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EXPERIMENTAL ARTICLES

Thiobacillus sajanensis sp. nov., a New Obligately Autotrophic Sulfur-Oxidizing Bacterium Isolated from Khoito-Gol Hydrogen-Sulfide Springs, Buryatia

N. M. Dul'tseva[a,1], T. P. Tourova[a], E. M. Spiridonova[b], T. V. Kolganova[c], G. A. Osipov[d], and V. M. Gorlenko[a]

[a] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*
[b] *Moscow State University, Vorob'evy gory, Moscow, 119899 Russia*
[c] *Bioengineering Center, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 1, Moscow, 117312 Russia*
[d] *Bakulev Research Center of Cardiovascular Surgery, Russian Academy of Medical Sciences, Moscow, Russia*

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Abstract—Four strains of rod-shaped gram-negative sulfur-oxidizing bacteria were isolated from Khoito-Gol hydrogen-sulfide springs in the eastern Sayan Mountains (Buryatia). The cells of the new isolates were motile by means of a single polar flagellum. The strains were obligately chemolithoautotrophic aerobes that oxidized thiosulfate (with the production of sulfur and sulfates) and hydrogen sulfide. They grew in a pH range of 6.8–9.5, with an optimum at pH 9.3 and in a temperature range of 5–39°C, with an optimum at 28–32°C. The cells contained ubiquinone Q-8. The DNA G+C content of the new strains was 62.3–64.2 mol %. According to the results of analysis of their 16S rRNA genes, the isolates belong to the genus *Thiobacillus* within the subclass *Betaproteobacteria.* However, the similarity level of nucleotide sequences of the 16S rRNA genes was insufficient to assign the isolates to known species of this genus. The affiliation to the genus *Thiobacillus* was confirmed by DNA–DNA hybridization of the isolates with the type strain of the type species of the genus *Thiobacillus*, *T. thioparus* DSM 505^T (= ATCC 8158^T). Despite the phenotypic similarity, the hybridization level was as low as 21–29%. In addition, considerable differences were revealed in the structure of the genes encoding RuBPC, the key enzyme of autotrophic CO_2 assimilation, between the known *Thiobacillus* species and the new isolates. Based on molecular-biological features and certain phenotypic distinctions, the new isolates were assigned to a new *Thiobacillus* species, *T. sajanensis* sp. nov., with the type strain 4HG^T (= VKM B-2365^T).

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Key words: sulfur-oxidizing bacteria, phylogeny, structure of RuBPC genes, sulfur springs.

Since 16S rRNA gene sequence analysis has shown that sulfur-oxidizing bacterial species assigned to the genus *Thiobacillus* represent subgroups of alpha-, beta-, and gammaproteobacteria, reclassification of thiobacilli has been undertaken [1]. Based on the priority principle, the original generic name *Thiobacillus* was retained for sulfur-oxidizing bacteria belonging to betaproteobacteria, and *T. thioparus* remains the type species of the genus. In addition to *T. thioparus*, the genus *Thiobacillus* currently includes the valid species *T. aquaesulis, T. denitrificans*, and *T. delicatus*; the nonvalid species *"T. plumbophilus"* and *"T. prosperus"* should also be mentioned [2]. The cells of *Thiobacillus* representatives are gram-negative rods motile by means of flagella. The known species are mesophilic and neutrophilic or alkalitolerant. Their electron-transport chains contain ubiquinone Q-8. They are chemolithoautotrophs (*T. thioparus, T. denitrificans, "T. plumbophilus," "T. prosperus"*), facultative chemolithoautotrophs (*T. delicatus*), or facultative chemolithoheterotrophs (*T. aquaesulis*) which utilize reduced sulfur compounds.

Most autotrophic organisms, including *Thiobacillus* representatives, assimilate CO_2 via the Calvin cycle, whose key enzyme is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC, EC 4.1.1.39). Studies conducted over the last years have shown that the RuBPC gene structure varies among different autotrophic organisms, and the information on this variability can be used to resolve evolutionary pathways and to determine the taxonomic positions of chemo- and photoautotrophs [3]. The primary structure of the RuBPC genes is currently known for only one *Thiobacillus* representative, namely *T. denitrificans.* The

[1] Corresponding author; e-mail: dultseva@mail.ru

Table 1. Results of physicochemical analyses at sampling sites

Spring	Sampling site	H_2S, mg/l	O_2, mg/l	HCO_3^-, mg/l	*T,* °C	pH	Eh, mV
Hg1							
	above mat	20.4	0.2	555.1	34.2–34.4	7.3–7.8	–6…+4
	inside mat	20.4	0.4	555.1	34.2–34.4	–	–
Hg3							
	above mat	23.8	2.0	579.5	31.2–33.7	7.6–8.0	17–33
	inside mat	–	2.4	567.3	31.2–33.7	–	–
Hg5							
	above mat	28.9	3.0	579.5	31.0	8.0	18
	inside mat	–	0.6	530.7	31.0	–	–

Note: The mats were composed mainly of *Thiothrix* representatives. "–" means lack of data.

genome of this organism has been shown to contain two RuBPC genes; according to their structural characteristics, they belong to form I ("green-like") and form II [3].

The present work is devoted to the study of physiological, biochemical, chemotaxonomic, and genetic characteristics of a new *Thiobacillus* species, *T. sajanensis* sp. nov., isolated from Khoito-Gol hydrogen-sulfide springs in the eastern Sayan Mountains (Buryatia). The phylogenetic characterization includes the analysis of 16S rRNA and RuBPC genes.

