ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 1, pp. 76–83. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © E.V. Gridneva, M.Yu. Grabovich, G.A. Dubinina, E.Yu. Chernousova, V.N. Akimov, 2009, published in Mikrobiologiya, 2009, Vol. 78, No. 1, pp. 89–97.

EXPERIMENTAL ARTICLES

Ecophysiology of Lithotrophic Sulfur-Oxidizing *Sphaerotilus* Species from Sulfide Springs in the Northern Caucasus

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Received February 15, 2008

Abstract—Six strains of sulfur-oxidizing bacteria of the known organotrophic species *Sphaerotilus natans* were isolated from two North Caucasian sulfide springs. Similar to known colorless sulfur bacteria, all the strains accumulated elemental sulfur when grown in media with sulfide. Unlike previously isolated *S. natans* strains, new isolates had higher temperature growth optimum (33–37°C) and variable metabolism. All the strains were capable of organotrophic, lithoheterotrophic, and mixotrophic growth with sulfur compounds as electron donors for energy metabolism. Variable metabolism of new *Sphaerotilus* isolates is a highly important adaptation mechanism which facilitates extension of their geographic range and supports their mass development in new habitats, e.g. sulfide springs. Within the cluster of new isolates, the physiological heterogeneity was shown to result from the inducible nature of the enzymes of oxidative sulfur metabolism and from their resistance to aerobic cultivation.

Key words: colorless sulfur bacteria, *Sphaerotilus natans,* ecophysiology, microaerophily, metabolism of sulfur compounds, sulfide springs.

DOI: 10.1134/S002626170901010X

The genus *Sphaerotilus*, belonging to the *Betaproteobacteria*, comprises gram-negative organoheterotrophic aerobic bacteria widespread in various freshwater, natural and anthropogenic environments (bog water, springs, ponds, household and industrial sewage, and activated sludge of sewage disposal plants). Their mass development in the biocenoses of sewage treatment facilities is one of the main causes of sludge bulking; this phenomenon suggested numerous investigations on the physiology and taxonomy of these bacteria [1, 2]. In natural environments not contaminated with fresh organic matter, *Sphaerotilus* forms filamentous and flaky growth. Iron oxides are often accumulated in the sheaths and *Sphaerotilus* is considered a typical representative of heterotrophic iron bacteria [3]. The taxonomy and physiology of numerous *Sphaerotilus* isolates obtained from various ecosystems in various geographical locations was studied in some detail; they form a homogeneous group and belong to the single species *Sphaerotilus natans* [4].

Stable bacterial communities predominated by *Sphaerotilus* together with *Thiothrix* sulfur bacteria were recently discovered in the sulfur mats of several sulfide springs in the region of deep subterranean water discharge in the Northern Caucasus [5]. Unlike *Thiothrix*, the filaments had the typical *Sphaerotilus* structure, i.e., chains of cells without a common cell wall, covered with an outer sheath. Elemental sulfur was accumulated within *Sphaerotilus* cells. The *Sphaerotilus* strains from the sulfur springs, as well as the phylotypes of the clone library obtained from the same samples, exhibited a very high similarity in the 16S rDNA nucleotide sequence (99.9%) to the type strain *S. natans* DSM 6575; phylogenetic analysis and the study of their morphological and physiological characteristics supported their classification within the cluster of strains of this species [5].

The goal of the present work was a comparative investigation of the metabolism and adaptive mechanisms of resistance of these new *S. natans* strains to unusual conditions of sulfide springs and determination

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of the functional role of reduced sulfur compounds in their metabolism.

MATERIALS AND METHODS

Strains and cultivation techniques. Five *S. natans* strains (D-501, D-502, D-504, D-505, and D-507) were isolated from the sulfur mats of the Petushok spring; two strains (BV-1 and BV-2) were obtained from the Besstyzhie Vanny spring. The isolation techniques and medium composition were given in [5]. For comparison, type strain *S. natans* DSM-6575 from the German Collection of Microorganisms and Cell Cultures (DSMZ) was used. The cultures were maintained and their physiological and biochemical characteristics investigated in the medium containing the following (mg/l): $(NH_4)_2SO_4$, 100; $CaCl_2$, 50; $MgSO_4 \cdot 7H_2O$, 100; sodium lactate, 200; sodium thiosulfate, 1000 or $Na_2S \cdot 9H_2O$, 100; 10% phosphate buffer, 10 ml; distilled water, 1 l; vitamins and trace elements [6]; pH 7.5. In the medium used for biomass accumulation, lactate content was increased to 0.5 g/l. In order to determine the effect of oxygen on bacterial growth and metabolism, the cultivation was carried out in sealed 1-l bottles with 100 ml of the medium and the argon–air gas phase containing 5, 10, or 20% oxygen.

