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EXPERIMENTAL ARTICLES

Capacity for Nitrate Respiration as a New Aspect of Metabolism of the Filamentous Sulfur Bacteria of the Genus *Thiothrix*

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Abstract—Capacity of *Thiothrix* species (*T. lacustris* strains AS and BL^T, *T. caldifontis* G1^T, *T. unzii* A1^T, and *T. eikelboomii* AR3^T) for anaerobic respiration in the presence of nitrate was discovered. The dynamics of nitrate reduction to nitrite was studied and the coupling of this process to thiosulfate oxidation was shown. The investigated *Thiothrix* representatives performed anaerobic thiosulfate-dependent reduction of nitrate only to nitrite. The presence of the *narG* gene, encoding the α-subunit of respiratory nitrate reductase NarGHI, was revealed in the cells. The induction of this gene expression was shown for the *T. lacustris* strain AS under anaerobic conditions of growth. The activity of several enzymes involved in the conversion of reduced sulfur compounds was determined.

Keywords: Thiothrix, anaerobic respiration, nitrate respiration, nitrate reductase, *narG* gene

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Colorless sulfur bacteria are a group of microorganisms widespread in freshwater and marine environments, in areas of oceanic hydrothermal vents, and in anthropogenic ecosystems. They occupy ecological niches where dynamic gradients of molecular oxygen are established or oxygen is absent. The vast majority of colorless sulfur bacteria are aerobes but, under microaerobic or anaerobic conditions, they experience stress which induces alternative respiration systems [1, 2].

Facultatively aerobic sulfur bacteria, such as *Beggiatoa, Thioploca*, and *Thiomargarita*, contain vacuoles where nitrates functioning as terminal electron acceptors accumulate at high concentrations; these bacteria were shown to contribute significantly to total marine nitrate reduction. Thus, bacteria of these genera turned out to be an important link between sulfur, nitrogen, and carbon cycles.

For sulfur bacteria of the genus *Thiothrix*, the capacity for nitrate respiration has not been shown previously. However, the possibility of this process may not be excluded, since the habitats of *Thiothrix* are characterized by regular daily rhythm of aerobic and anaerobic conditions in the tidal zones of sea littorals or in running waters with high sulfide content. In such habitats, the capacity to switch from aerobic to anaerobic respiration would be of profound ecological adaptational importance.

The aim of this work was to prove the possibility of anaerobic respiration in representatives of the genus *Thiothrix* and to study this process from the point of view of molecular biology and biochemistry of the reactions proceeding in bacterial cells.

MATERIALS AND METHODS

The subjects of study were colorless filamentous sulfur bacteria of the genus *Thiothrix* isolated from various sulfide-rich habitats: *T. lacustris* BL^T = DSM 21227^T (isolated from a low-temperature lake), *T. lacustris* AS (isolated from a microbial overgrowth in the contact zone between a freshwater brook and sea water of the White Sea littoral), *T. caldifontis* G1^T = DSM 21228^T (isolated form a moderately thermal sulfide spring), and two strains obtained from ATCC, *T. unzii* A1^T = ATCC 49747^T and *T. eikelboomii* AP3^T = ATCC 49788^T (isolated from sludge of domestic wastewater treatment plants).

Composition of culture media and conditions of cultivation were as follows. Armbruster medium [3] was

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supplemented before inoculation with solutions of sodium thiosulfate (to 1 g/L), sodium lactate, sodium acetate (both to 300 mg/L), vitamins, and microelements [4] (pH 7.5–7.8). For cultivation of *T. lacustris* AS, 10 g/L NaCl was introduced into the medium prior to inoculation.

For anaerobic cultivation, Hungate tubes and 0.5-L flasks with polybutyl rubber stoppers and metal screw caps were used. Flasks and tubes were filled to the top with freshly boiled sterile medium supplemented with 0.5 g/L $NaNO_3$. Cultivation was at 22–27°C.

Cell suspensions and enzyme preparations were obtained after centrifugation of bacterial cultures at 12 000 *g* and 4°C for 15 min. Cells were washed with 0.1 M Tris-HCl buffer (pH 7.5) and precipitated by centrifugation under the same conditions for 10 min.