MATERIALS AND METHODS

Isolation source and isolation methods. The Khoito-Gol mineral springs are located at 52°37′23.01″ N, 99°00′51.34″ E in the eastern Sayan Mountains (Buryatia). This group of springs includes file outlets (Hg1, Hg2, Hg3, Hg4, Hg5) of thermal waters with a temperature of 30.5–34.4°C and pH 7.3–8.0. Hg1 is the uppermost spring on the mountain slope, and Hg5 is the lowermost one. Carbon dioxide predominates among the gases dissolved in the outgoing water; its content varies, at different outlets, from 31.8 to 57.2 mg/l [4]. The sulfide content in the outgoing water varies from 28.9 to 20.4 mg/l, gradually declining down the stream flows to 0.05 mg/l [5].

Samples of microbial mats and ground were taken in the end of July and beginning of August at spring outlets in sites where massive growth of colorless sulfur bacteria of the genus *Thiothrix* and deposition of elemental sulfur were observed. The *Thiothrix* mats appeared as abundant white strands on stream bottoms and basin walls. The physicochemical characteristics of the water in the sites of collecting samples that yielded the new isolates are presented in Table 1. The concentrations of sulfide, oxygen, and bicarbonate were determined by the methods described in the manual by Reznikov et al. [5]. Temperature, pH, and Eh were determined with the following portable instruments: a Prima sensor electrothermometer (Singapore), a PRO pH meter (Singapore), an ORP redox potentiometer (Portugal); the values determined were analogous to those earlier published [4].

Sample aliquots were plated onto solid medium of the following composition (g/l): NH_4NO_3, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.03; K_2HPO_4, 0.022; KH_2PO_4, 0.017; $Na_2S_2O_3 \cdot 5H_2O$, 2; Difco agar, 20; a solution of vitamins and trace elements according to Pfennig [6], 1 ml; distilled water, 1 l. The pH value was adjusted to 9.0 with a 10% solution of $NaHCO_3$. Several transfers of single colonies onto solid medium in petri dishes allowed us to obtain four pure cultures, strains 1HG, 4HG, 9HG, and 10HG. Strains 9HG and 10HG were isolated form spring Hg1, from the *Thiothrix*-dominated mat at the upstream boundary of its growth. Strain 4HG was isolated from spring Hg3, and strain 1HG from spring Hg5. The isolates were very similar to each other. Strain $4HG^T$ was studied in greater detail. The type strain *Thiobacillus thioparus* DSM 505^T (=ATCC 8158^T) was used in comparative studies.

Morphological and physiological characteristics. The morphology of cultures was studied under a Carl Zeiss (Jena) light microscope equipped with a phase-contrast device. The size of live bacterial cells was determined using an ocular micrometer. The fine structure of cells was examined in ultrathin sections under a JEM-100 (Jeol, Japan) electron microscope at an accelerating voltage of 80 kV [7, 8].

The ability of bacteria to utilize sulfur compounds was tested in liquid medium of the above-specified composition. The medium was supplemented with one of the sulfur compounds tested (thiosulfate, sulfide, or sulfite) at a concentration of 0.03%. The ability of bacteria to use various carbon sources was tested in a mineral medium containing 0.03% thiosulfate and 0.05% carbon source. Cultivation was performed in 500-ml flasks with 200 ml of medium. Exponential-phase cells grown autotrophically in medium with thiosulfate were

used as the inoculum (10%). The utilization capacity was judged after three successive culture transfers in medium with a particular compound. The number of bacterial cells was counted in specimens with a fixed volume under a microscope with a phase-contrast device. Elemental sulfur inside and outside cells was identified under light microscope by characteristic refraction in polarized light.

The growth of bacteria was assessed from protein yield determined with Coomassie Blue [9].

The concentrations of reduced inorganic sulfur compounds were determined by iodometric titration [5]. Sulfates were determined with $BaCl_2$ by the nephelometric method. Nitrites were determined colorimetrically with sulfanilic acid and α-naphthyl ethylenediamine [10].

The resistance of bacteria to antibiotics was tested on agarized medium of the above-specified composition. The antibiotics were added in the following concentrations (μg): rifampicin, 5; fusidin, methicillin, ampicillin, gentamycin, benzylpenicillin, oxacillin, and doxycycline, 10; lincomycin, oleandomycin, and erythromycin, 15; carbenicillin, 25; and ristomycin, streptomycin, neomycin, polymyxin, levomycetin, tetracycline, and kanamycin, 30.

Analysis of the fatty acid composition was performed on an HP-5973D chromatograph–mass spectrometer (Agillent Technologies (former Hewlett Packard), United States). Samples for analysis were prepared as described by Karavaiko et al. [11].

Determination of the composition of isoprenoid quinones. Wet biomass of strain 4HG and *Thiobacillus thioparus* DSM 505^T was ground in liquid nitrogen in a porcelain mortar. Extraction was performed with cold acetone. The extract was evaporated until dry. The residue was subjected to chromatography on a plate with a fixed layer of Silufol UV-254 silica gel in a hexane–diethyl ether (85 : 15) system. The band with an R_f of 0.24–0.19 was extracted with methanol. Mass spectra of quinones were recorded under standard conditions (70 eV) on a Finnigan-MAT 8430 (Bremen, Germany) mass spectrometer [12].

Determination of the nucleotide composition of DNA was performed by the thermal denaturation method, and DNA–DNA–hybridization was studied by the optical reassociation method as described in [13].

