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EXPERIMENTAL ARTICLES

Sulfobacillus sibiricus sp. nov., a New Moderately Thermophilic Bacterium

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Abstract—In the course of pilot industrial testing of a biohydrometallurgical technology for processing gold-arsenic concentrate obtained from the Nezhdaninskoe ore deposit (East Siberia, Sakha (Yakutiya)), a new gram-positive rod-shaped spore-forming moderately thermophilic bacterium (designated as strain N1) oxidizing Fe^{2+}, S^0, and sulfide minerals in the presence of yeast extract (0.02%) was isolated from a dense pulp. Physiologically, strain N1 differs from previously described species of the genus *Sulfobacillus* in having a somewhat higher optimal growth temperature (55°C). Unlike the type strain of *S. thermosulfidooxidans*, strain N1 could grow on a medium with 1 mM thiosulfate or sodium tetrathionate as a source of energy only within several passages and failed to grow in the absence of an inorganic energy source on media with sucrose, fructose, glucose, reduced glutathione, alanine, cysteine, sorbitol, sodium acetate, or pyruvate. The G+C content of the DNA of strain N1 was 48.2 mol %. The strain showed 42% homology after DNA–DNA hybridization with the type strain of *S. thermosulfidooxidans* and 10% homology with the type strain of *S. acidophilus*. The isolate differed from previously studied strains of *S. thermosulfidooxidans* in the structure of its chromosomal DNA (determined by the method of pulsed-field gel electrophoresis), which remained stable as growth conditions were changed. According to the results of the 16S rRNA gene analysis, the new strain forms a single cluster with the bacteria of the species *Sulfobacillus thermosulfidooxidans* (sequence similarity of 97.9–98.6%). Based on these genetic and physiological features, strain N1 is described as a new species *Sulfobacillus sibiricus* sp. nov.

Key words: Sulfobacillus, moderate thermophiles, iron-oxidizing bacteria, sulfide-oxidizing bacteria.

Gram-positive acidophilic iron-oxidizing bacteria are widespread in sulfide ore deposits, coal fields, and hot vents. Three species of these bacteria were previously described and attributed to the genus *Sulfobacillus* (*S. thermosulfidooxidans*, type strain VKM B-1269 (DSM 9293) [1]; *S. acidophilus*, type strain NAL [2]; and *S. disulfidooxidans*, type strain SD-11 [3]), and one species was assigned to the genus *Acidimicrobium* (*A. ferrooxidans*) [4]. It was also proposed that two subspecies be included in the species *S. thermosulfidooxidans*: *S. thermosulfidooxidans* subsp. *asporogenes*, strain 41 [5], and *S. thermosulfidooxidans* subsp. *thermotolerans*, strain K1 [6]. The taxonomic position of strain 41 as a representative of the species *S. thermosulfidooxidans* was eventually confirmed, while that of strain K1 is not yet definite. According to the results of 16S rRNA gene sequencing, strain K1 and strain SD-11 happen to be closer to the genus *Alicyclobacillus* [7].

A distinctive feature of *Sulfobacillus* representatives is their ability to use, as a source of energy, iron, sulfur, and sulfide minerals, oxidized in the presence of yeast extract (0,02%) or other organic substances. These organisms can also grow under organotrophic conditions. With regard to temperature, bacteria of the genus *Sulfobacillus* are divided into moderately thermophilic (*S. thermosulfidooxidans* and *S. acidophilus*) and basically mesophilic (*S. disulfidooxidans* and *S. thermosulfidooxidans* subsp. *thermotolerans*).

The goal of this work was to isolate a pure culture of the new strain N1, admittedly belonging to the genus *Sulfobacillus*, study its morphological, biochemical, and genotypic characteristics, and determine its taxonomic position.