Cell suspensions and enzyme preparations in the supernatant of disintegrated cells were obtained as described [7].

Analytical techniques. Protein content was determined by the Lowry method. The protein of lysed cells was determined in the supernatant after the undamaged cells were removed by centrifugation for 20 min at 10000 *g* (4°C).

Analysis of inorganic sulfur compounds. When $S_2O_3^{2-}$, and $S_4O_6^{2-}$, were simultaneously present in the medium, they were determined by separate iodometric titration [8]. Sulfate was determined by the chloranilate method [9]. Intracellular elemental sulfur was identified by its birefringence under a polarizing microscope. Sulfide content in the spring water and dissolved oxygen in the presence of sulfide were determined by the modified Winkler method [8].

Polarographic O_2 determination. The rate of oxygen consumption by suspensions of respiring cells was determined with a closed Clark electrode and registered with a Rekord-4 polarograph (joint production of Institute of Biophysics, Russian Academy of Sciences and Pilot Plant, Pushchino). The polarograms were analyzed according to the accepted procedure [10]. The results were calculated with the Rekord-4 software package. For the experiments, the cells were grown to the exponential phase in the medium containing 0.2 g/l lactate and 1 g/l thiosulfate; the concentration of sulfite (or thiosulfate) in the cell was 12 mM. The respiration rate with sulfite (thiosulfate) was determined by subtracting the endogenous respiration and chemical oxidation of sulfite (thiosulfate).

Inhibitor analysis. For investigation of the functioning of the electron-transport chain (ETC), inhibitor analysis was used [7]. The rate of oxygen consumption by cell suspensions in the presence of sulfur compounds was determined polarographically after addition of one of the following inhibitors (μmol/ml): 8-hydroxyquinoline-N oxide (inhibitor of the flavin–quinone–cytochrome *c* part), 30.0; myxothiazol (inhibitor of the quinone–cytochrome *b* part), 25.0; antimycin A (inhibitor of the cytochrome *b* part), 90.0 and 180.0; carbonyl cyanide phenylhydrazone and carbonyl cyanide chlorophenyl hydrazone (decouplers of oxidative phosphorylation), 0.06 and 0.05, respectively.

Activity of the enzymes of dissimilatory sulfur metabolism was determined in the supernatant of disintegrated cells (2-day culture in the middle of the exponential growth phase). Activity of thiosulfate oxidoreductase (EC 1.8.2.2), sulfite oxidoreductase (EC 1.8.3.1), and APS reductase (EC 1.8.99.2) were determined spectrophotometrically as described [11].

Rhodanese (EC 2.8.1.1) activity was determined by the modified Sorbo method [12].

All experiments were carried out at least two–five times; the figures depict the results of the representative experiments. The data presented in the tables are averages of three measurements; the data spread did not exceed 10–15%.

RESULTS AND DISCUSSION

Habitat conditions. *Sphaerotilus* strains were isolated from the sulfur mats of two sulfide springs in the Crimea–Caucasian region of deep spreading sulfide waters. The Petushok spring flows into the Psekups River in Goryachii Klyuch, Krasnodar Region. The runway at the outflow, along the watercourse, and in the site of inflow into the Psekups River is covered with bacterial growth in the form of sulfur mats (Fig. 1a). The temperature was 34–37°C. Sulfide content at different times of observation varied from 2 to 11 mg S^{2-}/l, depending on dilution by a weak flow of the subterranean water from the upper water-bearing formation and the yield of the sulfur spring; the latter was affected by the water head in nearby production wells. Oxygen ingress into the water occurred at the contact with air; oxygen content varied from 0.1 to 0.5 mg/l; pH was 7.5–7.6. Another sulfide spring, Besstyzhie Vanny, is located in Pyatigorsk, Stavropol' Region. It is a seeping of natural sulfide waters from the higher Lake Proval through the rocks and down along the slope. Unlike the first spring, sulfide content was constant throughout the observation period (1.5–2 mg S^{2-}/l). The thin water layer was aerated due to turbulent mixing with air; oxygen content, however, did not exceed 2–2.5 mg/l. The water temperature was 28–30°C, on hot days it went up to 35°C. The total mineralization was 1.45–1.6 g/l. The sulfur mats developed on the slope along the flow. Unlike the mats from the first spring, local development