Amplification and sequencing of 16S rRNA genes and RuBPC genes were performed as described in [14]. Primary analysis of the nucleotide sequences of 16S rRNA genes was performed using data and software available in GenBank (http://www.ncbi.nlm.nih.gov). Sequence editing was done using the BioEdit software package (http://jbrown.mbio.ncsu.edu/BioEdit/bioedit.html). The newly determined gene sequences were aligned with closely related sequences using the CLUSTALW v 1.75 software package. Unrooted phylogenetic trees were constructed using algorithms implemented in the TREECONW software package (http://bioc-www.uia.ac.be/u/yvdp/treeconw.html).

Deposition of sequences. The nucleotide sequences of the 16S rRNA genes of strains 4HG and 1HG were deposited in GenBank under accession numbers DQ390445 and DQ390446; the sequences of the RuBPC genes of strains *T. thioparus* DSM 505^T (= ATCC 8158^T), 4HG^T, and 1HG were deposited in GenBank under accession numbers DQ390449, DQ390447, and DQ390448.

RESULTS

Four bacterial strains (1HG, 4HG, 9HG, and 10HG) morphologically similar to *Thiobacillus* representatives and staining gram-negative were isolated on mineral medium with thiosulfate as the energy source. Our isolates were components of the microbial community dominated by *Thiothrix* representatives, which formed typical mats at the sites of sampling (Table 1). The isolates were very similar to each other. Strain 4HG was studied in greater detail.

Morphology and fine structure. All of our isolates were gram-negative rods motile by means of a single polar flagellum; cells occurred singly or in short chains (Figs. 1a, 1b). The cells were 0.9–1.65 μm long and 0.3–0.4 μm thick. Multiplication occurred by binary fission with a constriction. Growth on medium with thiosulfate was accompanied by deposition of sulfur, mainly outside cells and occasionally in the periplasmic space. In the latter case, the sulfur appeared as intracellular under light microscope. Sulfur deposition in the periplasmic space of thionic bacteria was first discovered by Pivovarova and Karavaiko [15].

In ultrathin sections (Figs. 1c–1f), it can be seen that the cell wall of the sulfur-oxidizing bacterium consisted of several layers typical of gram-negative bacteria (Fig. 1e); the outer membrane had a wavy surface (Figs. 1d, 1e). The cells were covered with a slimy microcapsule (Figs. 1d–1f). Within the microcapsule, spheric three-layered membranous structures occurred, most probably involved in the transport of elemental sulfur. The three-layered cytoplasmic membrane occasionally formed invaginations looking like big loops and bags in which sulfur was deposited. In ultrathin sections, they resembled hollows because sulfur had been extracted with ethanol in the course of specimen processing.

In the central part of the cells, a fibrillar nucleoid was easily discernible (Figs. 1d–1f). In the nuclear zone of the cytoplasm, large round electron-dense inclusions occurred, which are usually identified as polyphosphates (Figs. 1c, 1f).The cytoplasm contained hexagonal inclusions (0.0625 × 0.0625 μm), surrounded by a membrane monolayer (Fig. 1f). Such a structure is typical of carboxysomes, containing RuBPC.

Physiological, biochemical, and cultural properties. The growth of the new isolates of sulfur-oxidizing

