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EXPERIMENTAL ARTICLES

# *Singulispaera mucilagenosa* sp. nov., a Novel Acid-Tolerant Representative of the Order *Planctomycetales*

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**Abstract**—Two novel strains of budding bacteria, Z-0071$^T$ and Z-0072, were isolated from dystrophic humified waters formed by xylotrophic fungi in the course of spruce wood degradation. The cells of both strains are coccoid (0.95–1.80 μm), nonmotile, single or arranged in pairs. The cells have a complex system of intracellular membranes and are covered with fimbriae and surrounded by a mucous capsule up to 0.3 μm thick. Both strains are aerobic organoheterotrophic, mesophilic, and acid-tolerant microorganisms that are able to grow under microaerobic conditions. They utilize N-acetyl-glucosamine, carbohydrates, and lactate as growth substrates. The strains grow in a pH range of 4.0–7.5 with an optimum at 6.0–6.5. The temperature range for growth is 4–30°C, with an optimum at 25–28°C. Strains Z-0071$^T$ and Z-0072, inhabitants of dystrophic low-mineral waters, are NaCl-sensitive: the NaCl content in the media above 0.5 g/l inhibited growth. The main fatty acids of strains Z-0071$^T$ and Z-0072 are $C_{16:0}$, $C_{18:1\omega9c}$, and $C_{18:2\omega9c,\ 12c}$. The DNA G + C base content is 51.2–51.7 mol %. The sequences of the 16S rRNA gene fragments (1310 bp) of strains Z-0071$^T$ and Z-0072 were found to be identical. The obtained sequences showed a 94.3% similarity with the sequences of the type strain of the most closely related species *Singulisphaera acidiphila* MOB10≅T. The phenotypic and phylogenetic properties of strains Z-0071$^T$ and Z-0072 support classification of these strains within the genus *Singulisphaera* as a new species *Singulisphaera mucilagenosa* sp. nov., with the type strain Z-0071$^T$ (VKM B-2626).

*Keywords*: xylotrophic community, planctomycetes, wood decomposition, low-mineral waters, acid-tolerant bacteria.

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Planctomycetes are a widespread group of microorganisms inhabiting natural ecosystems [1–3]. Planctomycetes isolated in pure cultures comprise the family *Planctomycetaceae*, order *Planctomycetales*, which currently consists of nine genera—*Planctomyces, Pirellula, Gemmata, Isosphaeara, Schlesneria, Singulisphaera, Zavarzinella, Rhodopirellula*, and *Blastopirellula* [1, 3–6]. Molecular biological studies have confirmed the results of previous visual observations and revealed planctomycetes in terrestrial, marine, and freshwater ecosystems, as well as in industrial areas, and showed the relationships between these bacteria and other organisms [1, 7]. However, their ecological role is poorly understood, since the physiological and biochemical properties of planctomycetes in cultures have not yet been studied adequately.

The goal of the present work was to study strains Z-0071$^T$ and Z-0072, novel representatives of the order *Planctomycetales*, as well as to determine their phylogenetic position.

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## MATERIALS AND METHODS

**Subject of study and source of isolation.** Strains Z-0071$^T$ and Z-0072 were isolated from dystrophic low-humified acidic (pH 4.3) low-mineral water (conductivity 140 μS) from a microlysimeter in which spruce wood was degraded by a xylotrophic fungal community.

**Composition of the media and cultivation conditions.** Strains Z-0071$^T$ and Z-0072 were isolated on low-mineral PC medium containing the following: Hutner's basal salt solution, 5 ml/l [1]; fungal culture liquid as a carbon and energy source, 10 ml/l; and yeast extract as a growth factor, 0.05 g/l [8]. The pure cultures were maintained in liquid mineral MC medium containing the following: Hutner's basal salt solution, 20 ml/l [1] (pH 6.0); sucrose as a substrate, 1.0 g/l; and yeast extract as a growth factor, 0.05 g/l [8].