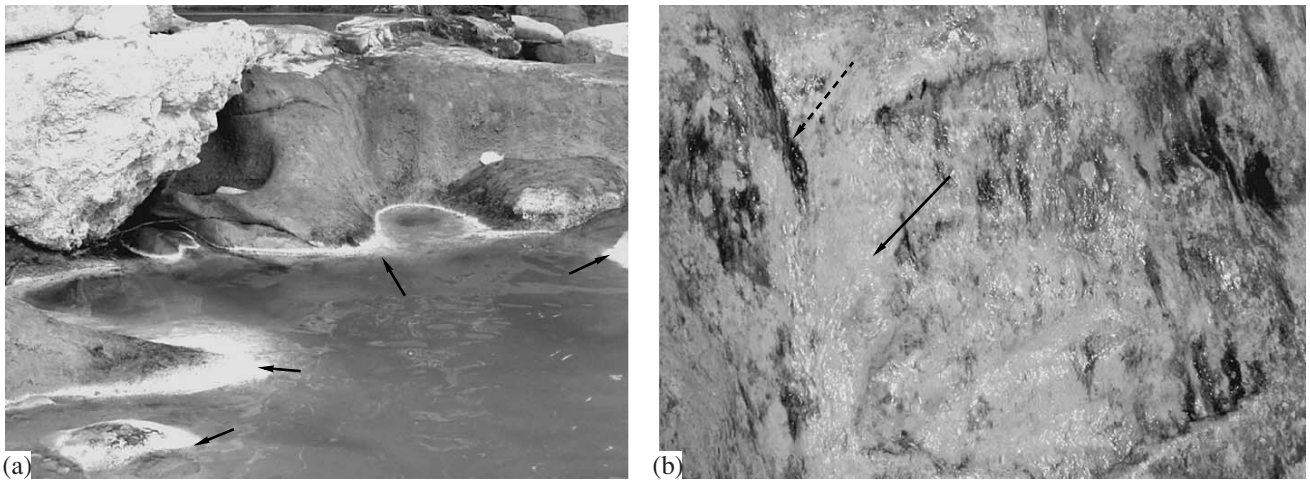

Fig. 1. Photographs of the sulfur mat in North Caucasian sulfide springs: Petushok spring, Goryachii Klyuch, Krasnodar Region (a); Besstyzhie Vanny spring, Pyatigorsk, Stavropol' Region (b).

of cyanobacterial communities occurred along with colorless sulfur bacteria (Fig. 1b).

Cultural and morphological characteristics. Fig. 2 demonstrates the temperature and pH growth ranges for *S. natans* isolates. All isolates grew within the range from 15 to 45°C, with an optimum at 32–37°C. The pH range for their development was from 6.3 to 8.5 with an optimum at pH 7.5 (Fig. 2). These growth parameters are close to the physicochemical conditions in sulfide springs and differ from those for the known *S. natans* strains isolated from other ecosystems. Those strains for which such parameters have been determined had optimal growth temperatures from 20 to 30°C and a pH growth range from 5.4 to 9.0 with an optimum at 6.5–7.0 [2, 3].

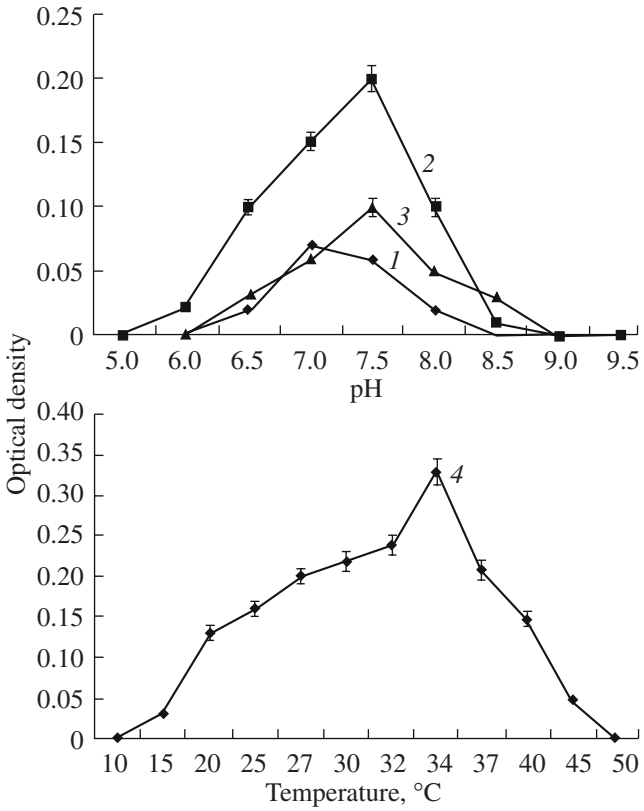

Fig. 2. Ranges of temperature and pH for *S. natans* strains: DSM 6575 (*1*), D-507 (*2*), D-504 (*3*), and D-501 (*4*).

