ChLG 7	
2	ChLG 7	63.7		100	
	ChLG 8	64.8		68	
	ChLG 17	64.4		59	
					ChLG 10
3	ChLG 5	61.7		26	97
	ChLG 10	62.5		37	100
4	ChLG 11	60.0		6	

3.4 Influence of Thiosulfate on Respiratory Chain and ATP Synthesis

Spectrophotometric analysis of cell-free extracts of S-HSOB cells identified the presence of cytochromes $c_{551–552}$, b_{558} and type aa_3 cytochrome *c* oxidase. Thiosulfate addition to the cell-free extracts resulted in rapid partial reduction of the cytochrome *c* pool and cytochrome *c* oxidase. In *S. pontiacus*, such an effect was observed in the presence of sulfite. The potential ability

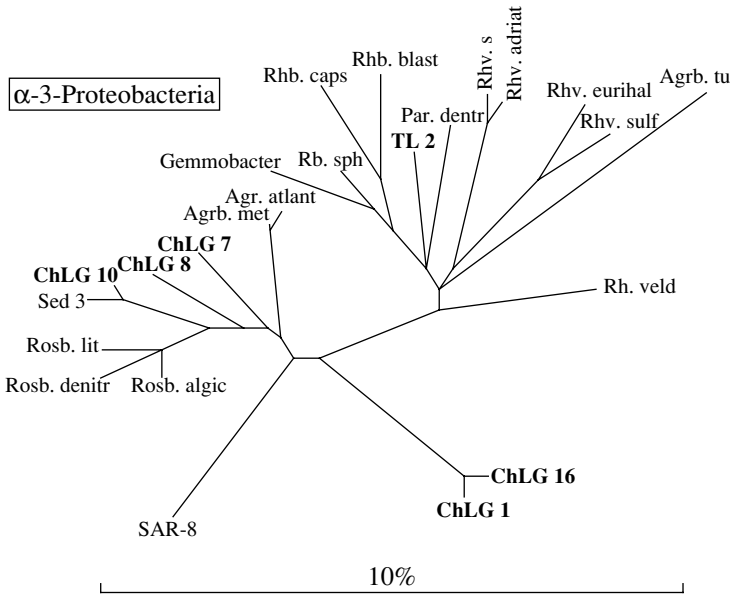


Fig. 4. Unrooted phylogenetic tree showing the position of S-HSOB strains isolated from the redox layer of the Black Sea [12, 36]. The 16 S rDNA sequencing and phylogenetic analysis were performed by F. Rainey.

of S-HSOB to utilize the energy of thiosulfate oxidation was confirmed by measurements of ATP synthesis in starved washed cells of strain ChLG 1 grown in continuous culture with acetate and thiosulfate. While the acetate-dependent rate of ATP synthesis exceeded the endogenous level by 9 times, thiosulfate caused a 6.5-fold increase and acted much faster than acetate.

Overall, the growth and activity data clearly demonstrated the potential of the investigated S-HSOB for lithoheterotrophy with the use of inorganic SC as an additional energy source.

Similar to T-HSOB described above, S-HSOB also increased their level of dark anaplerotic CO_2 assimilation by 20–200% in the presence of thiosulfate. However their endogenous activity level was much higher (10–23 against 1–5 nmol CO_2/(mg protein min)). Strain ChLG 1 possessed a high activity of pyruvate carboxylase. Pyruvate also significantly stimulated the activity of CO_2 assimilation. In favor of the important role of pyruvate carboxylase in the thiosulfate-dependent stimulation of the CO_2 assimilation was also the fact of growth stimulation of the investigated S-HSOB by biotin, which is a cofactor of pyruvate carboxylase. The stimulatory effect of thiosulfate on the CO_2 assimilation mediated by pyruvate carboxylase might be explained by its demand for ATP according to the following scheme [30]:

$$\text{pyruvate} + HCO_3^- \longrightarrow \boxed{\textit{pyruvate carboxylase}} \longrightarrow \text{oxalacetate}$$
$$[Mg^{2+}, \text{ATP, biotin}]$$
$$\uparrow$$
$$S_2O_3^{2-} \xrightarrow{\text{ATP}} SO_4^{2-}$$

3.5 A Unique Case of Lithoheterotrophy in S. pontiacus

Given its exceptionally high activity of sulfite oxidation, *S. pontiacus* was attempted to be grown with sulfite to determine its growth parameters and the possibility of lithoheterotrophy. Until now, this kind of growth has never been described, either for organotrophic or for autotrophic SOB, probably because of the toxicity and chemical instability of sulfite in the presence of oxygen. Nevertheless, *S. pontiacus* proved to be able to grow in a chemostat culture under acetate and sulfite limitation. The culture demanded prolonged adaptation to a relatively low sulfite load before it was possible to increase the influent sulfite concentrations to 60–80 mM. Above this level, the culture started to wash out. During the gradual increase of sulfite loading, a significant increase of the biomass yield was registered, while the sulfite-dependent respiration and the activity of the AMP-independent sulfite dehydrogenase reached maximal levels known for bacteria. At the same time, the activity of acetate-dependent respiration significantly decreased [37]. Such a behavior is typical for lithoheterotrophic growth when organic substrate is utilized mainly as the carbon source, while inorganic cosubstrate is used as the electron donor. The potential of strain ChLG 10 for utilization of the energy from sulfite oxidation was supported by the fact of active reduction of cytochrome c_{551} and cytochrome *c* oxidase aa_3 in the presence of sulfite and by stimulation of ATP synthesis in washed cells by sulfite. The dynamics of ATP synthase activity strongly correlated with the influent sulfite concentration in the continuous culture of ChLG 10. The acetate-dependent activity decreased almost to zero upon a sulfite concentration increase from 10 to 80 mM, whereas the sulfite-dependent activity sharply increased, correlating with the trend of respiratory activity.