**Microscopic investigations.** Cell morphology was studied under a light microscope with a phase-contrast device (Amplival, Germany) and by electron microscopy (JEM 100C, Japan) of negatively stained prepa-

rations and ultrathin sections. The cells were stained with 1% uranyl acetate. To obtain ultrathin sections, the cells were prefixed with glutaraldehyde with subsequent fixation with osmic acid in cacodylate buffer and embedded in an epoxy resin. Ultrathin sections were obtained with an LKB ultramicrotome, stained with lead citrate, and additionally stained with a 3% aqueous solution of uranyl acetate.

**Physiological properties of strains Z-0071$^T$ and Z-0072.** The range of substrates utilized by the novel strains as carbon sources was determined on liquid MC medium. N-acetyl-glucosamine, sugars (arabinose, cellulose, galactose, glucose, fructose, lactose, maltose, mannose, raffinose, starch, sucrose, xylan, and xylose,), alcohols (glycerol, mannitol, and sorbitol), salts of organic acids (acetate, benzoate, butyrate, citrate, formate, fumarate, malate, oxalate, oxaloacetate, propionate, pyruvate, and succinate), primary alcohols (ethanol and methanol), amino acids (aspartate, cysteine, glutamate, leucine, and methylalanine), and methylamines were tested as carbon and energy sources. The tested substrates were added to a concentration of 1.0 g/l.

The respiration rate during growth on cellulose was determined from changes in the $CO_2$ concentration in the gas phase of the experimental vials using an INFRALYT 4 infrared gas analyzer (GUNKALOR–DESAU, Germany). The peak value of the $CO_2$ concentration in the samples was compared with that of the standard gas. The rate of $CO_2$ production (respiration rate) was expressed in mg/(l h) and calculated from the sample volume.

The capacity of the isolates for oligotrophic growth was studied using sucrose (0.2–40.0 g/l) as a growth substrate. The growth was assessed nephelometrically by the optical density ($OD_{600}$) of the cell suspension measured on a UNICO 2100 spectrophotometer at 28°C for 14 days.

The growth rate of the strains within a pH range of pH 3.0–8.0 was determined by supplementing the medium with 0.05 M solutions of $Na_2HPO_4$ and $KH_2PO_4$. Growth within the pH range of 3.0–4.8 was determined in the medium acidified with 0.1 N HCl to the required pH level, which was determined potentiometrically using an Expert 001 pH/ion meter (Russia).

Growth of strains Z-0071$^T$ and Z-0072 was investigated within a temperature range of 2–37°C.

The dependence of growth on NaCl concentrations was determined in media supplemented with NaCl (0.02–1.5%).

The temperature and pH optima for growth of strains Z-0071$^T$ and Z-0072, as well as the dependence of growth on NaCl concentration in the media were determined with sucrose as a substrate.

The capacity for lithoautotrophic growth was assessed by measuring optical density ($OD_{600}$) of the cell suspension and by monitoring hydrogen utilization during cultivation of strains Z-0071$^T$ and Z-0072 in liquid medium with the gas phase containing $H_2$, $O_2$, and $CO_2$ (7 : 2 : 1). The hydrogen concentration was measured on an LKhM-80 gas chromatograph (Russia) with a katharometer detector. The separation was carried out on a column packed with a 5A molecular sieve.

The ability of the cultures to grow under microaerobic conditions was tested on a semisolid agar medium (1%) [9].

The ability of the culture to grow with various nitrogen sources was tested in liquid mineral medium containing the following (g/l): $KH_2PO_4$, 0.45; $K_2HPO_4$, 6.84; $MgSO_4$, 0.30; and sucrose. The following nitrogen sources (0.05%) were tested: ammonium sulfate, potassium nitrate, potassium nitrite, urea, methylamine, trimethylamine, N-acetyl-glucosamine, yeast extract, and peptone, as well as amino acids (alanine, glutamate, leucine, serine, threonine, tryptophan and tyrosine).

