ISSN 0026-2617, Microbiology, 2013, Vol. 82, No. 3, pp. 271–279. © Pleiades Publishing, Ltd., 2013.
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EXPERIMENTAL ARTICLES

Capacity of *Azospirillum thiophilum* for Lithotrophic Growth Coupled to Oxidation of Reduced Sulfur Compounds

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Received July 2, 2012

Abstract—Capacity for lithotrophic growth coupled to oxidation of reduced sulfur compounds was revealed in an *Azospirillum* strain, *A. thiophilum* BV-S^T. Oxygen concentration in the medium was the major factor determining the type of energy metabolism (organotrophic or lithotrophic) in the presence of thiosulfate. Under aerobic conditions, metabolism of *A. thiophilum* BV-S^T was organoheterotrophic, with thiosulfate oxidation to tetrathionate resulting from the interaction with reactive oxygen species, mostly H_2O_2, which was formed in the electron transport chain in the course of oxidation of organic electron donors. Under microaerobic conditions (2 mg/L O_2 in liquid medium), *A. thiophilum* BV-S^T carried out lithoheterotrophic (mixotrophic) metabolism; enzymes of the dissimilatory type of sulfur metabolism were responsible for thiosulfate oxidation to tetrathionate and sulfate. Two enzyme systems were found in the cells: thiosulfate dehydrogenase, which catalyzes incomplete oxidation of thiosulfate to tetrathionate and the thiosulfate-oxidizing Sox enzyme complex, which is involved in complete oxidation of thiosulfate to sulfate. The genetic determinant of a Sox complex component in *A. thiophilum* BV-S^T was revealed. The *soxB* gene was found, and its expression under microaerobic conditions was observed to increase 32-fold compared to aerobic cultivation.

Keywords: lithotrophy, thiosulfate oxidation, Sox complex, thiosulfate: ferricytochrome *c* oxidoreductase, gene expression, aerobic and microaerobic conditions

DOI: 10.1134/S0026261713030053

The genus *Azospirillum* comprises of associative nitrogen-fixers. They usually develop in the root system of herbs [1], although *A. largimobile* [2, 3] and *A. thiophilum* [4] inhabit aquatic ecosystems. All known azospirilla possess the aerobic type of metabolism. The optimal growth conditions develop, however, at low partial pressure of oxygen in the medium, ~0.2 kPa O_2 [5]. Still lower oxygen concentrations are required to activate nitrogen fixation and to enhance nitrogenase activity. Thus, nitrogen-fixing azospirilla may be considered microaerophiles. While azospirilla are usually organoheterotrophs, some *A. lipoferum* strains are known to be capable of hydrogen-dependent autotrophic growth. Capacity for lithotrophic growth in the presence of H_2, reduced sulfur compounds, etc. should not be ruled out for other species of this genus.

The strain BV-S^T isolated from a sulfide spring and described as the new species *A. thiophilum* accumulated intracellular globules of elementary sulfur in its natural habitat and in the cultures grown in the presence of sulfide. Cultivation of strain BV-S^T in the medium with thiosulfate and an organic substrate resulted in thiosulfate oxidation [4]. The mechanism of oxidation of sulfur compounds and the functional role of these processes in the metabolism have not been investigated previously.

The goal of the present work was to investigate the mechanism of oxidation of sulfur compounds and their role in the energy metabolism of *A. thiophilum*.

MATERIALS AND METHODS

Strains and cultivation techniques. The subject of investigation was strain BV-S^T of the new species *Azospirillum thiophilum*. The strain was isolated from the microbial community of a moderately thermophilic sulfide spring in the northern ridge of the Caucasus range (Pyatigorsk, Stavropol krai, Russia) [4]. Phylogenetically related *Azospirillum* strains *A. picis* DSM 19922^T, *A. lipoferum* ATCC 29707^T, and *A. doebereinerae* DSM 13131^T were used for comparison.

Bacteria were grown under aerobic or microaerobic conditions.

For cultivation of azospirilla, the modified semiliquid PSS medium was used containing the following (g/L distilled water): $(NH_4)_2SO_4$, 1.0; $CaCl_2 \cdot 2H_2O$, 0.03; $MgSO_4 \cdot 7H_2O$, 1.0; Na succinate, 1.0; peptone,

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2.0 [6]. Prior to inoculation, solutions of trace elements and vitamins [7] were added to the medium (1.0 mL/L), and pH was adjusted to 7.5–7.8. For investigation of sulfur metabolism, the medium was supplemented with 1 g/L sodium thiosulfate.