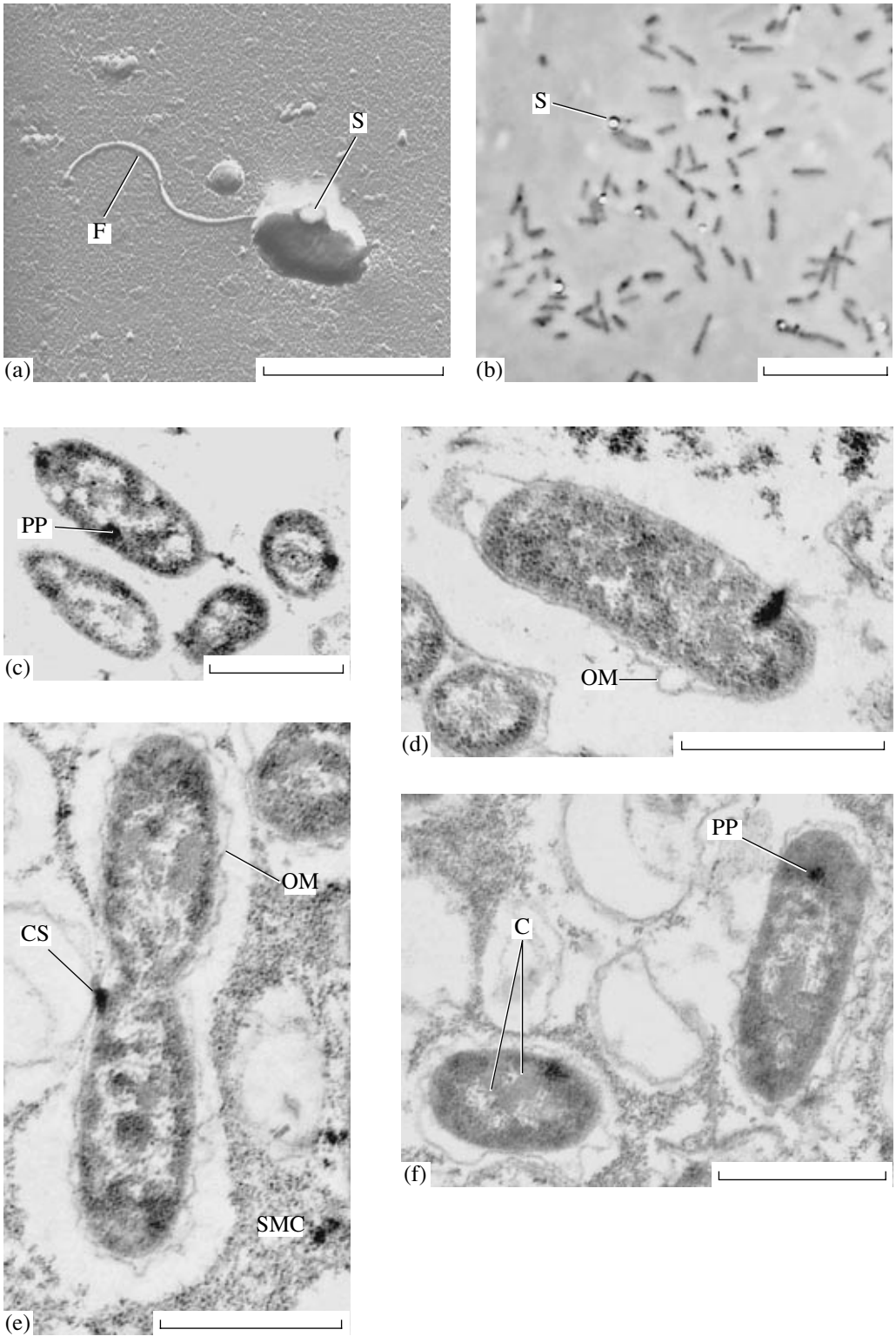

Fig. 1. Morphology and fine structure of strain 4HG^T cells: (a) Electron micrograph of whole cells grown on medium with thiosulfate; staining with ammonium molybdate; bar, 0.5 μm; F, flagellum; S, sulfur; (b) phase-contrast micrograph of cells grown on medium with thiosulfate; bar, 5 μm; (c–f) electron micrographs of ultrathin sections; PP, polyphosphates; OM, outer membrane; CS, cellular septum; SMC, slimy microcapsule; C, carboxysomes.

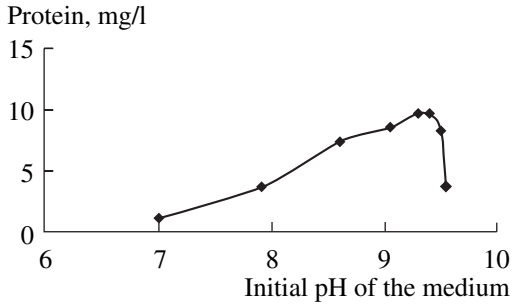

Fig. 2. Dependence of strain 4HG^T biomass yield on the initial pH value of the medium during autotrophic growth on medium with thiosulfate.

bacteria was studied as dependent on the cultivation conditions. The optimal cultivation temperature for strains 1HG, 4HG, 9HG, and 10HG was found to be 28–32°C. The growth temperature range was 5–39°C. The bacteria preferred low mineralization of the medium: the highest concentration of NaCl at which weak growth still occurred was 20 g/l. The isolates grew in media with initial pH values of 6.8–9.5, with an optimum at pH 9.3–9.4 (Fig. 2).

All of the new isolates were obligate aerobes and chemolithoautotrophs. They could grow on mineral medium with $S_2O_3^{2-}$, SO_3^{2-}, HS^-, or S^0. On media with sulfide or thiosulfate, sulfur and sulfates were the final products. The curves of strain 4HG growth on thiosulfate are shown in Fig. 3. Under optimal conditions, the generation time was 17 h. Maximal biomass (about 15 mg protein/l) was accumulated by strain 4HG on the fourth day of growth.

Table 2. Fatty acid composition of membrane lipids of strain 4HG^T cells

Fatty acid	% of total fatty acids
12:0	3.90
h10	0.72
14:0	0.36
h12	0.21
15:1	0.20
15:0	0.49
16:1ω7c	42.10
16:0	36.36
17cyc	0.52
18:2	0.08
18:1ω11	0.35
18:1ω9	11.14
18:1ω7	0.90
18:0	2.64
Total	100.0

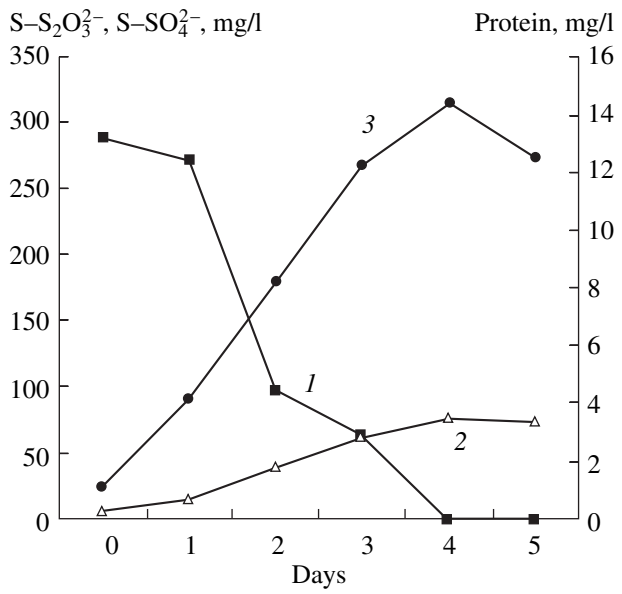

Fig. 3. Dynamics of biomass accumulation and thiosulfate oxidation during growth of strain 4HG^T on medium with thiosulfate: *1*, S-$S_2O_3^{2-}$, *2*, S-SO_4^{2-}, *3*, protein.