The ability of the culture to grow on low-mineralized media was tested on media with various contents of inorganic salts on the basis of their conductivity. During the experiments, MC medium was diluted in the ratios of 1 : 2; 1 : 4; and 1 : 10, which correlated to the conductivity values of 1760, 779, 410, and 145 μS.

The sensitivity of strains Z-0071$^T$ and Z-0072 to antibiotics (lincomycin, 10 μg; novobiocin, 30 μg; ampicillin, 10 μg; chloramphenicol, 30 μg; neomycin, 10 μg; gentamycin, 10 μg; kanamycin, 30 μg; and streptomycin, 10 μg) was determined by the diameter of growth inhibition zones surrounding antibiotic discs (Oxoid) on the agar surface.

Catalase activity was assayed by monitoring the formation of gas bubbles on addition of a 3% hydrogen peroxide solution to the cells; the presence of oxidase was detected by changes in the colony pigmentation when the reactant REF-55635 was applied.

**Fatty acid composition** of the lipids of both strains was analyzed on a Sherlock gas chromatograph (Microbial Identification System, MIDI Inc., Newark, United States) according to the protocol described in [10]. The separated fatty acids were identified using an Agilent Technologies AT-5971 SMART mass spectrometer.

**Quinone composition** was determined after their extraction from the cells. The quinones were extracted, purified, and analyzed using an LCQ ADVANTAGE MAX mass spectrometer (Finnigan, Germany) according to the protocol described in [11].

**Molecular genetic analysis.** DNA isolation and purification, as well as determination of the DNA G + C base content, were performed as described earlier [12].

Determination of the nucleotide sequences of the 16S rRNA gene of strains Z-0071$^T$ and Z-0072 was performed as follows. DNA was extracted by the phe-

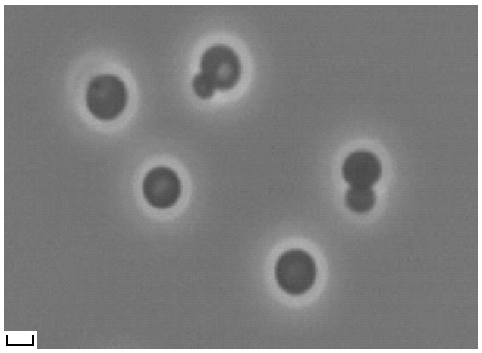

**Fig. 1.** Phase contrast microphotograph of the cells of strain Z-0071$^T$ of *Singulisphaera mucilaginosa* sp. nov. (10-day culture). Scale bar, 0.5 μm.

nol method [14]. PCR amplification of the 16S rRNA gene was carried out using the universal eubacterial primers 27f and 1492r on a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, United States). Sequencing of amplification products was performed on a CEQ2000 XL automatic sequencer (Beckman Coulter, United States) according to the manufacturer's instructions. For identification of strains closely related to strain Z-0071$^T$ the GenBank database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) was used. The phylogenetic tree was constructed by the methods implemented in the TREECON software package [14]. The obtained 16S rRNA gene sequence of strain Z-0071$^T$ was deposited in the GenBank under the accession number HM748856.

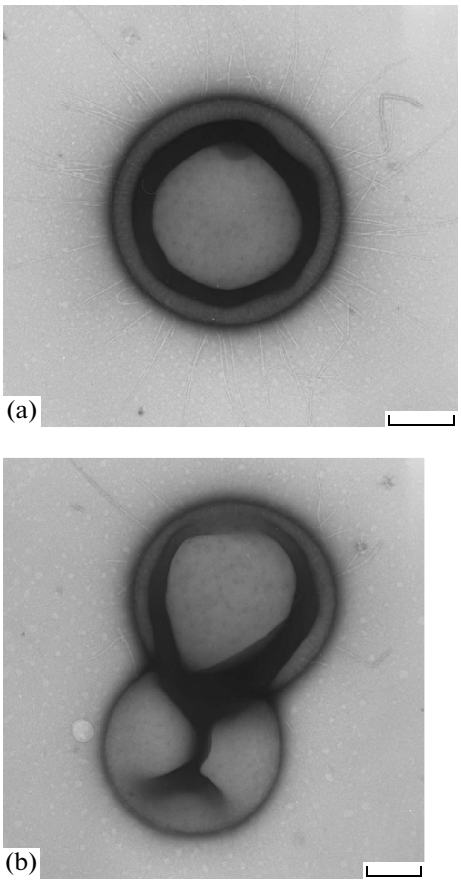


**Fig. 2.** Electron microphotographs of the cells of strain Z-0071$^T$. The preparations were stained with 1% uranyl acetate. Scale bar, 0.5 μm.