For microaerobic cultivation, bacteria were grown in screw-capped 0.5-L vials with 50 mL of the medium sealed with butyl rubber stoppers. To obtain the gas phase which contained 5% O_2, the vials were filled to capacity with freshly boiled medium, a specified volume was replaced with argon, and the required amount of air was injected. The air to argon ratio in the gas phase was 1 : 3. In all experiments the gas phase was filter sterilized (Millipore, 0.2 μm). Inoculum volume was 1 mL per 100 mL of the medium. To prevent significant O_2 decrease in the gas phase resulting from consumption by bacteria, the ratio of the liquid and gas phase was 1 : 10.

Analytical techniques. When present together in the medium, $S_2O_3^{2-}$, $S_4O_6^{2-}$ were determined by separate iodometric titration [8].

Intracellular elementary sulfur was identified using its characteristic birefringence in polarized light under a Nu-2 phase contrast microscope (Zeiss, GDR).

Cell extracts (homogenates) were obtained by sonication of bacterial cells in an UZDN-2T disintegrator at 500 W and 22 kHz (5 times for 30 s on an ice bath). The supernatant was obtained by centrifugation of the homogenate for 20 min at 8000 rpm. Protein in the cell extracts was determined according to Lowry [9] or Bradford [10]. Prior to analysis, alkaline hydrolysis of whole cells in 1 N NaOH was carried out for 10 min at 90°C.

Determination of activities of the enzymes of the dissimilatory sulfur cycle. The enzymatic activities were determined in the supernatant of the cell extract from a two-day (mid-exponential phase) culture. Activity of thiosulfate: ferricytochrome **c** oxidoreductase (Thiosulfate dehydrogenase = TDH) (EC 1.8.2.2) [11] and sulfite: ferricytochrome *c* oxidoreductase (EC 1.8.3.1) [12] were determined spectrophotometrically from the rates of reduction of $K_3[Fe(CN)_6]$ and cytochrome *c* in the presence of the oxidized substrate. Activity of the Sox complex was measured similarly to thiosulfate dehydrogenase activity, but at pH 6.5 [11]. The peaks of activity for thiosulfate: ferricytochrome *c* oxidoreductase, sulfite: ferricytochrome *c* oxidoreductase, and the Sox complex were observed at pH 4.5, 7.5, and 6.5, respectively. Spectrophotometric measurements with $K_3[Fe(CN)_6]$ and cytochrome *c* were carried out at 420 and 550 nm, respectively.

APS reductase (EC 1.8.99.2) was determined in the reaction mixture used for sulfite: ferricytochrome *c* oxidoreductase, supplemented with 1 μM adenosine monophosphate (AMP) [13].

Polarographic determination of O_2. The rate of oxygen consumption by a suspension of respiring cells was measured using a Clark electrode and registered with a Rekord-4 polarograph (Russia). The polarograms were recorded according to the standard procedure. The results were treated using the Rekord-4 bundled software [14]. The measurements were carried out with the exponential-phase cells in the medium containing (g/L): lactate, 0.2; peptone, 0.2; and thiosulfate, 1.0; thiosulfate concentration in the polarographic cell was 12 mM. The respiration rate with thiosulfate was calculated by subtracting the values for endogenous respiration and chemical thiosulfate oxidation.

Inhibitor analysis. The following inhibitors were used for investigation of the activity of the electron transport chain (ETC) in bacteria (μM): HQNO (inhibitor of the flavin–quinone–cytochrome *c* site), 0.1; antimycin A (inhibitor of the cytochrome *b* site), 0.5; ClCCP (uncoupler of oxidative phosphorylation), 0.06; rotenone (NADH inhibitor), 50.0; and KCN, 10.0.

Isolation of chromosomal DNA. Genomic DNA was extracted from the cells of *A. thiophilum* BV-S^T using the Genomic DNA Purification Kit (Fermentas, Lithuania) according to the manufacturer's recommendations.

Isolation of the total cell RNA was carried out as follows. The cells were homogenized by grinding with liquid nitrogen in a porcelain mortar; the resultant mass was transferred into TRIzol preheated to 34–42°C, mixed for 5 min, supplemented with chloroform, mixed for 5 min, and centrifuged for 10 min at 15000 *g*. The subsequent isolation was carried out using the SV Total RNA Isolation System (Promega) according to the manufacturer's recommendations.

Qualitative analysis of the DNA and RNA preparations was carried out by non-denaturing electrophoresis in 1% (wt/vol) agarose gel at 7 V/cm. The gel was stained with 0.1% aqueous ethidium bromide solution.