MATERIALS AND METHODS

Isolation of a pure culture and cultivation conditions. Strain N1 was isolated by serial dilutions from the pulp produced in pilot industrial testing of mesophilic (30°C) biohydrometallurgical processing of gold–arsenic concentrate obtained from the ore of the Nezhdaninskii deposit. The concentrate content of the pulp was 20 wt %. For dilutions, a modified 9K medium [8] supplemented with 0.02% yeast extract was used. This medium differed from the standard 9K medium in that it has a lower content of ferrous iron (2 instead of 9 g/l) and a lower pH of 1.8 (instead of 2.0–2.1). The isolation was carried out at 42°C. Strain N1 was maintained at 55°C on the modified 9K medium supple-

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mented with 0.02% yeast extract and 1 mM sodium thiosulfate.

The purity of the culture was checked by microscopic examination, by inoculation of media containing organic substrates, media containing ferrous iron and sulfur and free of organic carbon, media with 0.02% yeast extract and 1 mM thiosulfate or sodium tetrathionate, and by molecular typing, namely, by comparing restriction profiles of the chromosomal DNA of biomass grown on media with Fe^{2+} (at 30 and 55°C) and S^0 with the corresponding profile for strain N1, determined earlier [9].

In all tests, the strain was cultured in 250-ml Erlenmeyer flasks containing 100 ml of the medium on a rotary shaker operated at 180 rpm.

Monitoring bacterial growth. Concentrations of cells in all tests were determined by direct count in a Goryaev chamber using an Amplival microscope (Carl Zeiss) equipped with a phase contrast device. The concentration of Fe^{2+} and Fe^{3+} was determined by trilonometric titration [10]. In studies of cell growth on sulfur and sulfide minerals, the oxidation of substrate was judged from the decrease in the pH value and the formation of sulfates, determined turbidimetrically [11].

Electron microscopic studies. Whole cell specimens for electron microscopy were negatively stained with 1% phosphotungstic acid (pH 1.7). For ultrathin sectioning, cells were fixed in 1% osmic acid in a phosphate buffer (66 mM, pH 6.2) for 18 h at 5–7°C, dehydrated with ethanol applied in stepped-up concentrations, and embedded in Epon-812. Sections were obtained using an LKB-4800 ultramicrotome. The sections were negatively stained with a 3% water solution of uranyl acetate for 20 min and then with lead citrate by the Reynolds method for 10 min [12]. The preparations were examined under a JEM-100C electron microscope (Japan).

Physiological assays. The influence of temperature and pH on the growth of strain N1 was studied using a modified 9K medium supplemented with 0.02% yeast extract and 1 mM sodium thiosulfate.

To investigate the effect of inorganic source of energy on bacterial growth, we used a 9K medium devoid of Fe^{2+} and supplemented with yeast extract (0.02%) and one of the following compounds: pyrite (FeS_2), chalcopyrite ($CuFeS_2$), galena (PbS), zinc blende (ZnS), antimonite (Sb_2S_3), elemental sulfur (10 g/l), sodium thiosulfate, or tetrathionate (1 mM).

The effect of organic compounds on the growth of strain N1 was studied using a modified 9K medium supplemented with 1 mM sodium thiosulfate and one of the following organic substrates: glucose, fructose, sucrose, sorbitol, sodium acetate, sodium pyruvate, alanine, cysteine, or reduced glutathione in quantities equivalent to a 0.01% carbon content in the medium. We also studied the growth of bacteria on a 9K medium devoid of ferrous iron and containing one of the above-listed organic compounds (0.01% by carbon) or yeast extract (0.02 or 0.05%) as the source of energy.

Sulfide minerals and organic substrates were sterilized separately at 0.5 atm. The pH value was adjusted with 10 N H_2SO_4 to 2.5 for growth on S^0; to 1.9–2.0 for growth on sulfide minerals, thiosulfate, tetrathionate and organic compounds; and to 1.7–1.8 for growth on Fe^{2+}. When S^0, $S_2O_3^{2-}$, or $S_4O_6^{2-}$ was used as a sole inorganic source of energy, the medium was supplemented with $FeSO_4 \cdot 7H_2O$ (10 mg/l).