Cell extracts were obtained by bacterial cell disruption in 0.1 M Tris-HCl buffer (pH 7.5) in a UZDN-2T ultrasonic disintegrator at 500 W and 22 kHz for 2 min on an ice bath, followed by removal of cell debris by centrifugation of the homogenate at 12000 *g* and 4°C for 10 min.

Protein determination was performed by the Lowry method on an SF-56 spectrophotometer (λ = 750 nm) [5] after alkaline hydrolysis of whole cells in 1 N NaOH for 10 min at 90°C.

Determination of the activity of sulfur catabolism enzymes was performed in extracts of cells from a two-day culture in the mid-exponential growth phase. Activities of thiosulfate:ferricytochrome *c* oxidoreductase (thiosulfate dehydrogenase, EC 1.8.2.2) [6] and sulfite:ferricytochrome *c* oxidoreductase (EC 1.8.3.1) [7] were determined spectrophotometrically in cell extracts by the rate of $K_3[Fe(CN)_6]$ and cytochrome *c* reduction in the presence of the oxidized substrate at pH 4.5 and 7.5, respectively. Spectrophotometry that used $K_3[Fe(CN)_6]$ and cytochrome *c* was performed at wavelengths of 420 and 550 nm, respectively.

Adenylyl phosphosulfate (APS) reductase (EC 1.8.99.2) was determined in the reaction mixture of the same composition as sulfite:ferricytochrome *c* oxidoreductase, but supplemented with adenosine monophosphate (AMP, 1 μM) [8].

Determination of nitrate reductase activity was performed in 0.2 M sodium phosphate buffer, pH 7.3. An aliquot of 5 μL of cell extract was added to 800 μL of buffer supplemented with 0.01 M $NaNO_3$. Methyl viologen (1 mM) was used as an electron donor. The reaction was initiated by introduction of 0.1 M sodium dithionite. The exposure lasted 10 min [9]. Enzyme activity was assessed from the concentration of the nitrite formed.

Chemical analyses. Concentrations of thiosulfate and tetrathionate were determined by separate iodometric titrations [10]. Sulfate concentration was determined by the chloranilate assay [11]. Elemental sulfur was determined by the Morris method [12]. Nitrite concentration was determined by a modified Griess–Ilosvay method [11]; nitrate concentration, by titration with chromotropic acid in the presence of H_2SO_4 [11].

To determine ammonium ions, 250 μL of the Nessler reagent was added to 2 mL of sample. The mixture was kept for 10 min at room temperature, and centrifuged at 12000 *g* for 4 min to precipitate insoluble admixtures. Concentration of ammonium ions was determined in the supernatant on an SF-53 spectrophotometer at λ = 400 nm [13].

Genomic DNA isolation was performed with a Genomic DNA Purification Kit (Fermentas, Lithuania) according to the manufacturer's protocol with modifications (samples were incubated with lysis buffer at 85°C and centrifugation duration and rates were increased to 4 min and 16000 *g*, respectively, at the stages of DNA precipitation and reprecipitation and protein removal). The quality of the isolated genomic DNA was evaluated by electrophoresis in 1% agarose gel with 1% ethidium bromide.

PCR amplification of genomic DNA was performed using a standard PCR mixture and the primer pairs narG 1960F (5'-TAY GTS GGC CAR GAR AA-3') and narG2659R (5'-TTY TCR TAC CAB GTB GC-3') and *nrfA*F1 (5'-GCN TGY TGG WSN TGY AA-3') and *nrfA*7R1 (5'-TWN GGC ATR TGR CAR TC-3') [14]. PCR amplification of *narG* gene fragments was performed in the following mode: primary denaturation for 5 min at 95°C; 35 cycles of denaturation for 30 s at 95°C, primer annealing for 30 s at 60°C, and extension for 30 s at 72°C; and final extension for 2 min at 72°C. For amplification of *nrfA* gene fragments, the protocol proposed by Smith et al. [14] was used.