In the course of the adaptation to sulfite, the culture decreased its acetate dissimilation to complete cessation and passed to purely lithoheterotrophic growth, when the organic cosubstrate is utilized only as a carbon source. This conclusion was supported by measurements of ^{14}C-acetate metabolism by the *S. pontiacus* cells obtained from different stages of the culture adaptation to sulfite. It was also demonstrated that the main reason for the observed changes in the carbon balance during the cultivation of ChLG 10 with sulfite was its direct influence on the level of synthesis and activity of the enzymes of the citric acid cycle. While the presence of sulfite in the medium at concentrations above 30 mM did not influence the activity of hydratases, it inhibited the activity of dehydrogenases such as malate, succinate, isocitrate and, especially, 2-ketoglutarate dehydrogenases, which suggested a decrease in the role of the citric acid cycle in acetate metabolism with parallel activation of acetate utilization for amino acid biosynthesis via pyruvate [44]. Thus, the growth of *S. pontiacus* with sulfite is a so far unique example of lithoheterotrophy with a toxic inorganic cosubstrate whose influence on the metabolism of the organic substrate can reach complete inhibition.

Two possible functions of the *Sulfitobacter*-type S-HSOB in natural microbial communities might be suggested. Lithoheterotrophs actively oxidizing toxic sulfite could develop in consortia with autotrophic SOB which excrete sulfite and organic compounds during

autotrophic growth on nontoxic SC. Another possible source of sulfite in the environment is sulfonates, natural and artificial organosulfur compounds with the general formula RSO_3H, which have lately attracted substantial attention due to their increasing release into the increasing [45–47]. Most of the bacteria mineralizing sulfonates release sulfite into the medium, and only few are able to further oxidize sulfite by means of highly active sulfite dehydrogenase after hydrolytic cleavage of sulfite moiety from the organic molecule. The latter organisms include *Ralstonia* sp. [48], *Comamonas acidovorans* [49], and methylotrophic bacteria capable of growth on methylsulfonate (CH_3SO_3H) [50]. They can be considered as representatives of S-HSOB. It might be speculated that the organotrophic bacteria specialized on sulfite oxidation, such as *Sulfitobacter*, could facilitate microbial degradation of sulfonates by effectively removing the sulfite released in consortia by bacteria that are unable to oxidize sulfite themselves. Recent data demonstrated that the bacteria of the *Sulfitobacte–Roseobacter* cluster represent one of the dominant group of organotrophs in the marine littoral ecosystems [51]. From such habitats two new species of *Sulfitobacter*, *S. mediterraneus* [52], and *S. brevis* [53] have recently been isolated. Unfortunately, a solid evidence on their potential for sulfite oxidation was presented.

4. DOUBTFUL CASES OF HETEROTROPHIC OXIDATION OF SC

Many reports about heterotrophic oxidation of SC by cultures of gram-positive bacteria [54] and micromycetes [55] can be found in older literature. These reports are mostly based on growth experiments where chemical analyses detected the accumulation of oxidized products, such as thiosulfate, tetrathionate and sulfate. Complete absence of such microorganisms among our isolates of organotrophic SOB prompted us to examine the ability to oxidize SC in several strains of gram-positive bacteria isolated from preparations of elemental sulfur and in some collection yeast strains [56]. Most of these microorganisms formed significant amounts of thiosulfate from crystalline sulfur (up to 20 mM), which apparently suggested the ability to oxidize SC. However, the true reason for thiosulfate formation by gram-positive bacteria and basidial yeasts was unspecific sulfur reduction to sulfide which actively proceeded in the presence of oxygen. The sulfide produced from sulfur was oxidized abiotically to thiosulfate in the presence of trace metals. None of these organisms was capable of further thiosulfate oxidation. Evidently, the same process, which has nothing to do with the biological oxidation of SC, was described for streptomycetes [54]. It is necessary to stress here that our experiments were carried out at neutral pH in buffered media where thiosulfate is chemically stable. This cannot be said about the experiments conducted with soil micromycetes which were claimed to be able to oxidize elemental sulfur through thiosulfate to sulfate [57–60]. If we assume that those "sulfur-oxidizing" micromycetes conducted aerobic sulfur reduction, then the produced thiosulfate might well be chemically decomposed in the acidic medium used in these experiments with the final formation of sulfate and sulfur. Unfortunately, respiratory experiments on the oxidation of SC by micromycetes are missing. Therefore, although the ability of eukaryotic microorganisms to oxidize SC cannot be ruled out, the data existing at the moment are not sufficient for a positive conclusion. The same holds for gram-positive bacteria, except for the single adequately proved example of the genus *Sulfobacillus* [61].

CONCLUSION

Our investigation revealed broad distribution of organotrophic bacteria with a potential to oxidize SC in saline environments. They are mostly represented by two groups of gram-negative bacteria of different origin and with different mechanisms of SC oxidation. The representatives of both groups have the potential to utilize the energy of SC oxidation, which, under certain conditions, can allow the lithoheterotrophic mode of growth. The new data on the ability of the T-HSOB group to oxidize sulfide and elemental sulfur in addition to thiosulfate, on the potential of some members to perform anaerobic sulfide and thiosulfate oxidation during denitrification, and on their potential for thiosulfate-dependent ATP synthesis can be considered an important addition to the previous knowledge about these bacteria. The knowledge on the S-HSOB group has also significantly broadened. The phylogenetic analysis of the new marine and soda lake representatives clearly demonstrated their close relationship to nonsulfur purple bacteria. These bacteria undoubtedly belong to the SC-dependent lithoheterotrophs.

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