## RESULTS

**Cell morphology and ultrastructure.** When grown under optimal cultivation conditions on the medium with sucrose (1.0 g/l) as a substrate, strains Z-0071$^T$ and Z-0072 were nonmotile cocci (0.95–1.8 μm) (Fig. 1). The cells were covered with fimbriae and surrounded by a mucous capsule up to 0.1–0.3 μm thick. Craterlike structures were detected on the cell surfaces. The cells did not produce resting forms and reproduced by budding. The cells in the culture were single or arranged in pairs; no cell aggregates were detected. Ultrathin sections revealed a cell structure typical of planctomycetes, such as bacteria of the genus *Isosphaera* [3, 15]. The cell wall was ~10 nm thick and consisted of two electron-dense layers separated by an electron-transparent layer (Fig. 3). The outer membrane of the cell wall was covered by a thick mucous layer. The surface of the cell wall contained recesses adjacent to the paryphoplasm were detected, a structure characteristic of planctomycetes (Fig. 3a). The inner cytoplasmic membrane divided the cell, forming intracellular compartments in which numerous ribosome-like particles and the nucleoid were detected (Fig. 3).

**Cultural properties.** On agarized MC medium, both strains produced slimy, smooth, rounded, convex, opaque, milky-yellow colonies (up to 4 mm in diameter) with even edges and dense consistency after incubation at 28°C for 3–4 weeks. During growth in liquid medium, the cultures produced a slimy sediment.

**Physiological properties.** Bacteria of strains Z-0071$^T$ and Z-0072 were aerobic, mesophilic, and acid-tolerant heterotrophs. The strains were able to grow under microaerobic conditions. No growth was detected under anaerobic conditions. The strains grew within a temperature range of 4–30°C, with an optimum at 25–28°C. The pH range for growth was 4.0–7.5 with an optimum at 6.0–6.5 (Table 1).

The isolates were NaCl-sensitive: the NaCl content in the media above 0.5 g/l inhibited growth. The NaCl concentrations of 0.3 and 0.2% induced 50% growth inhibition of strains Z-0071$^T$ and Z-0072, respectively.

The strains utilized N-acetyl-glucosamine, arabinose, glucose, fructose, lactate, lactose, maltose, mannose, sucrose, trehalose, and xylose as carbon and energy sources. Both strains were able to hydrolyze xylan, but not pectin or dextrin; the strains varied in

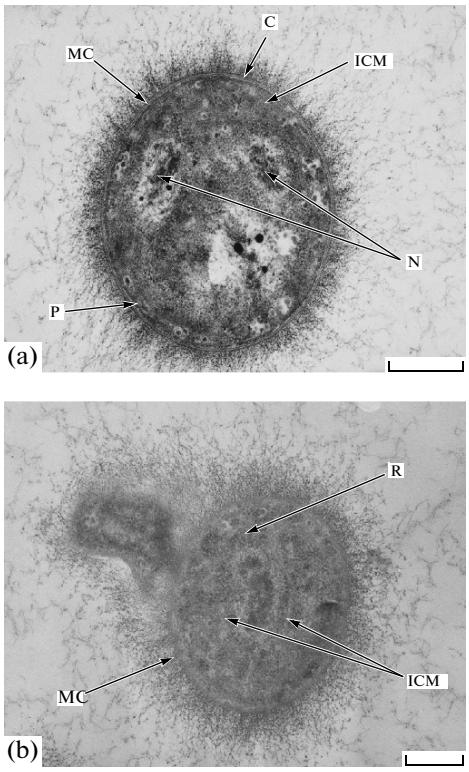


**Fig. 3.** Electron microphotograph of ultrathin sections of the cells of strain Z-0071$^T$. CW, cell wall; ICM, inner cytoplasmic membrane; P, paryphoplasm; N, nucleoid; R, ribosomes; MC, mucous capsule; C, craterlike structures. Scale bar, 0.5 μm.