On lactate-containing medium, all the strains were morphologically similar to *Sphaerotilus* members. During the early exponential phase, bacteria grew as isolated motile swarmer cells; later, filaments developed as cell chains covered with a common sheath. When cultured with shaking, the filaments often had adhesive disks at the distal end. Similar to the known *Sphaerotilus* strains, new isolates utilized a broad spectrum of organic mono- and polymeric compounds [4]. Transfer to the medium with sulfide resulted in an abundant intracellular accumulation of elemental sulfur (Fig. 3); these inclusions were also observed in the sulfur mat samples. The role of reduced sulfur compounds in the metabolism of new isolates was therefore the central issue in their investigation. Since oxygen content was low in the water of the springs under study, effect of this factor on growth and metabolism of aerobic *S. natans* strains was determined.

Dependence of thiosulfate oxidation on oxygen content. Under all the tested oxygen concentrations, from 1 to 20% in the gas phase, none of the strains oxidized thiosulfate in the absence of organic matter in the medium. Thiosulfate oxidation occurred only when lactate, acetate, or succinate (200 mg/l) was introduced. The rate of thiosulfate oxidation, the products of its decomposition, and effect of catalase on thiosulfate oxidation were determined for all organoheterotrophic strains (D-501, D-502, D-504, BV1, and BV2) and for

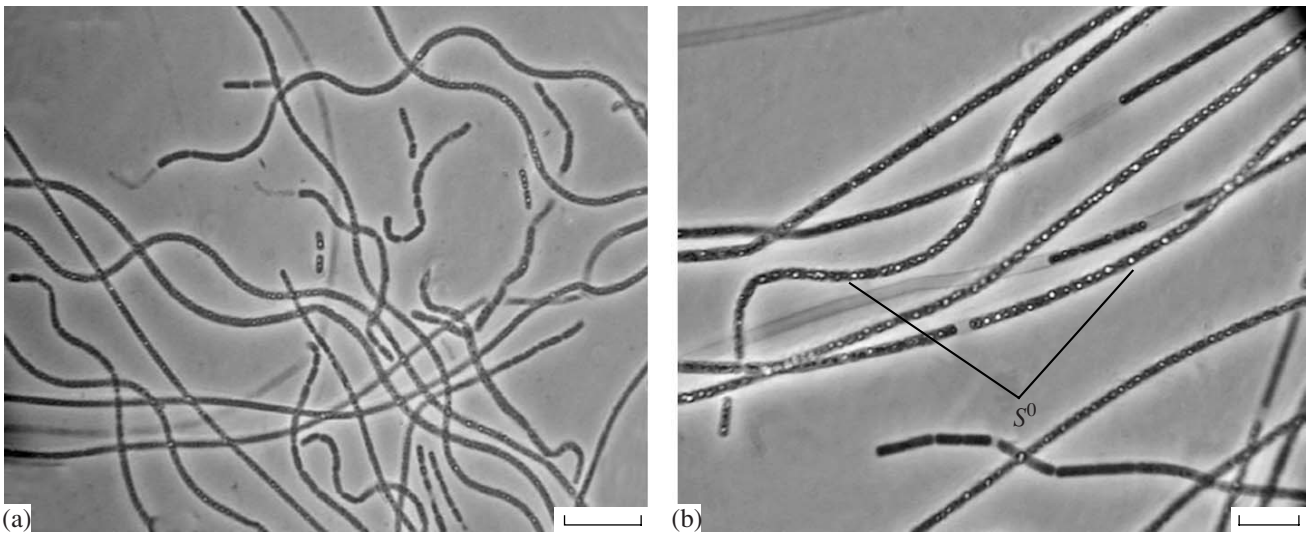

Fig. 3. Morphology of new *S. natans* isolates: a, under aerobic conditions on the medium with thiosulfate and lactate (0.5 g/l)l strain D-501, intracellular accumulation of polyhydroxybutyrate; b, cells with elemental sulfur inclusions on medium with sulfide (20 mg/l S/S^{2-}) and lactate (100 mg/l), strain D-507. Phase contrast microscopy. Scale bar, 5 μm. S^0, elemental sulfur.