The strains were incapable of organotrophic growth. In the absence of thiosulfate, the bacteria did not grow on any of the organic substrates tested: alanine, arabinose, aspartate, citrate, glucose, histidine, maltose, malate, serine, succinate, sucrose, or xylose.

Nor were the strains capable of mixotrophic growth. Their growth was not stimulated by the addition to the medium with thiosulfate of such compounds as tyrosine, malate, xylose, fructose, glucose, arabinose, maltose, citrate, succinate, sucrose, cellobiose, or histidine.

Complete inhibition of the growth of the isolates was observed after the addition to the thiosulfate-containing medium of such compounds as lactose, rhamnose, phenylalanine, proline, phenol, ribose, lactate, pyruvate, yeast extract, casein hydrolysate, benzoate, glutamate, glycerol, raffinose, and fumarate.

Neither of the four strains was capable of anaerobic growth at the expense of nitrate reduction. For stains 9HG and 10HG, production of nitrites (up to 5 μM) from nitrates was recorded under aerobic conditions.

The effect of antibiotics was studied for strain 4HG^T. Ampicillin, tetracycline, doxycycline, lincomycin, methicillin, gentamycin, ristomycin, levomycetin, rifampicin, oxacillin, fusidin, benzylpenicillin, and carbenicillin did not affect growth, which was inhibited by neomycin, kanamycin, oleandomycin, erythromycin, streptomycin, and polymyxin.

Chemotaxonomic characteristics. The analysis of fatty acids showed the predominance of unsaturated ones containing 16 or 18 carbon atoms, 16:1ω7c and 18:1ω9, and the saturated palmitic acid, 16:0 (Table 2).

Based on these results, we can state the relatedness of strain 4HG to known species of *Thiobacillus* (not only the coincidence of the dominant fatty acids is important, but also the presence of hydroxydecanoic acid, h10, inherent in all known *Thiobacillus* species [2]).

The chromatogram obtained in the analysis of isoprenoid quinone composition exhibited ubiquinone bands with an R_f of 0.24 for *T. thioparus* DSM 505^T and an R_f of 0.19 for strain 4HG. Mass-spectrometric analysis revealed two characteristic peaks: a peak with *m/z* 235, typical of ubiquinones, and a peak with *m/z* 726, typical of ubiquinone Q-8 with a formula $C_{49}H_{74}O_4$.

DNA analysis. Strains 4HG, 9HG, and 10HG were virtually identical in their DNA base pair composition (Table 3), whereas strain 1HG was characterized by a somewhat different value. In accordance with these data, strains 4HG, 9HG, and 10HG exhibited a high value of DNA–DNA homology (95–97%), and strain 1HG showed a considerably lower DNA–DNA hybridization level with this group of strains (45–51%). All of the new isolates showed a low level of DNA–DNA hybridization (21–29%) with the type strain *T. thioparus* DSM 505^T.

Phylogenetic analysis of the 16S rRNA genes. To establish the phylogenetic position of the new isolates of thiobacilli, we determined nearly complete sequences of the 16S rRNA genes of strains 4HG^T and 1HG (about 1400 nucleotides, approximately corresponding to *Escherichia coli* positions 20–1480). These sequences proved to be virtually identical (99.8% similarity). Screening in the GenBank database showed that both sequences belonged to the genus *Thiobacillus* within betaproteobacteria. In the phylogenetic tree (Fig. 4a), the 16S rRNA sequences of both strains fell, with a bootstrap support level of 99, into the monophyletic cluster of *Thiobacillus* species. The new isolates were most close to *T. thioparus* strains, including the type strain (97.6–97.8% sequence similarity), and to the type strain of *T. denitrificans* (97.4–97.7%). Approximately the same similarity level (97.7–98.1%) was recorded between *T. thioparus* and *T. denitrificans* representatives, which agreed with earlier published data [16]. The level of relatedness of the new isolates with the *T. aquaesulis* (the last of the validated *Thiobacillus* species) was considerably lower (93.1–93.2%).

Phylogenetic analysis of the RuBPC genes. An earlier designed system of oligonucleotide primers [17] was used to amplify RuBPC genes of the type strain *T. thioparus* DSM 505^T and of strains 4HG^T and 1HG. In all of the strains that we studied, PCR yielded products that corresponded to fragments of genes of the green-like form I RuBPC and form II RuBPC, amplified in positive control reactions.

Sequencing of the PCR products obtained with primers specific to green-like form I RuBPC yielded 750-nucleotide sequences for each of the strains studied. The sequences of the RuBPC genes of our isolates

Table 3. Genotypic characteristics of newly isolated thiobacilli

Strain	G+C, mol %	DNA–DNA homology, %				
		DSM 505^T	4HG^T	9HG	10HG	1HG
Thiobacillus thioparus DSM 505^T (reference)	65.1	100				
4HG^T	64.2	21	100			
9HG	64.1	–	95	100		
10HG	64.4	–	98	97	100	
1HG	62.3	29	45	47	51	100

Note: "–" means lack of data.

4HG^T and 1HG proved to be 100% identical. Screening in the GenBank database showed high similarity (87–89%) of the newly determined sequences with the *cbbL* gene sequences of other bacteria, thus confirming their affiliation with this gene family.

The conceptually translated amino acid sequences of corresponding proteins were aligned with sequences of green-like form I RuBPC of other bacteria available from GenBank, and 244 alignment positions were compared.