Assays of fatty-acid composition of lipids. Cell biomass (30 mg) grown on a medium with Fe^{2+} was dried in a nitrogen flow and subjected to acid methanolysis in 400 μl of 1.2 M HCl in methanol for 2 h at 80°C. Methyl esters of fatty acids and dimethylacetals formed as a result of methanolysis were twice extracted with 200 μl hexane, dried, and treated with 20 μl *N,O*-bis(trimethylsilyl)-trifluoroacetamide for 15 min at 80°C to obtain trimethylsilyl esters of hydroxy acids. The obtained mixture (2 μl) was fed into the injector of an HP-5973 gas chromatograph mass spectrometer (Hewlett-Packard, USA). Regular software supplied with the instrument was used for its control and data processing. Chromatographic separation of the mixture was performed on an HP-5ms methyl silica capillary column (Hewlett-Packard) (30 m; i.d., 0.2 mm). The initial temperature was set at 120°C for 2 min and then increased to 320°C at a rate of 5°C/min.

DNA analyses. DNA was isolated using Murmur's procedure [13]. The G+C content of the DNA was determined by thermal denaturation [14]. The level of DNA–DNA hybridization of bacteria was determined by the method of optical reassociation according to De Ley [15]. The genome size was estimated from the renaturation rate [16]. The stability of the structure of the chromosomal DNA of the strain under varied growth conditions was studied by analyzing *Not*I restriction digests of native DNA by pulsed-field gel electrophoresis [9]. Cell biomass grown on a medium with Fe^{2+} at 55 and 30°C and on a medium with S^0 was used for these analyses.

16S rRNA gene sequencing. Amplification and sequencing of the 16S rRNA gene was performed using primers that are universal for most prokaryotes [17] and a buffer composed of 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), and 0.001% gelatin. The reaction mixtures (100 μl) contained standard concentrations of dNTP and equimolar quantities of primers pA and pH'. Thirty amplification cycles were performed according to the following temperature profile: DNA denaturation at 94°C, 30 s; primer annealing at 40°C, 1 min; primer extension at 72°C, 2 min 30 s. Upon purification on low-gelling point agarose and on Promega columns, the 16S rRNA gene was sequenced in both directions with the use of forward and reverse universal primers and sequenases (Biochemicals, USA).

The 16S rRNA gene sequence of strain N1 was manually aligned with the corresponding sequences for the species of the *Alicyclobacillus–Sulfobacillus* group.