the type strain *S. natans* DSM 6575. Since the results differed insignificantly, the data will be further presented only for strain D-501 (similar to strains D-502, D-504, and D-505) and for strain D-507; the latter was physiologically different from the others (see below). Comparative study of thiosulfate oxidation and the composition of end products revealed physiological heterogeneity of the strains. Only for one strain, D-507, the rate of thiosulfate oxidation did not depend on oxygen concentration in the medium. Under microaerobic growth conditions, thiosulfate oxidation was accompanied by accumulation of up to 90 mg/l sulfate sulfur as the only end product. Under aerobic conditions, apart from sulfate, a small amount of tetrathionate was formed. In other strains, thiosulfate oxidation rate was 2.5–3 times lower under aerobic conditions; tetrathionate was then the only end product. However, under microaerobic growth conditions the thiosulfate oxidation rate increased and became similar to the values found in strain D-507; it also resulted in production of equimolar amounts of sulfate (Fig. 4).

Apart from thiosulfate, all strains grown with sulfide accumulated elemental sulfur inside the cells (Fig. 3b). In the suspension of cells grown on the medium with lactate and sulfide (0.52 mg protein/ml), the rate of sulfide oxidation was 9.6–10.0 μg S^{2-}/(min ml). Unlike the new strains, the type strain *S. natans* DSM 6575 carried out insignificant thiosulfate oxidation to tetrathionate (21–23 mg $S/S_2O_3^{2-}$ in 3 days) under all oxygen concentrations. The activity of the enzymes of dissimilatory sulfur metabolism was not revealed (Table 3). Growth of this strain with sulfide resulted in the formation of a small number of isolated sulfur globules.

Effect of thiosulfate and oxygen concentration on growth. Table 1 presents the results of experiments on the effect of thiosulfate on growth characteristics for different strains under aerobic growth conditions. For strain D-501, an approx. 20% increase in the growth rate and a decrease in the generation time were observed in the presence of thiosulfate. Effect of thiosulfate on strain D-507 was more pronounced; the growth rate increased by 30%, and the generation time decreased by 40%.

Experiments on the effect of different oxygen concentrations on growth of strain D-501 revealed dependence of the cell yield on the oxygen regime of cultivation. The highest yield was obtained under microaerobic conditions (5% oxygen in the gas phase); it was two times higher than the value obtained at free air access. This is in agreement with the data on the dependence of the growth parameters (specific growth rate and generation time) from the oxygen regime of cultivation (Table 2).

Activity of the enzymes of sulfur metabolism. The prevalence of sulfate among the end products of thiosulfate oxidation indicates an enzymatic character of

Table 1. Effect of thiosulfate on the growth parameters of *S. natans* strains

Strain	+ thiosulfate and lactate*		+ lactate**	
	μ_{max}, h^{-1}	t_d, h	μ_{max}, h^{-1}	t_d, h
D-501	0.132	5.23	0.114	6.07
D-507	0.163	4.25	0.125	5.55

Notes: μ_{max}, specific growth rate; t_d, generation time.
* 200 mg/l sodium lactate.
** μ_{max}, specific growth rate; t_d, generation time.