The topology of the phylogenetic tree constructed based on the comparison of RuBPC sequences (Fig. 4b) exhibited considerable deviations from the 16S rRNA-based tree. This fact correlates with the results obtained earlier for representatives of various phylogenetic groups of autotrophic bacteria [18] and apparently reflects peculiarities of the evolution of the RuBPC genes.

In the RuBPC-based tree, as distinct from the 16S rRNA-based tree, the representatives of the genus *Thiobacillus* did not form a monophyletic cluster. Moreover, different representatives of beta- and gammaproteobacteria proved to be their closest neighbors in the RuBPC-based tree. *T. denitrificans* clustered with the gammaproteobacterium *Acidithiobacillus ferrooxidans* (96.3% homology and a bootstrap value of 91); *T. thioparus* formed a separate branch with an unstable position in the tree (a bootstrap value of 28); and the new strains 4HG^T and 1HG formed a monophyletic cluster with representatives of another genus of sulfur betaproteobacteria, *Thiomonas intermedia* (96.2% homology and a bootstrap value of 98).

DISCUSSION

Our isolates of sulfur-oxidizing bacteria exhibit morphological, physiological, and genotypic properties typical of the genus *Thiobacillus* (Tables 3, 4). Like representatives of the *Thiobacillus* – *T. aquaesulis*, *T. denitrificans* species *T. aquaesulis, T. denitrificans*,

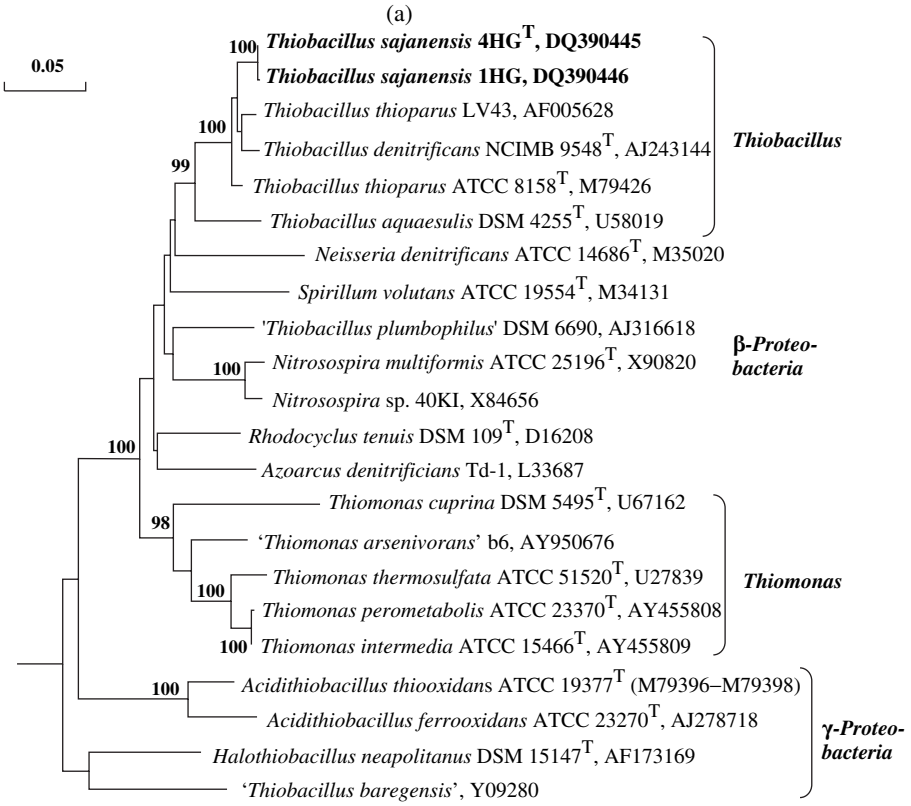

Fig. 4. Phylogenetic position of the new isolates of sulfur-oxidizing bacteria in unrooted phylogenetic trees constructed using the neighbor-joining algorithm. Genes sequenced in the present study are set in boldface. Numerals show the significance of the branching order as determined by bootstrap analysis of 1000 alternative trees (values above 90% were considered significant). (a) Phylogenetic tree constructed based on nucleotide sequences of the 16S rRNA genes; scale bar shows evolutionary distance corresponding to 5 substitutions per 100 nucleotides. (b) Phylogenetic tree constructed based on deduced amino acid sequences of RuBPC. Underlined are *Thiobacillus* representatives; scale bar shows evolutionary distance corresponding to 5 substitutions per 100 amino acid residues. GREEN and RED designate green-like and red-like RuBPC.

and *T. thioparus*, the new strains are gram-negative motile rods capable of chemolithoautotrophic growth on reduced sulfur compounds and elemental sulfur. Their chemotaxonomic properties (composition of fatty acids and isoprenoid quinones) and base pair composition of their DNAs agree with the affiliation of these strains with the genus *Thiobacillus* [1, 2, 16, 19].

The hybridization level of total genomic DNAs of strains 4HG^T, 9HG, and 10HG (no less than 95%) corresponds to the intraspecific level, suggesting that these strains belong to the same species. The hybridization level of this group of strains with strain 1HG was considerably lower (no higher than 51%); however, due to the lack of significant differentiating phenotypic characteristics and similarity of the nucleotide sequences of the 16S rRNA genes and RuBPC genes, we believe that strain 1HG should be assigned to the same species as strains 4HG^T, 9HG, and 10HG.