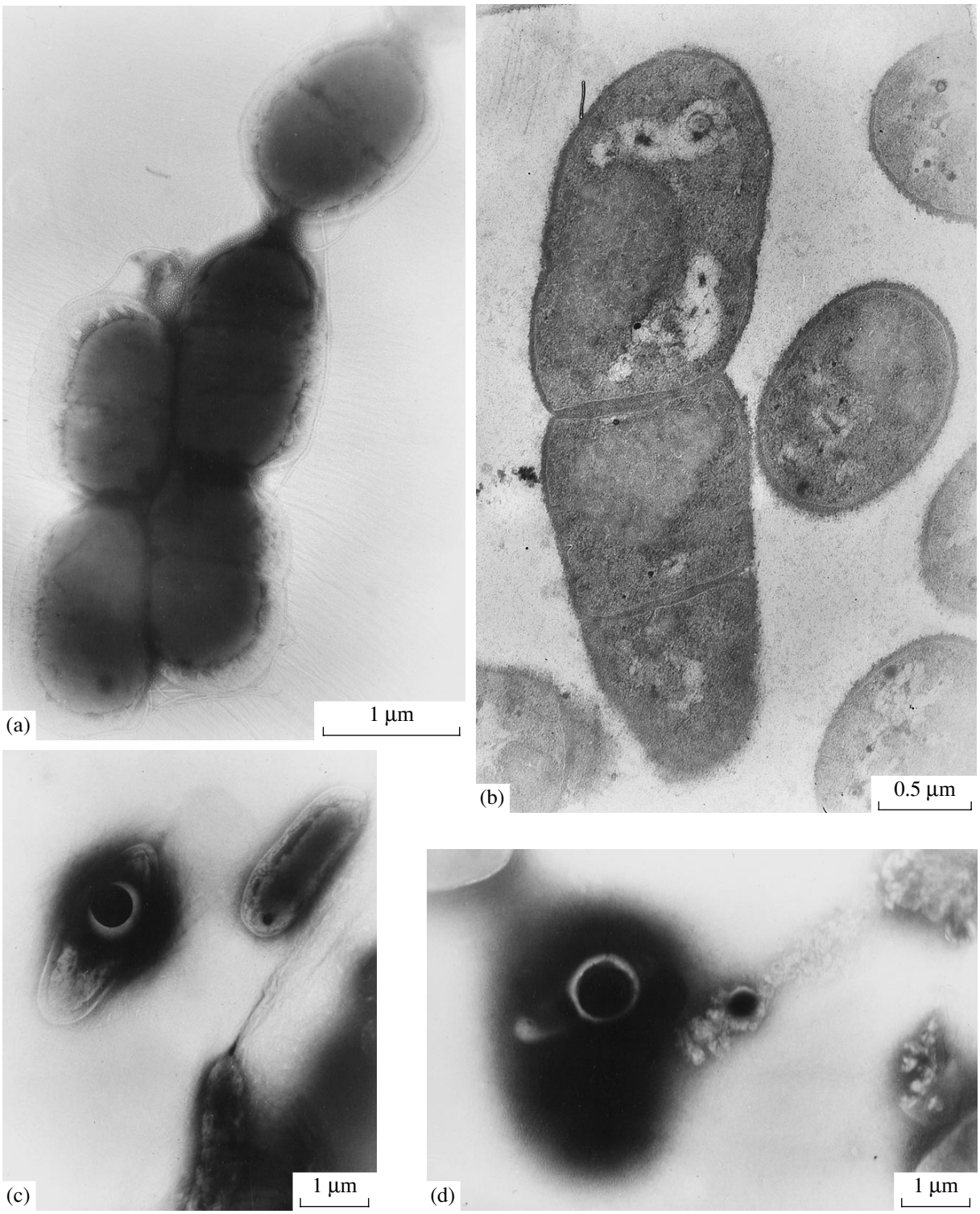

Fig. 1. Morphology of strain N1 cells: (a) a general view of cells; (b) ultrathin sections of cells; (c) and (d) cell culture with forespores.

A rootless phylogenetic tree for the bacteria studied was constructed using the TREECON software package [18]. The 16S rRNA gene sequence obtained was deposited in the GenBank database (accession no. AY079150).

RESULTS

Morphology and ultrastructure. Cells of strain N1 are nonmotile straight rods with oval-shaped ends measuring 0.7–1.1 × 1.0–3.0 μm (Fig. 1a). The culture

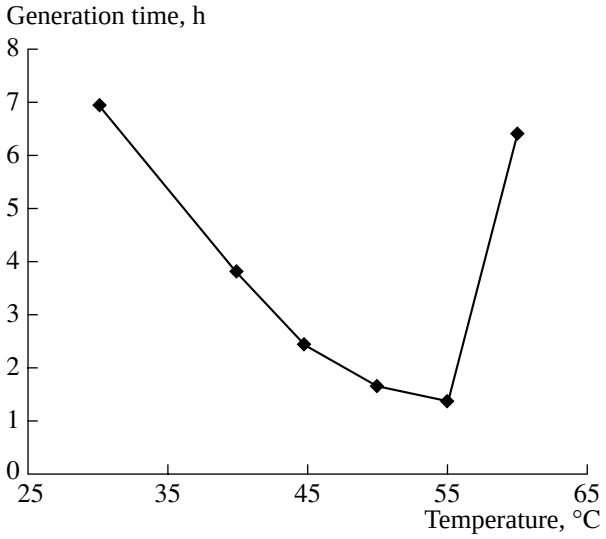

Fig. 2. Generation time of strain N1 cells as a function of temperature.