Obtaining cDNA copies (reverse transcription) was carried out using the M-MuLV reverse transcriptase (Fermentas) according to the manufacturer's protocol. After melting for 10 min at 68°C, the RNA mixture (2 μg) with the relevant primer (4 pmol) was supplemented with the reaction mixture containing the reverse transcriptase buffer (250 mM Tris–HCl, pH 8.3; 250 mM KCl, 20 mM $MgCl_2$, and 50 mM DTT), dNTP (to the final concentration in the sample 0.2 mM), and ribonuclease inhibitor. The test tubes were rapidly cooled on ice, and 40 U of the M-MulV reverse transcriptase (Revert Aid™) was added. The reaction was carried out for 40 min at 42°C and terminated by heating for 5 min at 85°C.

Polymerase chain reaction. The degenerate primers used for detection of the genes *rDsr, sqr, aprAB*, and *soxB* encoding reverse dissimilatory sulfite reductase, dissimilatory adenosine-5'-phosphosulfate reductase, sulfide:quinone oxidoreductase, and the SoxB component of the thiosulfate-oxidizing enzyme complex, respectively [15–18] are listed in Table 1. The reaction

Table 1. Primers used in the work

Gene	Primers	Annealing temperature, °C	Reference
*apr*A and *apr*B	AprB-1-FW TGC GTG TAY ATH TGY CC AprA-5-RV GCG CCA ACY GGR CCR TA	Touch-down 60–50	[15]
	AprA-1-FW TGG CAG ATC ATG ATY MAY GG AprA-5-RV GCG CCA ACY GGR CCR TA	Touch-down 60–50	
*sox*B	soxB432F GAY GGN GGN GAY CAN TGG soxB693F ATC GGN CAR GCN TTY CCN TA soxB1164B AAR TTN CCN CGN CGR TA soxB1446B CAT GTC NCC NCC RTG YTG	55	[16]
sqr	Sqr-G3-199F TBT AYS AGC CGG GWC TKC TBT Sqr-G3-566R GGY GCM ACS GGG CAT TTG	55	[17]
	Sqr-G2-145F TGG ACC CTG GTG GGC GSS GG Sqr-G2-490R TTC WKC GGC GCG CCS SCG CA	60	
rDsr	rDSR1Fa AARGGNTAYTGGAARG rDSR1Fb TTYGGNTAYTGGAARG rDSR1Fc ATGGGNTAYTGGAARG rDSR4Ra CCRAARCAIGCNCCRCA rDSR4Rb GGRWARCAIGCNCCRCA	48	[18]

was carried out as described in [12–15] on an MJ Mini™ amplifier (Bio-Rad Laboratories, United States).

Quantitative PCR. The following highly specific primers were developed to assess the level of the *soxB* gene expression based on the nucleotide sequence of a fragment of this gene from *A. thiophilum* BV-S^T:

(1) direct RT-F (AACTTCAACGGCAGCTT);

(2) reverse RT-R (GTGTTGGTAATAGGGAT).

OT-PCR was carried out on a DT-322 device (DNA-Technology, Russia) using the SYBR Green I stain (Invitrogen, United States), which intercalates into double-strand DNA. The amplification program was as follows: 1 cycle of preliminary melting of cDNA and the primers, 1 min at 94°C, and 30–40 cycles of melting, 20 s at 94°C; annealing, 23 s at 48–60°C; and 50–60 s at 72°C (elongation).

The fluorescence signal was measured for 15 s at the end of each cycle. The experiments were carried out using two independent DNA preparations in three repeats. In all cases, the quality of PCR products was accessed by electrophoresis in 5% PAG (210 V, 100 mA). The standard set of DNA fragments (QuickLoadTM 100 bp DNA Ladder, New England Biolabs, United States) was used as molecular mass markers. Quantitative analysis of gene expression was carried out using the qPCR software package (DNA-Technology). The relative amount of synthesized amplicons was calculated from the formula $2^{\Delta Ct}$.

Nucleotide sequences of the genes were determined in the All-Russian Complex for Gene Activity, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences using a CEQ2000XL automatic sequencer (Beckman Coulter, United States) according to the manufacturer's recommendations. The primary analysis of the similarity of the gene sequences obtained was carried out using the BLAST, ClustalW2, and TCoffee software packages (http://blast.ncbi.nlm.nih.gov/Blast.cgi, http://www.ebi.ac.uk/Tools/msa/clustalw2/, http://www.ebi.ac.uk/Tools/msa/tcoffee/).

Construction of the SoxB hydrolase phylogenetic tree. The phylogenetic tree was constructed using amino acid sequences of the SoxB hydrolase fragments. The tree was constructed with the MEGA 5.10 software package using the maximum likelihood algorithm and bootstrap analysis of 500 alternative trees.

All experiments were carried out in at least 3–5 repeats. The figures illustrate the results of individual experiments. The tables present average values of the experimental series. The data spread did not exceed 10%.