Isolation of total cellular RNA was performed as described in [15]. The quality of the isolated RNA was controlled by denaturing electrophoresis in 4% polyacrylamide gel with 8 M urea. RNA concentration in the preparations was determined on an ND-1000 spectrophotometer.

Obtaining cDNA by reverse transcription and RT-PCR. Obtaining of cDNA was performed according to the manufacturer's protocol (Fermentas, Lithuania). Mixture of RNA (2 μg) with relevant specific primer (4 pmol) in milliQH2O was annealed for 10 min at 68°C, after which it was supplemented with the master mix, containing revertase buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, and 50 mM DTT), dNTPs (0.2 mM), and ribonuclease inhibitor. The tubes were cooled on ice, 40 units of MMulV (Revert Aid™) reverse transcriptase was added, and the reaction was allowed to proceed at 42°C for 40 min. To inactivate the enzyme, the samples were heated at 85°C for 5 min. Further PCR amplification of cDNA was performed on a DT-322 thermal cycler (DNK-Tekhnologiya, Russia) according to a program similar to the one used for genomic DNA amplifica-

Table 1. Ratio between decrease in thiosulfate content and increase in nitrite and protein contents upon aerobic and anaerobic growth of *Thiothrix* representatives

Bacteria	Maximum protein yield, mg/L		$S_2O_3^{2-}$, oxidized, mM		Increase in specific rate of $S_2O_3^{2-}$, oxidation (mM/mg protein), %	NO_2^-, formed, mM
	1	2	1	2		2
T. lacustris BL^T	29.9	15.0	2.02	2.41	235	0.31
T. lacustris AS	25.8	13.2	2.48	2.84	229	2.10
T. caldifontis $G1^T$	20.1	12.5	1.89	2.23	191	0.58
T. unzii $A1^T$	17.8	10.6	1.65	1.92	194	0.50
T. eikelboomii $AP3^T$	22.6	13.1	1.53	2.48	279	0.49

Note: Duration of the experiment was four days. 1—aerobic growth, 2—anaerobic growth.

tion, but with a 1 min shorter primary denaturation time. The experiments were performed with two independent RNA samples in triplicates.

Nucleotide sequence determination was performed by VNTK Gennaya aktivnost', Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, on a CEQ2000 XL (Beckman Coulter, United States) automated sequencer according to the manufacturer's protocol.

Preparative electrophoresis. PCR products were separated by horizontal electrophoresis in 1% low gelling temperature agarose in Tris–borate buffer (17.8 mM Tris-HCl, 17.8 mM boric acid, 0.4 mM EDTA, pH 8.0). HyperLadder IV (Bioline, Russia, 100–1000 bp) was used as a marker. Electrophoresis was performed at 140 V voltage and up to 110 mA current for 20–25 min.

DNA isolation from gel and purification were performed using a Wizard® SV Gel and PCR Clean-Up System (Promega, United States) kits. Concentration of DNA fragments was assessed by electrophoresis.

Phylogenetic tree construction used amino acid sequences aligned with the ClustalW [16] software. Complete and partial sequences of the *narG* gene of 150 bacterial and archaeal isolates retrieved from FGPR database (http://fungene.cme.msu.edu//index.spr) were used as reference ones. The tree was constructed using the MEGA5 software [17].

Primary analysis of the obtained nucleotide sequences was performed using the online resources BLAST (http://blast.ncbi.nlm.nih.gov/) and AliBee Multiple Alignment (http://www.genebee.msu.su/services/malign_reduced.html).

All experiments were performed in at least 3–5 replicates. The values in tables are mean values of a series of measurements. Data scatter did not exceed 10%.