their ability to hydrolyze cellulose. Strain Z-0071$^T$ was able to hydrolyze cellulose. Unlike strain Z-0071$^T$, strain Z-0072 was able to hydrolyze starch. Both strains did not utilize acetate, aspartate, benzoate, butyrate, citrate, cysteine, ethanol, formate, fumarate, gluconate, glutamate, glycerol, leucine, malate, methanol, methylamine, oxalate, propionate, pyruvate, sorbitol, succinate, and trimethylamine as growth substrates.

The isolates grew at substrate concentrations ranging from 1.0 to 40.0 g/l and at a conductivity of the medium from 145 to 1760 μS.

No growth was detected on nitrogen-free media. The novel strains utilized ammonium sulfate, leucine, glutamate, tyrosine, threonine, serine, alanine, yeast extract, peptone, and N-acetyl-glucosamine as nitrogen sources. The strains were not capable of utilizing nitrate, nitrite, urea, tryptophan, and trimethylamines.

No growth was detected in the absence of yeast extract as a growth factor; vitamins stimulated growth.

The organisms were oxidase- and catalase-positive.

**Antibiotics.** Strains Z-0071$^T$ and Z-0072 were sensitive to chloramphenicol, lincomycin, ampicillin, streptomycin, neomycin, kanamycin, and gentamycin; they were resistant to novobiocin (Table 1).

**Fatty acid composition.** Table 2 shows the fatty acid composition of the lipids of the cell membranes of Z-0071$^T$ and Z-0072 (% of total fatty acids). The main fatty acids of the isolates were $C_{16:0}$, $C_{18:1\omega9c}$, and $C_{18:2\omega6c,\ 12c}$.

**Quinone composition.** Menaquinone MQ-6 was found in the cells of strains Z-0071$^T$ and Z-0072.

**Molecular genetic analysis.** The DNA G + C base content of strains Z-0071$^T$ and Z-0072 was 51.2 and 51.7 mol %, respectively.

The sequences of the 16S rRNA gene fragments (1310 bp) were determined for strains Z-0071$^T$ and Z-0072 and were found to be identical. The levels of 16S rRNA similarity between the studied strains and the phylogenetically close microorganisms *Singulisphaera acidiphila* MOB10$^T$, *"Nostocoida limicola"* III strain Ben223, and *Isosphaera pallid* DSM 9630$^T$ (Fig. 4) are 94.3, 93.4, and 89.6%, respectively.

## DISCUSSION

Comparative phylogenetic analysis of the 16S rRNA gene sequences demonstrated that strains Z-0071$^T$ and Z-0072 belonged to the order *Planctomycetales*. The highest similarity (94.3%) was observed with the type species of the genus *Singulisphaera, Singulisphaera acidiphila* MOB 10, isolated from acidic *Sphagnum* peat bogs of northern Russia [3]. The phenotypic properties of the studied strains revealed a number of characteristic traits of these bacteria. Their cells were smaller than those of *S. acidiphila* MOB10$^T$ (Table 1). The cells were covered with fimbriae and surrounded by a thick layer of mucus.