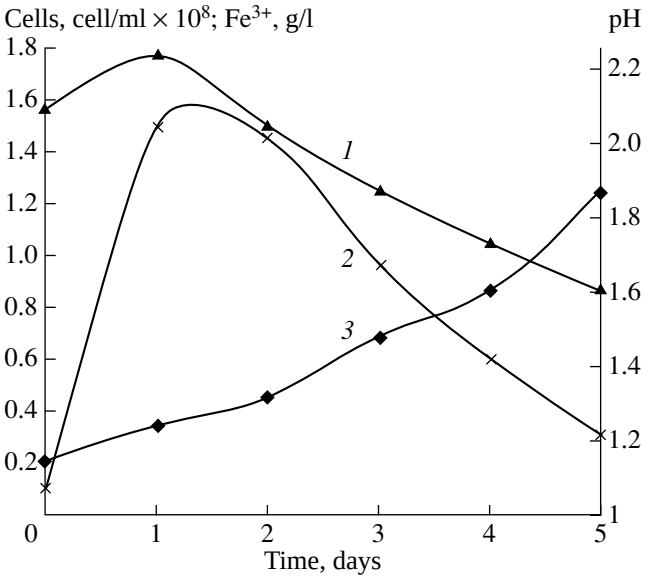

Fig. 3. Pyrite oxidation by strain N1: (*1*) pH; (*2*) cell concentration; (*3*) concentration of Fe^{3+}.

is represented by single cells, cells in pairs, and short chains of 3–5 cells (Figs. 1a, 1b). Immediately upon division, the shape of some cells can be close to coccoid. During active growth, transverse septa are often formed faster than daughter cells, and for this reason elongated cells with one to two transverse septa (visible only under an electron microscope) occur in the culture. The cell wall in strain N1 is of the type characteristic of gram-positive bacteria (Fig. 1b).

Strain N1 can form spores (Figs. 1c, 1d), but this process is not stable. The formation of spores was not observed during mixotrophic growth on media with Fe^{2+} and yeast extract (or reduced glutathione). The cause of instability of spore formation in strain N1 is not clear and requires special investigation.

Effect of temperature and pH on bacterial growth. As seen from Fig. 2, the optimal growth temperature for strain N1 is 55°C. Growth is possible at temperatures ranging from 17 to 60°C, and the bacterium is, therefore, moderate thermophile.

Oxidation of Fe^{2+} by strain N1 occurred at pH values ranging from 1.1 to 2.6. It is likely that the bacterium can grow at the expense of Fe^{2+} oxidation at even higher pH values. However, under the given conditions at an elevated temperature, the oxidation of Fe^{2+} was accompanied by precipitation of ferric oxide and jarosite, causing a fast decline of pH to 2.6. The optimal pH value on a medium with Fe^{2+} was 2.0, while on a medium with S^0, strain N1 grew at pH values ranging from 2.0 to 3.5, with optimal pH 2.2–2.5.

Utilization of inorganic electron donors. The inorganic donors of electrons for strain N1 are Fe^{2+}, S^0, and sulfide minerals. Active oxidation of ferrous iron was observed during the growth of the strain on a modified 9K medium supplemented with Fe^{2+} and yeast extract. The minimum generation time and the biomass yield were 1.3–1.4 h and 10^8 cells/ml, respectively. In growth on a medium with elemental sulfur and yeast extract, the biomass yield did not exceed 10^7 cells/ml, and the generation time was never lower than 3.5 h. On a medium 9K free of iron but supplemented with thiosulfate or tetrathionate, strain N1 showed growth only in the course of a few passages (up to six on thiosulfate and up to nine on tetrathionate). In the presence of yeast extract (0.02%), growth was also observed on all sulfides listed in the Materials and Methods section. The biomass yield was $1–2 \times 10^8$ cells/ml, depending on the

Fatty-acid composition of lipids in strain N1 (%)

Fatty acids**																
14	*i*15	*a*15	15*	*i*16	16 : 1*	16 : 0	*i*17	*a*17	17*	*i*18	2*hi*16*	18	2*h*16	*i*19*	2*h*17	Unident.
0.2	7.6	11.3	0.3	10.3	0.4	6.4	3.5	36.6	0.2	0.6	0.8	0.6	0.3	0.8	5.2	14.9

* Fatty acids not found in previously studied bacteria of the genus *Sulfobacillus*.

** Designations: in 16 : 1, 16 is the number of carbon atoms and the digit after the colon is the number of double bonds; *h* denotes hydroxy acid; and *a* and *i* at the beginning denote *anteso* and *iso* branching, respectively. For example, 2*hi*16 is 2-hydroxy-*iso*hexadecane acid.