RESULTS AND DISCUSSION

Dynamics of nitrate ion reduction in the process of anaerobic respiration. The capacity for anaerobic respiration was verified in representatives of two groups of *Thiothrix*: the "*T. nivea*" group (*T. lacustris* BL^T, *T. lacustris* AS, *T. caldifontis* $G1^T$, and *T. unzii* $A1^T$) and the "Eikelboom type 021N" group (*T. eikelboomii* $AP3^T$). All strains under study were capable of growth under anaerobic conditions with organic substrate (lactate + acetate) and thiosulfate in the presence of nitrate as terminal electron acceptor. Maximum protein yield was observed after 72 h and varied from 10.6 to 15.0 mg protein/L among the strains (Table 1, Fig. 1). No growth occurred under anaerobic conditions if organic substrates and thiosulfate were present but nitrate was not provided. Anaerobic growth was accompanied by a decrease in nitrate concentration in the culture medium and accumulation of nitrites in equimolar amounts (Table 1, Fig. 1). Upon accumulation of nitrites above 0.3–2.1 mM in the medium, they produced an inhibiting effect on growth, nitrate reduction, and thiosulfate oxidation.

No accumulation of ammonium ions in the culture medium was registered.

Molecular biological studies. To confirm at the molecular level the occurrence of nitrate respiration,

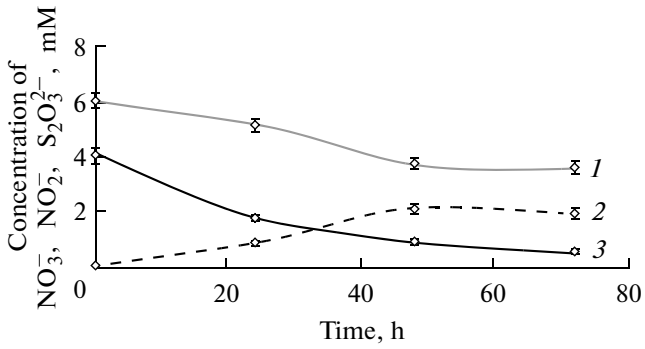

Fig. 1. Dynamics of nitrate reduction coupled to thiosulfate oxidation in *T. lacustris* AS. *1*—NO_3^-, *2*—NO_2^-, *3*—$S_2O_3^{2-}$. Similar patterns were observed for all *Thiothrix* strains under study.

Table 2. Nitrate reductase activity in *Thiothrix* representatives grown aerobically and anaerobically

Bacteria	Nitrate reductase activity, nmol min^{-1} mg $protein^{-1}$	
	aerobic growth	anaerobic growth
T. lacustris BL^T	9.94	34.47
T. lacustris AS	11.08	39.13
T. caldifontis $G1^T$	6.03	28.98
T. unzii $A1^T$	3.91	21.08
T. eikelboomii $AP3^T$	10.78	43.47

which involves nitrate reductase (NarGHI) [18], PCR with degenerate primers *narG* 1960 F/*narG* 2659 R was performed for the *Thiothrix* strains under study. For all strains, DNA fragments of expected length (650 bp) were revealed. Nucleotide sequences of the PCR products were determined and deposited in GenBank under accession numbers JX267821 (*T. caldifontis* $G1^T$), JX267822 (*T. eikelboomii* $AP3^T$), JX267823 (*T. unzii* $A1^T$), JX267824 (*T. lacustris* BL^T), and JX267825 (*T. lacustris* AS). Comparison of the obtained nucleotide sequences with those available in the NCBI database showed their high level of homology (75.5–76.3%) with a fragment of the *narG* gene coding for the α-subunit of the membrane-bound respiratory nitrate reductase of *Halomonas halodenitrificans* IFO 14912 (GenBank accession number AB076402.2).

In the phylogenetic tree (Fig. 2), the obtained sequences of the *narG* gene of *Thiothrix* strains formed a coherent group within the cluster comprising most *Gammaproteobactria* members represented in the tree. On the whole, our phylogenetic reconstruction, which employed 150 reference sequences, confirms an earlier conclusion of Gregory et al. [19] that phylogenetic trees constructed on the basis of the *narG* and 16S rRNA genes do not match. However, no significant deviations from the commonly acknowledged phylogeny were revealed for the *Thiothrix narG* genes, suggesting the absence of recent events of *narG* gene acquisition via horizontal transfer by the *Thiothrix* representatives under study [20].