RESULTS AND DISCUSSION

Effect of oxygen regime on *A. thiophilum* BV-S^T growth and thiosulfate oxidation. Since *A. thiophilum* was isolated from a spring with low oxygen content in the water (S/S^{2-} 1.5–2 mg/L, O_2 2–2.5 mg/L), the effect of oxygen regime on thiosulfate oxidation was investigated. The organism was grown under aerobic conditions and microaerobically at oxygen concentration in the liquid phase ~2 mg/L.

Microaerobic cultivation in the medium with lactate and thiosulfate resulted in doubling the cell yield and the rate of thiosulfate oxidation compared to aerobic growth with free oxygen access into the medium. The amount of thiosulfate oxidized in 72 h under aerobic and microaerobic conditions was 70 and 150–250 mg/L $S/S_2O_3^{2-}$ (Fig. 1).

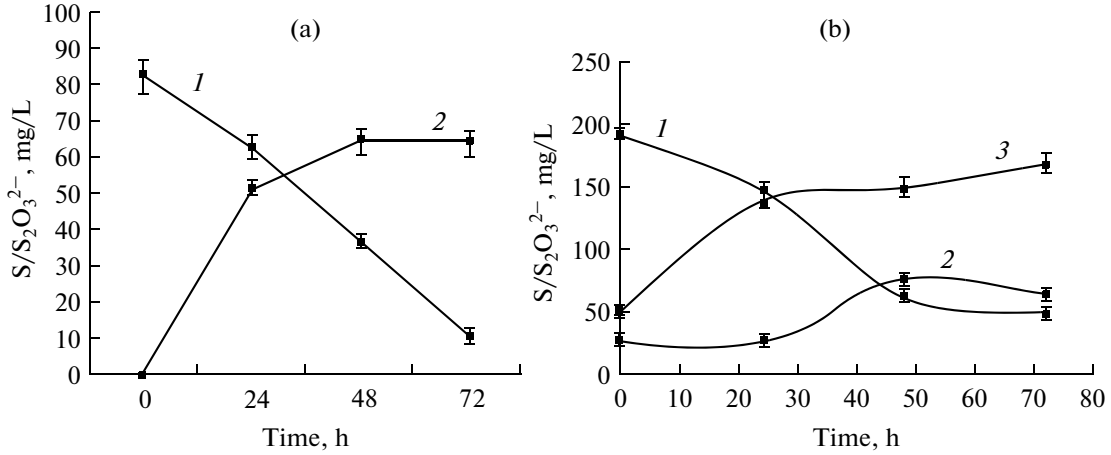

Fig. 1. Thiosulfate oxidation and accumulation of the end products in the culture of *A. thiophilum* BV-S^T at 20 (a) and 5% O_2 in the gas phase (b): $S/S_2O_3^{2-}$ (*1*), $S/S_4O_6^{2-}$ (*2*), and S/SO_4^{2-} (*3*). The medium contained 1 g/L NaS_2O_3 and 250 mg/L organic substrates (sodium lactate or succinate).

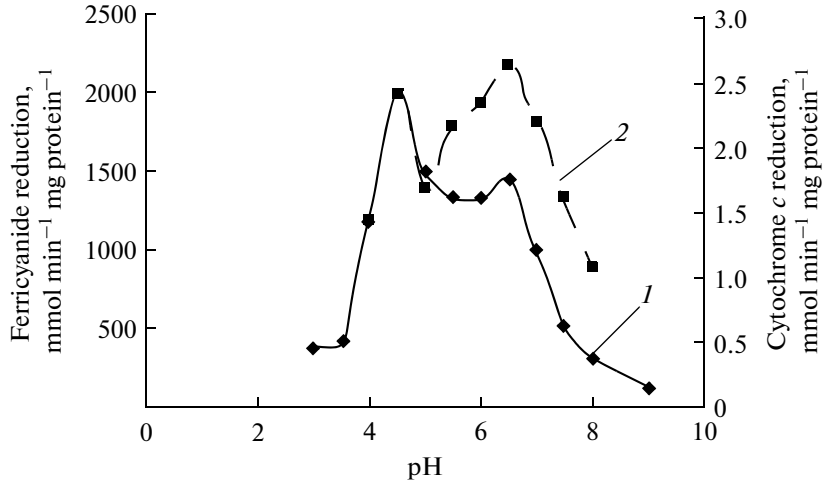

Fig. 2. Reduction of ferricyanide (*1*) and cytochrome *c* (*2*) during enzymatic thiosulfate oxidation at different pH values.

Analysis of the products of thiosulfate oxidation revealed differences in their composition. Aerobically grown bacteria formed only tetrathionate as the terminal product of thiosulfate oxidation, while under microaerobic conditions, apart from tetrathionate, sulfate was produced.