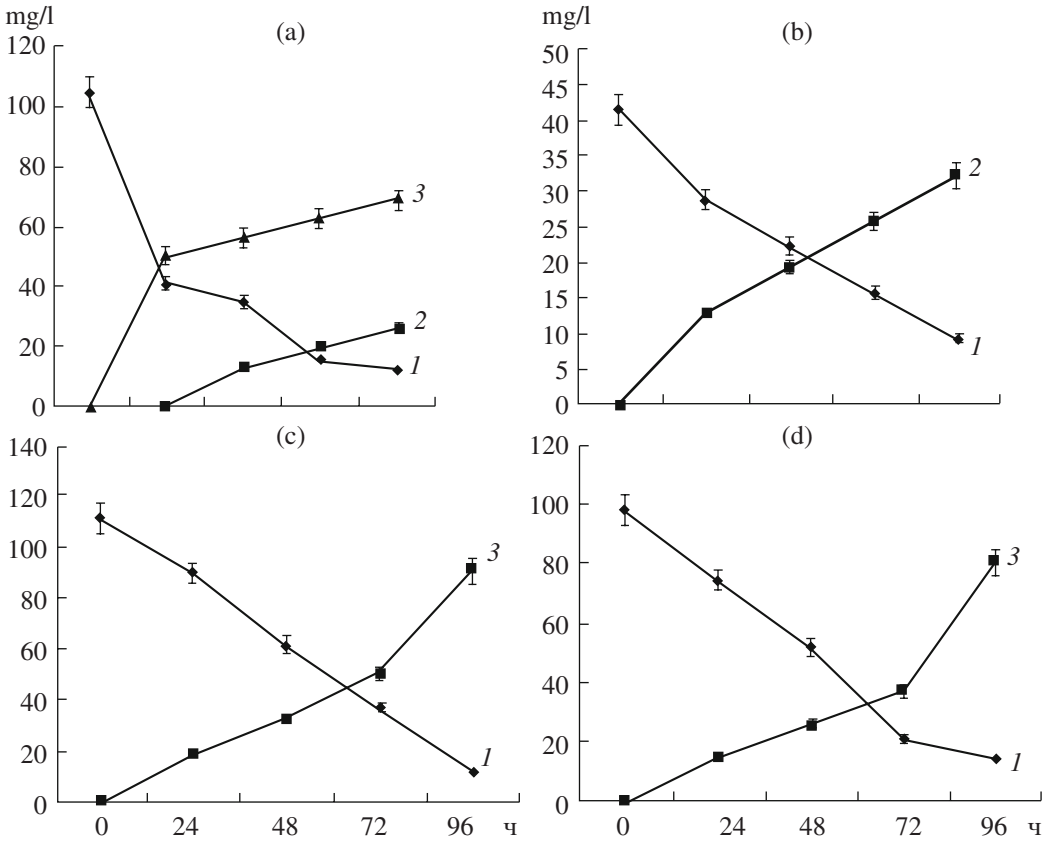

Fig. 4. Effect of oxygen regime on dynamics and products of thiosulfate oxidation by *S. natans* strains: a and b, at 20% O_2; c and d, at 5% O_2; a and c, strain D-507; b and d, strain D-501; S/S_2 O_3^{2-} (*1*); S/S_4 O_6^{2-} (*2*); S/S O_4^{2-} (*3*).

the oxidative reactions. For two groups of strains with different end products and rates of thiosulfate oxidation, activities of the key enzymes of oxidative sulfur metabolism were determined (Table 3). Strain D-507, which is capable of thiosulfate oxidation to sulfate independently of the oxygenation regime, high activity of two major enzymes of sulfite oxidation to sulfate was revealed, namely APS reductase and sulfite oxidoreductase; this activity was close to the level found in lithotrophic sulfur-oxidizing bacteria [13]. In another strain, D-501, no activity of these enzymes was found in aerobically grown cells, although it was revealed in the cells grown under microaerobic conditions. Activity of another enzyme, thiosulfate oxidoreductase, which participates in tetrathionate formation, was not revealed in any strain, independent of growth conditions. Rhodanese activity was detected in the cells of both strains; this enzyme participates only in preliminary sulfur metabolism (cleaving thiosulfate into sulfite anion and S^0) and is not related to the ETC functioning.

Table 2. Effect of oxygen regime on the growth parameters of strain D-50 grown on the medium with thiosulfate and lactate

Oxygen content in the gas phase, %	μ_{max}, h^{-1}	t_d, h
20	0.1	6.9
10	0.118	5.9
5	0.125	5.6

Note: μ_{max}, r^{-1} h^{-1} specific growth rate; t_d, r h generation time.

Relation between thiosulfate oxidation and ETC functioning. Suspensions of D-507 cells grown in the medium with thiosulfate and lactate were used in experiments on the relation between oxidation of sulfur compounds and the ETC; sulfite was used as an energy substrate. The respiration rate determined polarographically in the course of sulfite oxidation was high, 180 nmol/(min mg protein). Introduction of inhibitors in the reaction cell decreased the respiration rate by 23, 80, and 25–63% for myxothiazol, HQNO, and antimycin A, respectively. These results indicate electrons entering the ETC at the ubiquinone–cytochrome *b* level. Sulfite oxidation was sensitive to CCCP and C1CCCP, decouplers of respiration and oxidative phosphorylation. Introduction of these inhibitors resulted in a 2.5-fold decrease in the respiration rate. This is a direct indication for the coupling of sulfite oxidation and ETC functioning and therefore of sulfite acting as

Table 3. Activity of the enzymes of sulfur metabolism on oxygen content in the medium for *S. natans* strains [nmol/(min mg protein)]

Strain	Oxygen content in the gas phase, %				
	20			5	
	Rhodanese	Sulfite–ferricyanide oxidoreductase	APS reductase	Sulfite–ferricyanide oxidoreductase	APS reductase
507	75	929–1611	1150–2762	2554	2915
501	70–100	ND	ND	518	1104
505		ND	ND	1534	1223
DSM 6575		ND	ND	ND	ND

Note: ND stands for no activity detected.