Total RNA of one of the strains, *T. lacustris* AS, was purified, and cDNA was obtained by reverse transcription with a *narG* specific primer. In samples containing cDNA synthesized on the mRNA of an anaerobic culture, intense synthesis of amplicons was observed upon RT-PCR, while in samples corresponding to aerobic growth, no synthesis was observed (Fig. 3). Thus, it was demonstrated that under conditions of anaerobic growth, mRNA of the *narG* gene is present in the cells in large amounts while no *narG* expression occurs under aerobic growth.

The presence of *narG* gene in all strains under study and the rather high level of its expression in *T. lacustris* AS during anaerobic cultivation, as well as the capacity of this and other strains for anaerobic growth in the presence of nitrate and accumulation of nitrite in the culture media, allow the assumption to be made that the *narG* gene is expressed during anaerobic growth also in the other *Thiothrix* strains that we studied, leading to synthesis of the NarGHI respiratory nitrate reductase. These arguments support the hypothesis of nitrate respiration in the investigated *Thiothrix* representatives.

The reaction of nitrite reduction to ammonium is catalyzed by nitrite reductase NrfA. Amplification with primers *nrfA*F1 and *nrfA*7R1 did not yield the appropriate product of 520 bp in either of the strains under study, which is in accordance with the absence of ammonium ions in the culture media.

Activity of dissimilatory nitrate reductase. To verify the capacity of *Thiothrix* representatives for dissimilatory nitrate reduction and enzymatic generation of nitrites, total activity of nitrate reductases was determined in extracts of cells grown under aerobic and anaerobic conditions (Table 2).

Upon aerobic growth, the total specific activity of nitrate reductases in the strains studied did not exceed 3.91–11.08 nmol min^{-1} mg $protein^{-1}$, which is close to the known values of assimilatory nitrate reductase activity in many microorganisms. Upon anaerobic growth, the total specific activity of nitrate reductases increased fivefold, to values of 21.08–43.47 nmol min^{-1} mg $protein^{-1}$.

Two types of nitrate reductases are known to function in bacterial cells during aerobic growth. These are assimilatory cytoplasmic nitrate reductases and periplasmic nitrate reductases, e.g. NapAB involved in the maintenance of homeostasis upon cell transition from aerobic to anaerobic metabolism. It could be assumed that, in the strains under study, anaerobic conditions increase the activity of the same enzymes that function under aerobic conditions. However, our above-described molecular biological studies of *Thiothrix* representatives allow us to conclude that under anaerobic conditions the significant increase in the total activity of nitrate reductases occurs in *Thiothrix* cells due to synthesis of respiratory nitrate reductase. This conclusion is also supported by the decrease in the rate of anabolic processes and increase in the specific rate of thiosulfate oxidation under anaerobic conditions: the protein yield is almost two times lower under anaerobic conditions, and the thiosulfate oxidation rate per 1 mg protein is 2–2.7 times higher (Table 1).

Thus, our results provide evidence of the induction under anaerobic conditions of the nitrate reductase involved in respiration.

Thiosulfate oxidation and activity of enzymes involved in transformations of reduced sulfur compounds. Members of the genus *Thiothrix* are known to