The analysis of the 16S rRNA genes of strains 4HG and 1HG has shown that the isolates belong to the cluster of *Thiobacillus* species. At the same time, the moderate level of similarity with known species of this genus (no higher than 97.8%) and the low level of DNA–DNA hybridization (no higher than 29%) between the new strains and the type *Thiobacillus* species, *T. thioparus* do not allow our isolates to be assigned to the known *Thiobacillus* species.

In addition, notable phenotypic distinctions exist between the new isolates and the known *Thiobacillus* species (Table 4). *T. aquaesulis* is a moderate thermophile (the temperature optimum for its growth is 40–50°C). The new isolates, as well as the species *T. denitrificans* and *T. thioparus*, are mesophiles (the temperature optimum for their growth is 25–30°C). *T. denitrificans* and *T. thioparus* are capable of anaerobic growth at the expense of denitrification, whereas the new isolates lack this capacity. In addition, the new isolates differ from *T. thioparus* by the optimal value of initial pH of the medium: it is pH 9.3–9.4 for the new strains and 6.6–7.2 for *T. thioparus* [2]. Like *T. thioparus*, the new isolates contain carboxysomes in the cell cytoplasm and are obligately dependent on inorganic sulfur compounds as energy sources. *T. denitrificans* lacks carboxysomes.

Additional phylogenetic analysis, based on the comparison of the sequences of the genes of RuBPC, the

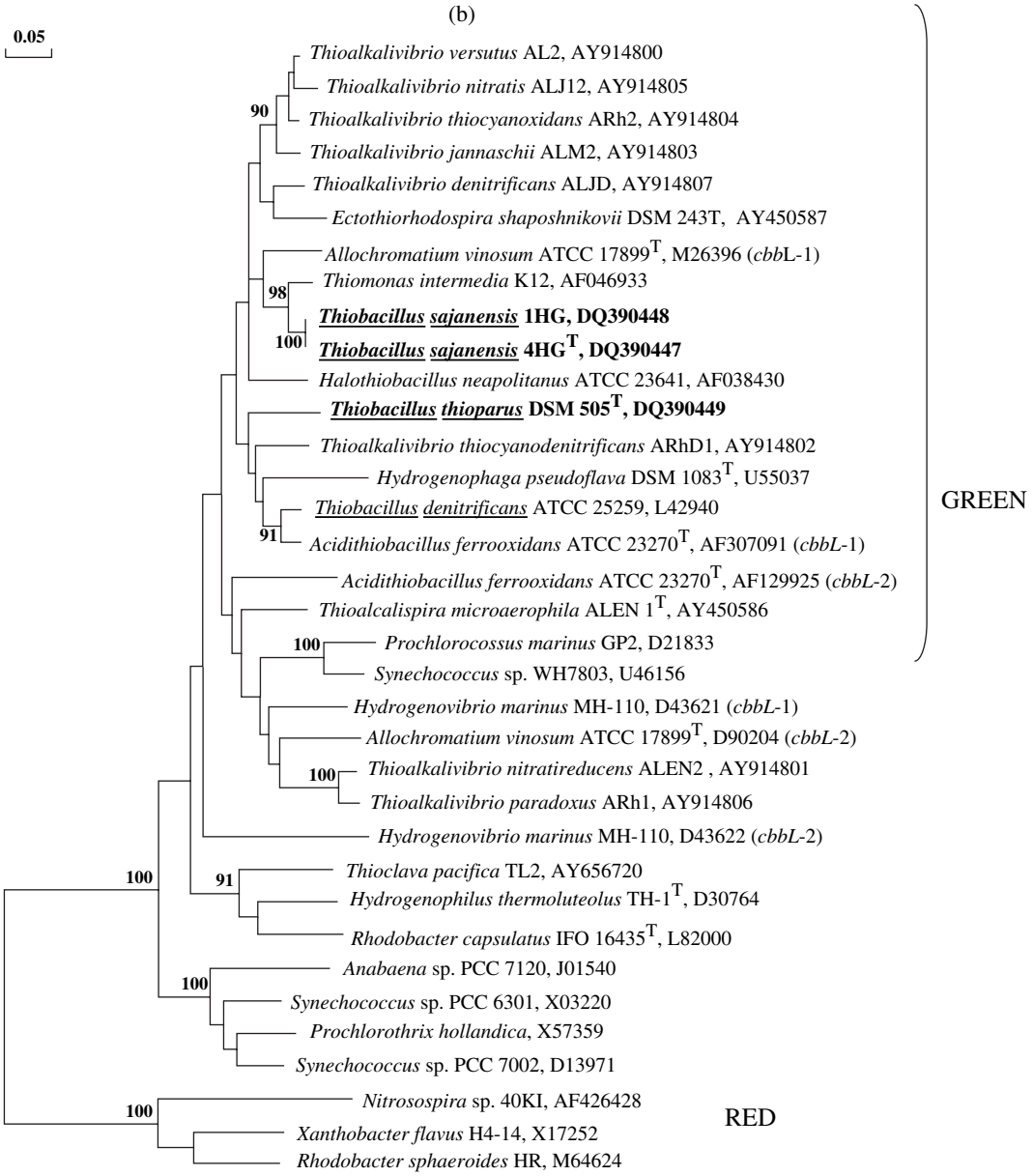

Fig. 4. (Contd.)

key enzyme of autotrophic carbon dioxide fixation, demonstrated independent origin of these genes in all of the examined *Thiobacillus* representatives, including the newly isolated strains. The phylogenetic proximity of these genes to the genes of different genera of both beta- and gammaproteobacteria suggests that the evolution of this group of chemoautotrophs involved multiple events of interspecies and intergeneric lateral transfer of RuBPC genes. An additional piece of indirect evidence in favor of this conclusion is the isolation of the strains from sites of massive development of *Thiothrix* representatives, which belong to gammaproteobacteria and posses RuBPC genes [20].