Table 4. Effect of rotenone on the activity of the oxidases of sulfur compounds, cell yield, and thiosulfate oxidation by strain D-507 under mixotrophic and lithoheterotrophic conditions

Parameters	Growth conditions*	
	Rotenone, 50 μM	Without rotenone
Biomass increase, mg protein/l	33.6	22.7
Oxidized S/S_2O_3, mg/l	185.8	95.8
Specific enzymatic activity, μmol/(min mg protein)		
Thiosulfate–ferricyanide oxidoreductase	ND	ND
Thiosulfate–cytochrome *c* oxidoreductase	ND	ND
Sulfite–ferricyanide oxidoreductase	4.3	3.4
APS reductase	ND	1.9

Note: ND stands for activity not detected.

* In the medium with thiosulfate (1 g/l) and lactate (0.1 g/l), the cells from late exponential phase.

an energy substrate for respiration. Addition of thiosulfate to the cell suspension resulted in low respiration rates not exceeding 60 nm/(min mg protein); this correlates with the low activity of thiosulfate oxidoreductase.

Conditions for mixotrophic and lithoheterotrophic growth. In order to determine the type of lithotrophic metabolism involved in the oxidation of sulfur compounds, the effect of rotenone, an inhibitor of the NADH dehydrogenase complex of the ETC, on growth and activity of the enzymes of sulfur metabolism was studied in strain D-507. The results are presented in Table 4. Addition of rotenone (50 μM) into the growth medium with thiosulfate resulted in a 1.5-fold increased cell yield and a 30% increase in sulfite oxidoreductase activity. Activity of another enzyme, APS reductase, was not detected in the presence of rotenone, although it was high in the cells grown without rotenone (Table 4). These results demonstrate that when the supply of reducing equivalents to the ETC from the HADH dehydrogenase complex is blocked, ATP accumulation occurs only via oxidative phosphorylation, but not via substrate-level phosphorylation, unlike mixotrophic growth without the inhibitor, when both phosphorylation pathways are functioning. Thus, inhibition of the initial stage of the ETC by rotenone results in strictly lithoheterotrophic bacterial metabolism; only sulfur compounds are used as electron donors, while organic compounds are used only for biosynthetic purposes.

Functional role and mechanisms of thiosulfate oxidation under aerobic growth. Although the cells of most strains exhibited no activity of the enzymes of oxidative sulfur metabolism when grown aerobically, oxidation of thiosulfate to tetrathionate resulted in an increased cell yield for all strains (Table 1, Fig. 4). Thiosulfate oxidation resulted from its interaction with H_2O_2, which is formed in the course of oxidation of organic compounds. Fig. 5 demonstrates the results of an experiment with the cell suspension of strain D-501; these results indicate the peroxide mechanism of thiosulfate oxidation. Addition of catalase or pyruvate

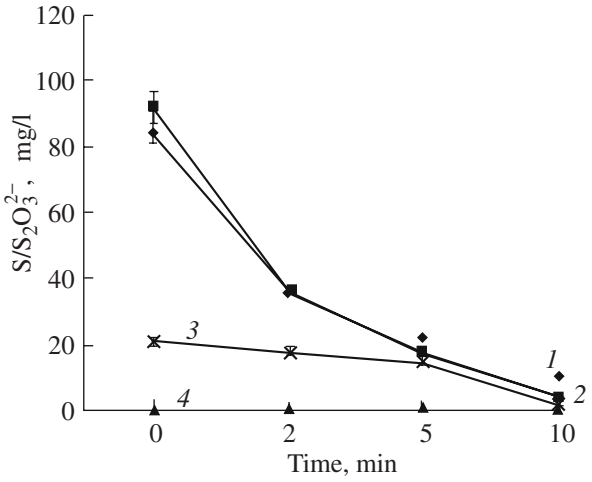

Fig. 5. Thiosulfate oxidation in the cell suspension of strain D-501 grown under organotrophic conditions. The suspension supplemented with: *1*, no supplements; *2*, glucose (0.5 mg/ml); *3*, catalase (10 μg/ml, activity 7000 U/mg, Serva); *4*, glucose (0.5 mg/ml) + sodium pyruvate (0.2 mg/ml).