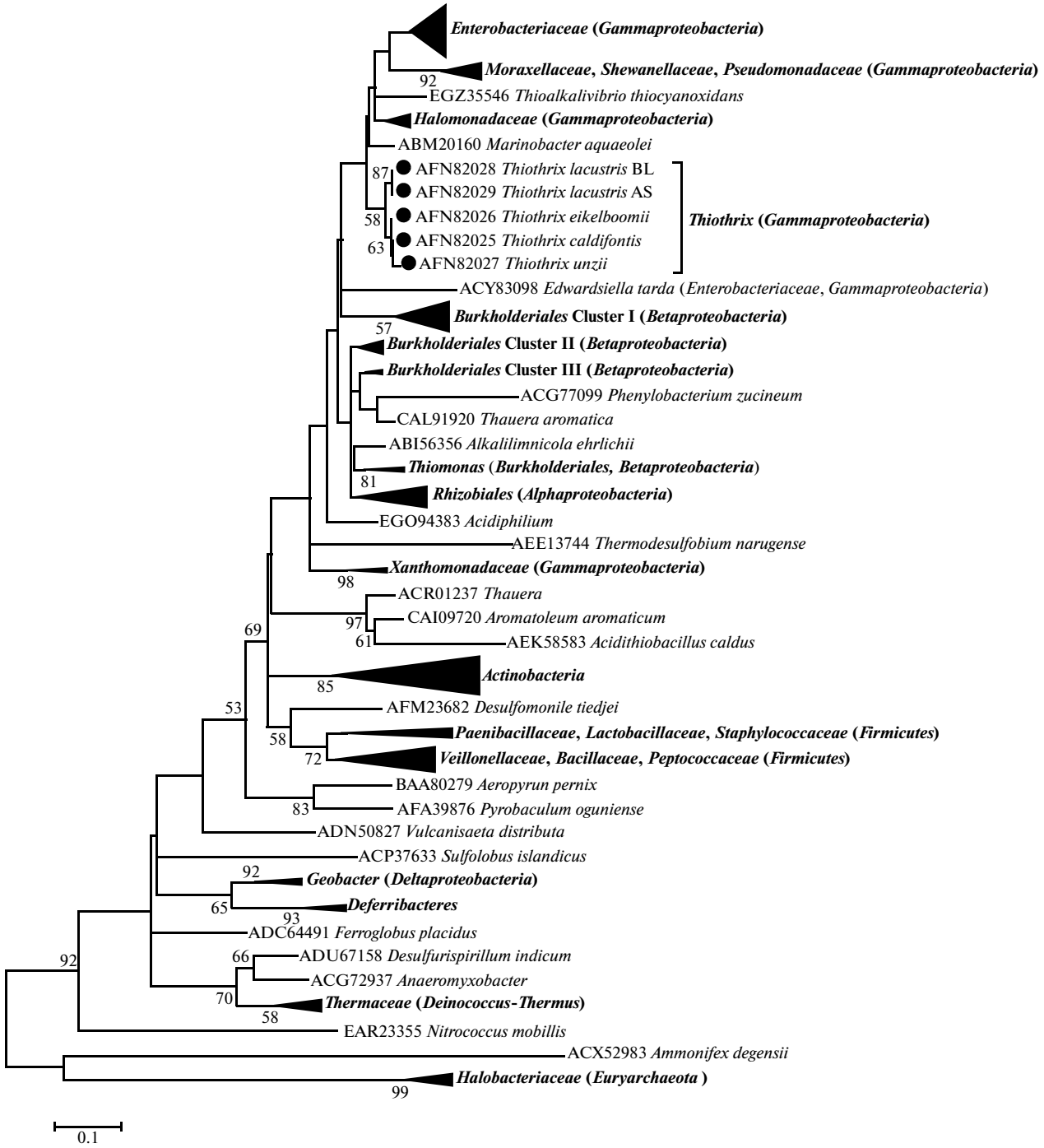

Fig. 2. Phylogenetic tree constructed on the basis of comparative analysis of *narG* gene fragments. Deduced amino acids sequences were used to construct the tree. Sequences determined in the course of this work are marked with black circles (●). Maximum likelihood algorithm, JTT model of amino acid substitution, and bootstrap analysis of 100 alternative trees were used. Statistical significance of the branching order is reported in percent at branching points of the dendrogram (only values above 50% are presented). Bar corresponds to 10% difference between amino acid sequences.

be capable of catabolic oxidation of reduced sulfur compounds under aerobic conditions [21–24]. In the *Thiothrix* strains under study, the amount of thiosulfate oxidized over 96 h under aerobic conditions varied from 1.5 to 2.5 mM. Under anaerobic growth, specific rate of thiosulfate oxidation (calculated per mg protein) was on average two times higher (Table 1). Only two final products, sulfate and elemental sulfur, were formed (in equimolar amounts) upon thiosulfate oxidation under both aerobic and anaerobic conditions;