Activity of the enzymes of sulfur metabolism. Activity of thiosulfate dehydrogenase and the Sox complex was measured in the extract from microaerobically grown cells using ferricyanide and cytochrome *c* as artificial electron acceptors. Two peaks were revealed (Fig. 2).

The first peak observed at pH 4.5 correlated with the activity of thiosulfate: ferricytochrome *c* oxidoreductase [11]. The second peak, at pH 6.5, was associated with the activity of the Sox complex [11]. At pH 4.5 activity of thiosulfate: ferricytochrome *c* oxidoreductase was 2.0 μmol min^{-1} mg $protein^{-1}$ when measured with ferricyanide and 9.7 nmol min^{-1} mg $protein^{-1}$ when measured with cytochrome *c*. The lower activity observed with cytochrome *c* as an electron acceptor resulted from the fact that affinity to cytochrome *c* isolated from horse heart was lower than to the endogenous cytochrome *c*.

Table 2. Effect of the respiratory chain inhibitors on thiosulfate-dependent oxygen consumption in *A. thiophilum* BV-S^T cell suspensions under microaerobic growth conditions

Respiration with thiosulfate (nmol O_2 min^{-1} mg $protein^{-1}$)—K_1, K_2	Inhibitor concentration, μM	Change in the respiration rate after inhibitor addition, nmol O_2 min^{-1} mg $protein^{-1}$	Inhibition, %
67.40	HQNO (10^{-2}–10^{-5})	32.22	52.2
58.03	HQNO (10^{-2}–10^{-5})	27.03	53.4
300.40	Antimycin A (0.5)	189.25	37
161.00	KCN (10)	0	100
108.58	KCN (10)	2.17	98

Note: K_1 and K_2 stand for chemical oxidation and endogenous respiration, respectively.

Activity of the Sox complex was 124.4 nmol min^{-1} mg $protein^{-1}$.

No activity of sulfite: ferricytochrome *c* oxidoreductase and APS reductase was found in the cells of *A. thiophilum* BV-S^T.

Relation between oxidation of sulfur compounds and activity of the electron transport chain. Effect of thiosulfate and the inhibitors of the respiratory chain on the respiration rates of the cells from aerobically and microaerobically grown cultures was investigated in order to determine the role of thiosulfate in energy metabolism of *A. thiophilum* BV-S^T and the level at which the electrons from reduced sulfur compounds enter the ETC.

The respiration rate of aerobically grown *A. thiophilum* BV-S^T cells in the presence of thiosulfate varied from 2.4 to 8 nmol min^{-1} mg $protein^{-1}$, while in the case of microaerobically grown cells it was 67 to 300 nmol min^{-1} mg $protein^{-1}$, i.e., 30–35 times higher. Endogenous respiration was sensitive to rotenone (50 μM/L) with 60 and 42.6% inhibition for aerobic and microaerobic growth conditions, respectively.

As will be shown below, low rates of oxygen consumption by the cells grown under aerobic conditions in the presence of thiosulfate result from chemical oxidation of thiosulfate by reactive oxygen species (ROS). All subsequent research on the effect of inhibitors on the respiration rate in the presence of thiosulfate was therefore carried out with suspensions of the cells grown under microaerobic conditions. The inhibitory effect of HQNO an antimycin A on the respiration rate was 53.4 and 37%, respectively (Table 2). Thiosulfate oxidation was sensitive to ClCCP, an uncoupler of respiration and oxidative phosphorylation. Addition of the uncoupler resulted in a 2.4-fold increase of the respiration rate under microaerobic conditions. This is a direct indication of association of thiosulfate oxidation with ETC functioning. In the presence of KCN (10 μM), the rate of oxygen consumption was inhibited by 85 and 98–100% under aerobic and microaerobic conditions, respectively, indicating the presence of terminal oxidases of the aa^3 type. Thus, inhibitor analysis revealed that thiosulfate oxidation was coupled to the functioning of the ETC and the electrons entered the ETC at the ubiquinone–cytochrome *b*–cytochrome *c* level (Fig. 3).