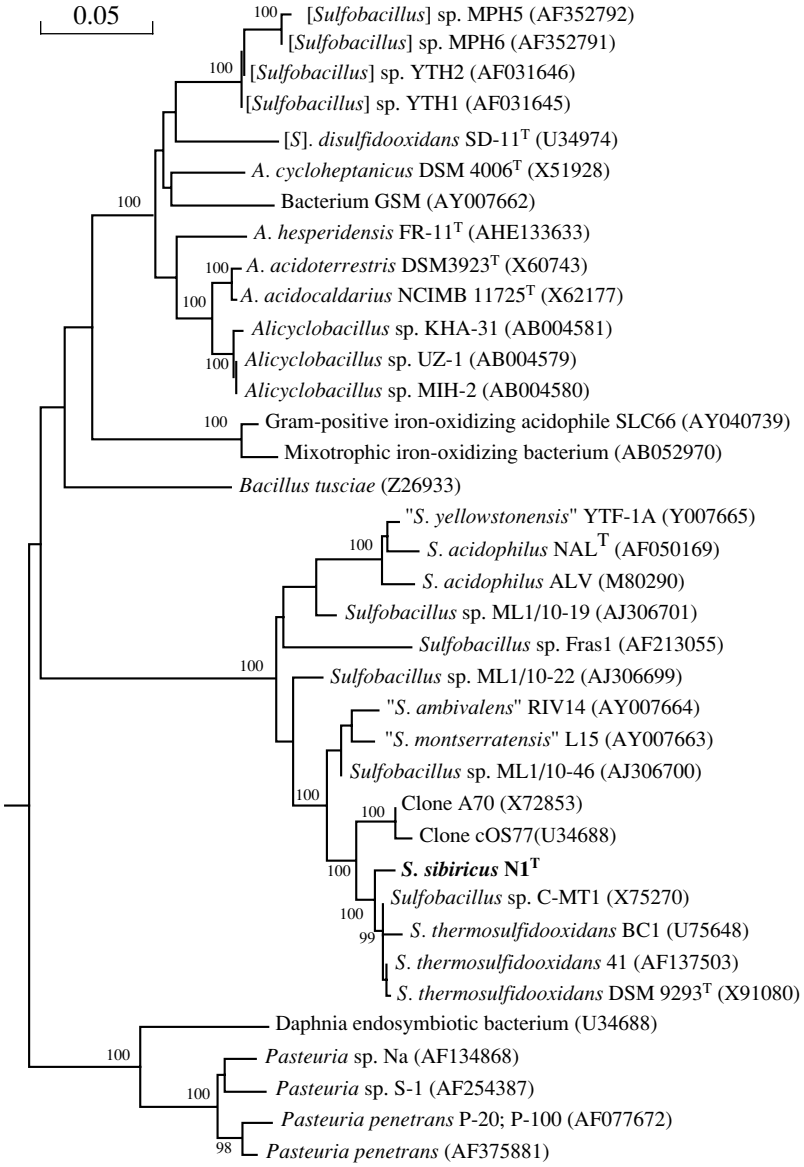

Fig. 4. Phylogenetic tree of representatives of the *Sulfobacillus–Alicyclobacillus* group showing the position of strain N1 and constructed based on a comparison of 16S rRNA gene sequences. The scale bar corresponds to 5 nucleotide replacements per 100 nucleotides. The figures at the branching points give the statistical confidence of the branching order determined by bootstrap analysis (only bootstrap values above 95 are assumed significant).

actual sulfide mineral used in the test. The oxidation of sulfide minerals was accompanied by the formation of sulfates (1–3 g/l) and H^+ and, in the experiment involving pyrite, Fe^{3+} (Fig. 3).

Strain N1 showed autotrophic growth on a modified 9K medium with Fe^{2+} only within two to three passages. No autotrophic growth was observed on a medium with elemental sulfur.