Based on the above-discussed phenotypic and genotypic distinctions, as well as on data of phylogenetic analysis, we propose that our isolates be assigned to a new species, *Thiobacillus sajanensis* sp. nov., with the following species description.

Description of Thiobacillus sajanensis sp. nov.

T. sajanensis (sa.ja.nen'sis; N.L. masc. adj. *sajanensis*, isolated from springs in the Sayan Mountains).

Cells are motile, non-spore-forming, gram-negative short rods (0.3–0.4 $\times$ 0.9–1.6 nm) covered with a microcapsule; they contain carboxysomes and polyphosphates.

Table 4. Comparison of the characteristics of *Thiobacillus* species and new isolates of sulfur-oxidizing bacteria

Characteristic	New isolates	*T. thioparus*	*T. denitrificans*	*T. aquaesulis*	*T. delicatus*	'*T. plumbophilus*'	'*T. prosperus*'
Size of the motile rods, μm	0.3–0.4 × 0.9–1.6	0.5 × 1.7	0.5 × 1.0–3.0	0.3 × 0.9	[a]; 0.4–0.6 × 0.7–1.6	0.25 × 3.0	0.3 × 3–4
Carboxysomes	+	+	–	ND	ND	ND	+
Ubiquinone Q-8	+	+	+	+	ND	+	+
pH range (pH optimum)	6.8–9.5 (9.3–9.4)	4.5–7.8 (6.6–7.2)	6–8 (6.8–7.4)	6.5–9.0 (7.5–8.0)	5.0–7.0 (5.5–6.0)	4.0–6.5 (4.0–6.5)	1.0–4.5 ND
Temperature range (temperature optimum)	5–39 (28–32)	25–30 (28)	25–32 (28–32)	30–55 (40–50)	15–42 (30–35)	21–41 (21–34)	37–41 (37)
G+C in DNA, mol %	62–64	62–63	63	66	66–67	66	64
DNA homology with *T. thioparus*, %	21–29	100	ND	ND	ND	ND	ND
Anaerobic growth with NO_3^-	–	+	+	+	+	–	ND
Metabolism type	Obligate chemolithoautotrophs, thiotrophs	Obligate chemolithoautotroph, thiotroph	Obligate chemolithoautotroph, thiotroph	Facultative chemolithoheterotroph, thiotroph	Facultative chemolithoautotroph and mixotroph, thiotroph	Obligate chemolithoautotroph, thiotroph	Obligate chemolithoautotroph, thiotroph
Reference		[1, 2]	[1, 2, 16]	[1, 2, 19]	[1, 2]	[1, 2]	[1, 2]

Note: "ND" stand for "no data"; "+" means that the character is positive; "–" means that the character is negative.[a] Nonmotile rods.

The metabolism is aerobic and chemolithoautotrophic. Oxidation of thiosulfate, sulfite, sulfide, or elemental sulfur provides energy for growth. Autotrophic growth occurs in a pH range 6.8–9.5, with an optimum at pH 9.3, and in a temperature range of 5–39°C, with an optimum at 28–32°C. Low mineralization of the medium is preferable. Carbon dioxide fixation occurs with the involvement of RuBPC. Colonies grown on agar medium with thiosulfate measure 1–2 mm in diameter; they are round and yellow due to sulfur deposition. In the stationary phase of growth in liquid medium with thiosulfate, deposition of sulfur occurs, and the medium becomes turbid due to cells and sulfur particles.

No organotrophic growth occurs on arabinose, glucose, xylose, maltose, sucrose, fructose, aspartate, malate, citrate, alanine, histidine, serine, or succinate. Nor is there mixotrophic growth with thiosulfate and such compounds as xylose, fructose, glucose, galactose, fructose, arabinose, maltose, sucrose, cellobiose, malate, citrate, succinate, histidine, tyrosine, serine, or cysteine.

Growth with thiosulfate is completely inhibited by lactose, raffinose, rhamnose, ribose, benzoate, lactate, pyruvate, fumarate, glutamate, yeast extract, casein hydrolysate, glycerol, proline, phenylalanine, and phenol.

Growth with thiosulfate is not affected by ampicillin, benzylpenicillin, gentamycin, doxycycline, carbenicillin, levomycetin, lincomycin, methicillin, oxacillin, ristomycin, rifampicin, tetracycline, or fusidin, but is inhibited by kanamycin, neomycin, oleandomycin, polymyxin, streptomycin, and erythromycin.

The DNA G+C content varies form 62.3 to 64.4 mol % among different strains, equaling 64.2 mol % in the type strain 4HG^T. The respiratory chain contains ubiquinone Q-8.

The position in the phylogenetic tree is in the *Thiobacillus* cluster within betaproteobacteria.

In terms of the 16S rRNA gene sequences, the closest relatives are *Thiobacillus thioparus* (97.6–97.8%) and *T. denitrificans* (97.4–97.7%).

In terms of the RuBPC gene sequences, clusterization is not observed with *Thiobacillus* representatives, but is observed with another sulfur-oxidizing betaproteobacterium, *Thiomonas intermedia* (96.2%).

The type strain is 4HG^T (= VKM B-2365^T).

The isolation source is Khoito-Gol hydrogen-sulfide mineral springs in the eastern Sayan Mountains (Buryatia, Russian Federation).

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