Conditions for mixotrophic and lithoheterotrophic growth. The effect of rotenone, an inhibitor of the NADH dehydrogenase complex, on *A. thiophilum* BV-S^T growth and activity of the enzymes of sulfur metabolism was investigated in order to determine the possible utilization of inorganic electron donors (thiosulfate) in energy metabolism in the presence of an organic substrate. The results are presented in Table 3. In the presence of rotenone (50 μM) in the medium with thiosulfate, the cell yield increased more than twofold, and activities of thiosulfate: ferricytochrome *c* oxidoreductase and Sox complex increased by 30 and ~40%, respectively. In the presence of rotenone the organic substrate was used mostly for constructive metabolism, while thiosulfate oxidation was responsible for dissimilatory processes, and thus both

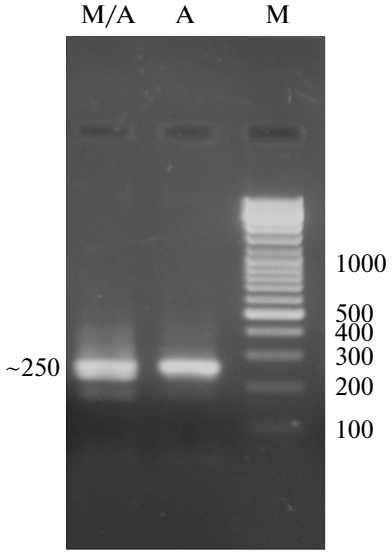

Fig. 3. OT-PCR on cDNA template with the RT-F and RT-R primers for the *soxB* gene.

Table 3. Effect of rotenone on activity of the enzymes of dissimilatory sulfur metabolism, cell yield, and thiosulfate oxidation by *A. thiophilum* BV-S^T under mixotrophic and lithoheterotrophic growth conditions

Parameters	Growth conditions	
	without rotenone	with 50 μM rotenone
Biomass increment, mg protein per 1 L	20.9	57.8
Thiosulfate oxidation, mg/L	163.4	333.2
Specific enzymatic activity, μmol min^{-1} mg protein^{-1}		
Thiosulfate: ferricytochrome *c* oxidoreductase	0.118	0.154
Sox complex	0.086	0.120

the cell yield and activities of the enzymes of sulfur metabolism increased. Stimulation by rotenone of specific activities of thiosulfate: ferricytochrome *c* oxidoreductase and the Sox complex was associated with suppression of the flavin components of NADH dehydrogenase resulting in uncoupling of the flow of reducing equivalents from the ETC donors to quinones. In this case, greater oxidation of the respiratory chain components facilitates electron flow from reduced sulfur compounds to the cytochrome site of the ETC. Thus, the bacterium grew as a lithoheterotroph and mixotroph in the presence and absence of rotenone, respectively. In the latter case, both organic and inorganic electron donors were used in energy metabolism.

Identification of the genes of dissimilatory sulfur metabolism in *A. thiophilum* BV-S^T. PCR analysis did not reveal the genes *rDsr, apr*AB, and *sqr*, which is in agreement with the absence of the relevant enzyme activities.

The combination of primers soxB693F–soxB1446B retrieved the product of expected length (~780 bp). Sequencing of a fragment of the *A. thiophilum* BV-S^T *soxB* gene (GenBank accession no. JN015012) was used to assess its homology to other bacterial species (66–78%).

OT-PCR with the primers specific for the *soxB* gene. As was shown above, oxygen concentration was the major factor determining the type of *A. thiophilum* BV-S^T metabolism in the presence of reduced sulfur compounds. Under microaerobic conditions, the rates of oxidation of reduced sulfur compounds and of respiration coupled to thiosulfate oxidation were significantly higher than under aerobic ones. For quantitative assessment of the *soxB* gene expression under microaerophilic and aerobic conditions, OT-PCR was carried out using the primers RT-F and RT-R. An OT-PCR electrophoregram is presented on Fig. 3. Expression of the *soxB* gene was shown to increase more than 32-fold under microaerobic conditions (Fig. 3).

Phylogenetic analysis of the amino acid sequence of the SoxB hydrolase. The phylogenetic tree constructed based on amino acid sequences of the SoxB hydrolase fragments is shown on Fig. 4. It can be seen that the SoxB hydrolase from *A. thiophilum* BV-S^T forms a cluster together with the enzymes of the *Betaproteobacteria*. This may probably be an indication of horizontal gene transfer from the *Betaproteobacteria* to *A. thiophilum* BV-S^T, which belongs to the class *Alphaproteobacteria* [4].

Mechanism of aerobic oxidation of reduced sulfur compounds in *A. thiophilum* and related *Azospirillum* species. *A. thiophilum* is phylogenetically closely related to other *Azospirillum* species: *A. picis, A. lipoferum*, and *A. doebereinerae* [4]. Sulfur metabolism within this group of closely related species was therefore investigated.