Utilization of organic compounds. Growth of strain N1 on media containing ferrous iron and sodium thiosulfate as an inorganic energy source was observed in the presence of yeast extract, reduced glutathione, glucose, fructose, sucrose, sorbitol, or alanine.

In the absence of inorganic sources of energy, strain N1 failed to grow on any of the organic compounds listed in the Materials and Methods section except for yeast extract. On yeast extract, it grew only in the course of seven to eight passages. On the rest of the organic compounds tested, strain N1 failed to grow even in the first passage.

Fatty-acid composition of lipids. The fatty-acid composition of strain N1 cells grown on a modified 9K medium containing 0.02% yeast extract and 1 mM sodium thiosulfate is presented in the table.

Results of DNA analysis. The G+C content of the genomic DNA of strain N1 was 48.2 mol %. The

homology of the genome with the type strain of *S. thermosulfidooxidans* was 42%, and with the type strains of the species *S. acidophilus* and *S. disulfidooxidans*, it was 10 and 19% respectively. The genome size of strain N1 was 3.5×10^9 Da. The *Not*I restriction profiles determined by pulsed-field gel electrophoresis after growth on a medium with ferrous iron (at 55 and 30°C) and on a medium with S^0 were identical to those previously obtained immediately after the isolation of the strain from the pilot plant and laboratory apparatus [9], and for this reason are not shown here.

Results of 16S rRNA gene sequence analysis. A comparative study of the 16S rRNA gene sequence showed a close similarity (97.9–98.6%) between strain N1 sequences and those of strains of *S. thermosulfidooxidans*, with which strain N1 makes up a single cluster on the phylogenetic tree with the highest possible bootstrap-analysis value (100) (Fig. 4). The gene sequence similarity with *S. acidophilus*, which is another species of the same genus, was notably lower (89.5–89.7%). Our analysis also covered several other strains of the genus *Sulfobacillus* belonging to different phylogenetic groups within this genus, and the similarity between their 16S rRNA sequences with that between strain N1 was found to range between 87.1 and 95.8%.

DISCUSSION

Our study showed that both morphologically and physiologically strain N1 is close to bacteria of the genus *Sulfobacillus* and is a moderately thermophilic, acidophilic, gram-positive spore-forming rod, utilizing ferrous iron, sulfur, and sulfide minerals as the source of energy. However, it differs from other species of the genus *Sulfobacillus* described up to date in having a higher optimum temperature for growth equal to 55°C (which is 50°C for *S. thermosulfidooxidans* and *S. acidophilus* and 35°C for *S. disulfidooxidans*). Strain no. 6 of the genus *Sulfobacillus* was reported to have an optimal temperature for growth of 58°C; according to the results of 16S rRNA sequencing, it does not belong to any of the previously described species [19]. However, the new species has not so far been described. According to the author of this paper, this strain is closest to bacteria of the species *S. acidophilus* (97% similarity of 16S rRNA sequences). Therefore, strain no. 6 is phylogenetically distant from our strain N1.

Morphologically, strain N1 differs from other representatives of the genus *Sulfobacillus* in that it has a somewhat greater width of cells (0.6–0.8 μm in *S. thermosulfidooxidans*, 0.5–0.8 μm in *S. acidophilus*, and 0.3–0.5 μm in *S. disulfidooxidans*).

Physiologically, strain N1 differs from the previously described species of *Sulfobacillus* in the instability of its growth on yeast extract and its failure to grow on the rest of the organic substrates tested in this study. Strain N1 also differs from *S. thermosulfidooxidans* in the instability of its growth on media with 1 mM thiosulfate or tetrathionate at pH values that are optimal for the growth of sulfobacilli and in its inability to grow on elemental sulfur at pH values lower than 2. The capacity of the new strain to oxidize reduced sulfur compounds is, apparently, not very strong. Strain N1 differs from strain 41 of *S. thermosulfidooxidans* subsp. *asporogenes* in exhibiting a spore-forming ability, which, however, as in strain 1269, is not stable. A summary table of morphological and physiological characteristics of bacteria of the genus *Sulfobacillus* can be found elsewhere [7].