(compounds decomposing H_2O_2) to the cell suspension inhibited the oxidation by 80–100%; addition of glucose enhanced thiosulfate oxidation. However, no thiosulfate oxidation occurred when glucose and catalase were added simultaneously, since enzymatic H_2O_2 decomposition took place. In aerobic cultures of strain D-507 with thiosulfate (this strain exhibited activity of the enzymes of sulfur metabolism in aerobic cultures), insignificant accumulation of tetrathionate (less than 30% of the oxidized thiosulfate) was prevented by an addition of catalase. In this case tetrathionate was not accumulated; sulfate, the product of enzymatic thiosulfate oxidation, was the only end product (Table 5).

Table 5. Effect of catalase on the rate of thiosulfate oxidation and end product composition in the culture of strain D-507

Mg/l	Growth conditions	
	+ catalase	without catalase
Accumulated $S/S_2O_3^{2-}$	70.5	70.5
Accumulated $S/S_4O_6^{2-}$	0	12.8–25.6*
Accumulated S/SO_4^{2-}	70.0	44–57*

Note: Experiment duration was 72 h; catalase content in the medium was 2 mg/l, activity 7000 U/mg (Sigma).

* In different experiments.

Organoheterotrophic growth of *S. natans* strains under aerobic conditions without thiosulfate was accompanied by cell lysis beginning from the early exponential phase. The protein of lysed cells constituted over 60% of the cell yield. Addition of thiosulfate to the medium resulted in less pronounced cell lysis; in different experiments, the protein of lysed cells did not exceed 25–30%. In the latter case it was possibly caused by accumulation of toxic H_2O_2 concentrations in the periplasmic space due to low activity of the enzymatic systems of antioxidant protection and to the slow influx of the detoxifier, thiosulfate, through the barrier of a dense mucopolysaccharide sheath. *S. natans* was previously shown to possess low catalase activity [4]. Thus, the stimulatory effect of thiosulfate on the growth processes of most strains, except for strain D-507, under aerobic conditions results from alleviation of the toxic effect of H_2O_2.

Our results indicate that sulfur compounds play a double role in *S. natans* inhabiting the springs. They may be used as electron donors for energy metabolism in the case of lithoheterotrophic or mixotrophic growth under microaerobic (or aerobic for some strains) conditions. They may also act as detoxifiers removing reactive oxygen species which are formed in the electron transport chain in the course of respiration. In both cases bacterial yield is increased, independently of the mechanism of the oxidative reactions. Lithoheterotrophic metabolism may occur in *S. natans* when growth processes are limited by low concentrations of available organic substrates; it is then used only for constructive metabolism as was recently demonstrated for *Leucothrix mucor*, one of the species of sulfur bacteria [14].

Thus, the capacity of *Sphaerotilus* for the oxidation of reduced inorganic sulfur compounds was demonstrated and the metabolic role of these compounds was determined. Utilization of reduced sulfur compounds in energy metabolism as electron donors and capacity for lithotrophic growth support introduction of the new *S. natans* ecotypes into the physiological group of lithotrophic colorless sulfur bacteria. The functioning of both mechanisms of oxidative processes, the enzymatic and the peroxide one, is determined by environmental conditions, primarily by the oxygen regime and probably by the availability of organic growth substrates. Variable metabolism and the mechanisms of adaptation to unstable conditions facilitates extension of the *Sphaerotilus* geographic range in new natural habitats and is crucial for their dominance in bacterial communities in the sulfur mats of sulfide springs.

It should be noted that the *S. natans* strains investigated in this work are closely related, as was revealed by analysis of the 16S rRNA gene sequences and by ERIC-PCR fingerprinting [5]. This is an indication of low population variability in *S. natans* strains in sulfide springs of the Northern Caucasus. Moreover, the present work demonstrated the existence of the meta-

bolic heterogeneity within this cluster. More detailed genetic, physiological, and biochemical study of the mechanisms for regulation of the enzymatic systems of oxidative sulfur metabolism in *Sphaerotilus* strains from various sources (and probably of other sulfur-oxidizing bacteria) is therefore required.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (projects nos. 07-04-00651, 05-04-48229, 06-04-63105k, and 06-04-49463) and the Molecular and Cell Biology program of the Presidium of the Russian Academy of Sciences.

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