Strains *A. doebereinerae* DSM 13131^T, *A. picis* IMMB TAR-3^T, and *A. lipoferum* ATCC 29707^T were capable of thiosulfate oxidation accompanied by accumulation of equimolar amounts of tetrathionate. The rates of thiosulfate oxidation by these strains were low (70–80 mg/L $S/S_2O_3^{2-}$). However, no activity of the enzymes of dissimilatory sulfur metabolism was detected under aerobic or microaerobic conditions. Moreover, unlike *A. thiophilum*, phylogenetically related species were not found to produce sulfate from thiosulfate under microaerobic growth conditions.

The rate of oxygen consumption in the presence of thiosulfate by *A. picis, A. lipoferum*, and *A. doebereinerae* cell suspensions was 0.5–2.0 nmol min^{-1} mg protein^{-1}, which was similar to the values for aerobically grown *A. thiophilum* (Table 4).

The activity of specific oxidases of sulfur compounds did not increase in *A. thiophilum* (under aerobic conditions) and *A. picis, A. lipoferum*, and *A. doebereinerae* (under both aerobic and microaerobic conditions), together with capacity for thiosulfate oxidation only to tetrathionate and low rates of oxygen consumption in the presence of thiosulfate, indicating that reduced sulfur compounds can not act as energy substrates for the studied strains under experimental conditions. Reduced sulfur compounds are oxidized via chemical interaction with ROS.

This conclusion was confirmed by experiments of the effect of catalase on aerobic respiration in the presence of an organic substrate (Table 4).

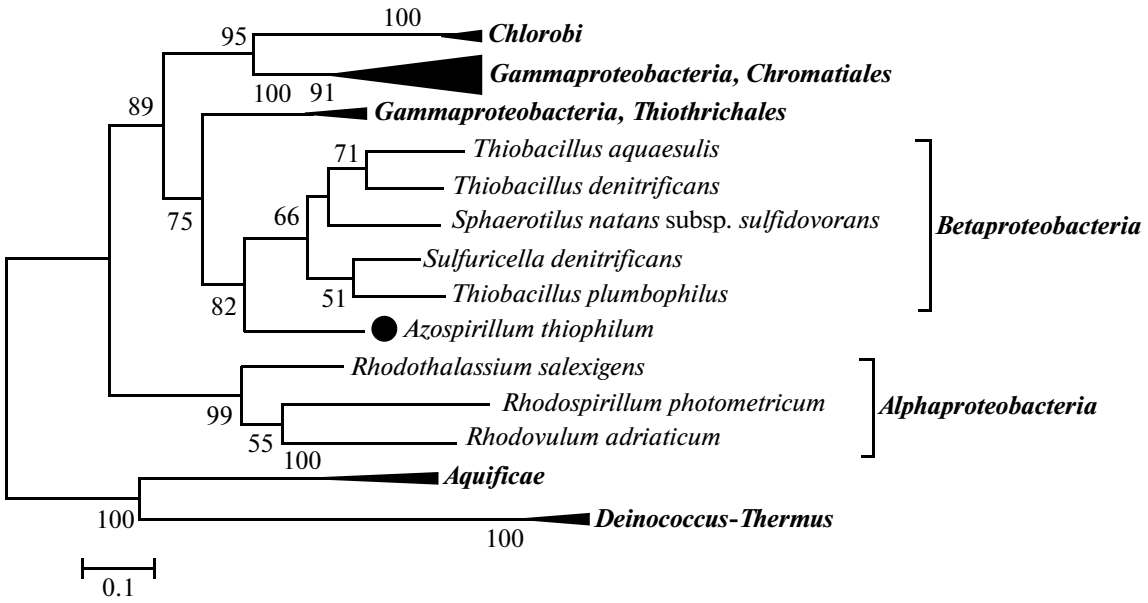

Fig. 4. Phylogenetic tree of SoxB hydrolases. The tree was constructed based on the fragments of amino acid sequences of SoxB hydrolases using the MEGA 5.10 software package. The maximum likelihood algorithm and bootstrap analysis of 500 alternative trees were applied.

The rate of H_2O_2 formation was determined polarographically from the rate of oxygen consumption after introduction of catalase into the cell suspension. It was shown that over 40% of oxygen consumed by respiring cell suspensions was reduced only to H_2O_2.

Thus, thiosulfate oxidation by *A. picis, A. lipoferum, A. doebereinerae*, and aerobically grown *A. thiophilum* is associated with the ETC activity only indirectly, due to the interaction with the products of incomplete oxygen reduction formed in the ETC. It is not coupled to energy metabolism.