It should be noted that there is no consensus in the literature as to the ability of the species *S. thermosulfidooxidans* to grow organotrophically. According to Norris *et al.*, all bacteria of the genus *Sulfobacillus* can grow on a medium with yeast extract as a sole source of energy, provided its concentration in the medium is 0.025% [2]. However, according to Karavaiko *et al.* [7], strains 1269 and 41 could grow on organic substrates only in the course of several passages. Our unpublished data show that growth of strain 1269 at a yeast extract concentration of 0.02% is limited to four to five passages, whereas when the concentration of yeast extract is 0.05% and under daily passages, it can grow infinitely long. Under these conditions, the culture contains many swollen cells, apparently attesting to unfavorable conditions for bacterial growth caused by medium deficiency in the energy source, as in our experiments described elsewhere [8].

Unlike strains 1269 and 41, the fatty-acid composition of which was described elsewhere [20], strain N1 grown on the given medium did not contain ω-cyclohexane fatty acids. However, such acids were also not detected in strain 1269 of *S. thermosulfidooxidans* grown on an iron-containing Manning's medium [21]. All strains of *S. thermosulfidooxidans* were found to contain maximum quantities of ω-cyclohexane fatty acids when grown heterotrophically, and under such conditions strain N1 failed to show stable growth. At the same time, the fatty-acid profile of strain N1 included five types of fatty acids that were never detected in previously described strains of *Sulfobacillus* on any of the media used in [20]. Two of them—2-hydroxy-*iso*hexadecanoic (2*hi*16 : 0) and *iso*nonadecanoic (*i*19 : 0) acids—can be considered specific and can be used for chemodifferentiation of strain N1.

As shown previously, the *Not*I restriction profile of strain N1 determined by pulsed-field gel electrophoresis differed significantly from those of strains 1269 and 41 [9]. In this work, it was established, in addition, that the *Not*I restriction profile of strain N1 did not change when iron as the energy substrate was replaced by sulfur. The structure of the chromosomal DNA in strain N1 was also found not to be affected by the cultivation temperature (55 and 30°C). This makes possible identification and monitoring of the new strain in the technological processes. The stability of the chromosomal

DNA with changed energy source was also noted in *S. thermosulfidooxidans* 1269 [9]. The coefficient of the fraction of conservative bands, calculated from the restriction profiles for strains 1269 and N1, was as low as 0.3. The procedure to calculate and analyze this coefficient for *Sulfobacillus* bacteria was described elsewhere [9]. For bacteria of the same species, this coefficient should not be lower than 0.4. Our calculation indicates, therefore, that the type strain of *S. thermosulfidooxidans* and strain N1 belong to different species.

Certain physiological features of the new strain, the degree of homology of its DNA with the DNA of the type strains of *S. thermosulfidooxidans* (42%), *S. acidophilus* (10%), and *S. disulfidooxidans* (19%), and the comparatively low phylogenetic affinity of strain N1 and new strains of the genus *Sulfobacillus* isolated from different environments and supposedly belonging to new species [19], warrant the assignment of strain N1 to a new species of the genus *Sulfobacillus*, *S. sibiricus*.

Description of *Sulfobacillus sibiricus* sp. nov.

Sulfobacillus sibiricus (si.bi.ri.cus) sp. nov. The species name derives from the name of the region where the Nezhdaninskoe deposit of gold-arsenic ores is situated.

Cells are aerobic gram-positive nonmotile rods (0.7–1.1 × 1.0–3.0 μm) tending to form chains of two to five cells in the process of growth. Spherical subterminal endospores are formed.

The temperature optimum for growth is 55°C; the temperature range for growth is 17–60°C. The pH range for growth on optimal iron-containing media is 1.1–2.6, with an optimum at 2. Stable growth is possible only with the simultaneous utilization of inorganic energy sources (ferrous iron, elemental sulfur, sulfide minerals) and organic carbon sources (yeast extract, reduced glutathione, sucrose, glutamic acid, etc.). The G+C content of the chromosomal DNA is 48.2 mol %. The type strain is N1.

The type strain of the species *S. sibiricus* $N1^T$ was deposited with the All-Russia Collection of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow oblast (collection number B-2280).

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