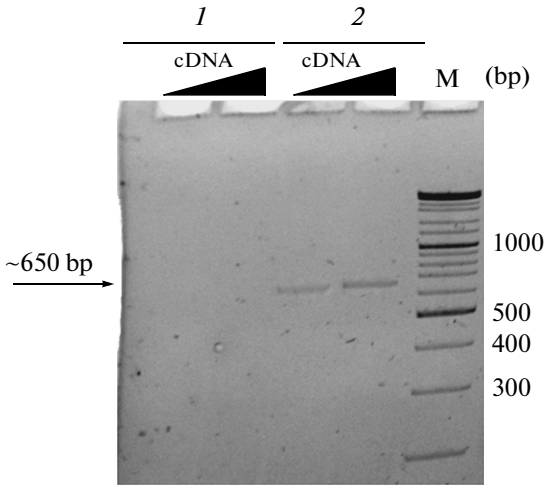

Fig. 3. Analysis of *narG* gene expression levels during aerobic and anaerobic growth. Results of electrophoresis of cDNA amplification products after reverse transcription of *narG* gene mRNA of *T. lacustris* AS. *1*, aerobic cultivation; *2*, anaerobic cultivation. Triangles show the increase in the template cDNA concentration in the PCR mixture. M, molecular weight markers.

no tetrathionate or sulfite were detected. In all *Thiothrix* strains under study, the average molar ratio between the final products of $S_2O_3^{2-}$ oxidation, SO_4^{2-} and S^0, was 1 : 1. The stoichiometry of enzymatic oxidation of thiosulfate and the products formed is to be established in our forthcoming studies.

The activities of thiosulfate : ferricytochrome *c* oxidoreductase, catalyzing thiosulfate oxidation to tetrathionate, and sulfite : ferricytochrome *c* oxidoreductase, catalyzing sulfite oxidation to sulfate, were not detected in the *Thiothrix* strains under study.

The activity of APS reductase, the enzyme involved in the series of reactions of sulfite transformation to sulfate, varied in *Thiothrix* strains from 265 to 865 nmol min^{-1} mg $protein^{-1}$ duing aerobic growth and from 246 to 685 nmol min^{-1} mg $protein^{-1}$ during anaerobic growth (Table 3). During heterotrophic growth, the activity of this enzyme decreased 3 to 5 times. In addition, in all *Thiothrix* strains under study, we detected the *soxB* gene, coding for the SoxB component of the thiosulfate oxidizing enzymatic complex (our unpublished data).

Table 3. Activity of APS reductase in *Thiothrix* representatives grown aerobically and anaerobically

Bacteria	APS reductase activity, nmol min^{-1} mg $protein^{-1}$	
	aerobic growth	anaerobic growth
T. caldifontis $G1^T$	865	685
T. lacustris BL^T	365	285
T. lacustris AS	325	318
T. eikelboomii $AP3^T$	265	246
T. unzii $A1^T$	357	255

Thus, the high rate of thiosulfate oxidation to sulfur and sulfates, high activity of APS reductase, and presence of the *soxB* gene confirm the capacity for anaerobic lithotrophic growth of all of the *Thiothrix* representatives under study.

In this work, a series of experiments was performed to demonstrate the occurrence of nitrate respiration in *Thiothrix* representatives and to prove coupling of thiosulfate oxidation and nitrate reduction. Incubation of the bacteria in the presence of organic substrates (acetate + lactate) and nitrate in the absence of thiosulfate resulted in lack of anaerobic growth and of nitrite production. The amount of thiosulfate oxidized under anaerobic conditions in the presence of nitrate as an electron acceptor varied in the *Thiothrix* strains between 1.9 and 2.8 mM, and the concentration of nitrites formed varied between 0.3 and 2.1 mM (Table 1).

Thus, in this work we were the first to demonstrate in *Thiothrix* representatives (*T. lacustris* BL, *T. lacustris* AS, *T. caldifontis* G1, *T. unzii*, and *T. eikelboomii*) the capacity for dissimilatory nitrate reduction to nitrite under anaerobic conditions and the coupling of this process with oxidation of reduced sulfur compounds.

The possible capacity of *Thiothrix* representatives for denitrification, i.e., for the reduction of nitrites to gaseous products, and the biochemical and enzymatic mechanisms of the oxidative reactions of sulfur metabolism in *Thiothrix*, will be the subjects of our further studies.

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