To conclude, it should be noted that capacity for the oxidation of reduced sulfur compounds has not been previously reported for diazotrophic bacteria of the genus *Azospirillum*. The differences in the functional role of the oxidative reactions in metabolic processes were revealed. Microaerobically grown *A. thiophilum* was shown to use reduced sulfur compounds in energy metabolism as electron donors. This was confirmed by biochemical and molecular biological analyses. Activity of the enzyme systems of dissimilatory sulfur metabolism (thiosulfate: ferricytochrome *c* oxidoreductase and the Sox complex) was revealed, as well as the presence of the genes encoding these enzymatic systems. Both expression of these genes and the enzymatic activity were found to depend on dissolved oxygen concentration. Analysis of the products of thiosulfate oxidation and data on the activity of the enzymatic systems involved in thiosulfate oxidation indicate the following possible scheme of thiosulfate oxidation (Fig. 5). Phylogenetic analysis of the amino acid sequence of the SoxB hydrolase suggests the possibility of horizontal gene transfer of the relevant gene

Table 4. Rates of oxygen consumption and H_2O_2 formation during aerobic respiration of azospirilla cells

Bacterial species	Experiment no.	Rate of O_2 consumption, nmol min^{-1} mg $protein^{-1}$	Rate of O_2 consumption in the presence of catalase, nmol min^{-1} mg $protein^{-1}$	Rate of H_2O_2 formation, nmol min^{-1} mg $protein^{-1}$	O_2 reduction to H_2O_2, % of consumed oxygen
A. thiophilum	1	120.0	75.0	3.1	37.5
A. lipoferum	1	137.3	82.8	3.7	39.7
	2	116.1	65.1	3.5	43.9
A. doebereinerae	1	67.0	29.0	2.6	56.7
A. picis	1	58.9	31.8	1.8	46.0
	2	62.2	38.0	1.6	38.9

Note: 1 and 2 indicate 1 g/L of lactate and succinate, respectively. The culture age was 48 h (late exponential phase).

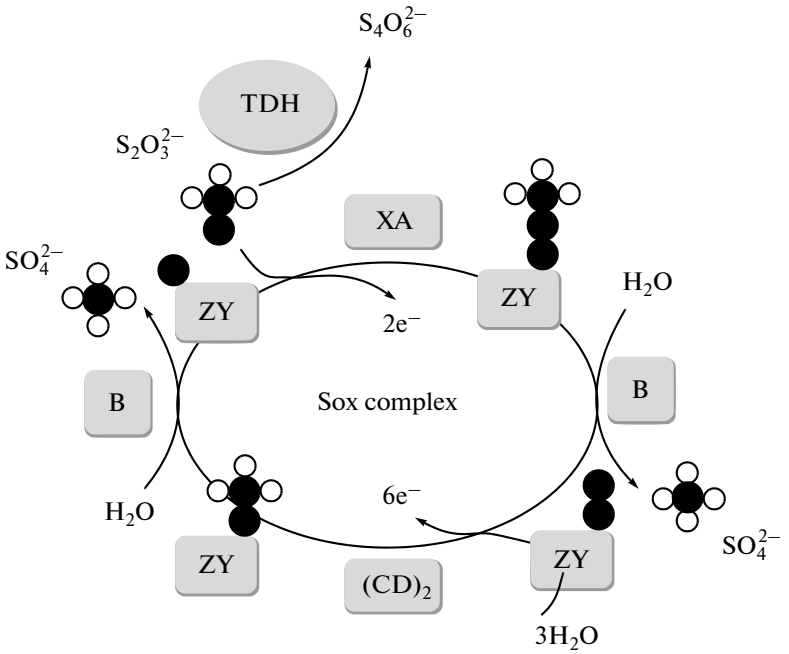

Fig. 5. Diagram of the enzymatic pathays of metabolism of reduced sulfur compounds in *A. thiophilum* BV-S^T: TDH, thiosulfate dehydrogenase (thiosulfate: ferricytochrome *c* oxidoreductase); SoxXA, SoxYZ, SoxB, and Sox$(CD)_2$, components of the Sox complex (functioning of the Sox complex presented according to [19]).

from *Betaproteobacteria* to *A. thiophilum* BV-S^T (*Alphaproteobacteria*).

In aerobically grown *A. thiophilum* and in other investigated azospirilla species, independent on the oxygen regime of cultivation (*A. picis, A. lipoferum*, and *A. doebereinerae*), sulfur compounds act as antioxidants involved in removal of the products of incomplete oxygen reduction. This phenomenon was previously reported for organoheterotrophic sulfur bacteria [20].

Thus, our results expand the physiological diversity of lithotrophic sulfur-oxidizing microorganisms.

ACKNOWLEDGMENTS

The authors are grateful to M.S. Tutukina (Institute of Cell Biophysics, Russian Academy of Sciences) and L.I. Yurevich (Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences) for their help in molecular biological analyses.

The work was supported by the Russian Foundation for Basic Research, project no. 10-04-01180a.

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Translated by P. Sigalevich