The strains were isolated from acidic, oligotrophic, dystrophic water and were found to be well adapted to these conditions. They were acid-tolerant microorganisms and are able to grow at the lowest pH level of 4.0; however, their growth optimum (pH 6.0–6.5) was higher than that of *S. acidiphila* MOB10$^T$. Both strains were obligate heterotrophs and utilized N-acetyl-glucosamine, carbohydrates, and lactate as carbon and energy sources. Unlike *S. acidiphila* MOB10$^T$, the studied strains were not capable of utilizing pyruvate and pectin as substrates; however, strain Z-0071$^T$ was able to utilize cellulose (Fig. 5; Table 1). Strain Z-0072 was able to hydrolyze starch. Like *S. acidiphila* MOB10$^T$, both microorganisms utilized ammonium, yeast extract, peptone, and N-acetyl-glucosamine; unlike strain MOB 10$^T$, they were capable of utilizing serine, leucine, and tyrosine, but not tryptophan (Table 1). The novel strains required yeast extract for growth.

The lipids of strains Z-0071$^T$ and Z-0072, like those of *S. acidiphila* MOB10$^T$, were characterized by predominance of the following fatty acids: $C_{16:0}$,

$C_{18:1\omega9c}$, and $C_{18:2\omega6c,\ 12c}$ [3]. However, the cells of both novel strains contained *iso*-$C_{16:0}$, $C_{17:1}$, and 10-OH-$C_{18:0}$ as minor components that were not detected in the fatty acid profile of strain MOB10$^T$. Like *S. acidiphila* MOB10$^T$, the studied strains contained menaquinone MQ-6 [1, 3, 5].

The characteristic trait of strains Z-0071$^T$ and Z-0072 that distinguished them from *S. acidiphila* MOB10$^T$ was their sensitivity to antibiotics (chloramphenicol, lincomycin, ampicillin, streptomycin, and kanamycin) (Table 1).

The DNA G + C base contents of strains Z-0071$^T$ and Z-0072 were 51.2 and 51.7 mol %, respectively, which differ significantly from the DNA G + C base content of *S. acidiphila* MOB10$^T$ (Table 1).

Although the studied microorganisms were isolated from low-mineral waters, both strains were able to grow on media with various concentrations of inorganic salts within a wide conductivity range (145–1760 μS), as well as in the presence of both high and low substrate concentrations, which demonstrates high adaptive capabilities of these microorganisms.

Hence, despite the fact that the morphological and phenotypic properties of the strains isolated from dystrophic acidic water of a microlysimeter differed from those of the phylogenetically close species *S. acidiphila* MOB10$^T$, they coincided with those of representatives of the genus *Singulisphaera*. These properties allowed us to classify strains Z-0071$^T$ and Z-0072 within the genus *Singulisphaera* as a novel species, *Singulisphaera mucilagenosa* sp. nov. with strain Z-0071$^T$ as the type strain.

*Description of Singulisphaera mucilagenosa sp. nov.*

*Mu.ci.la.'ge no.sa*, L. n. fem. *mucilage*, mucus; L. fern. adj. *genosa*, generating; N. L. fem. adj. *mucilagenosa*, mucus-generating .

The cells are gram-negative, coccoid (0.95–1.80 μm), nonmotile, covered with fimbriae and mucus, single or in pairs. The bacterium reproduces by budding.

The colonies are slimy, smooth, rounded, convex, opaque, milky-yellow, with even edges and dense consistency, up to 4 mm in diameter.

The organism grows in a pH range of 4.0–7.5 with an optimum at 6.0–6.5. The bacterium is a mesophile growing within a temperature range from 4 to 30°C with a growth optimum at 25–28°C. Active growth occurs at NaCl concentrations not exceeding 0.5 g/l.

The organism is an aerobe capable of growth under microaerobic conditions. N-acetyl-glucosamine, arabinose, fructose, glucose, lactate, lactose, mannose, maltose, sucrose, trehalose, and xylose are utilized as carbon and energy sources. The organism does not grow on organic acids (acetate, citrate, gluconate, malate, oxalate, and succinate). The organism is not

**Table 1.** Phenotypic properties of strains Z-0071$^T$, Z-0072, and *Singulisphaera acidiphila* MOB 10$^T$

| Phenotypic properties | Z-0071$^T$ | Z-0072 | *S. acidiphila* MOB10$^T$ |
|---|---|---|---|
| Cell size, mm | 0.95–1.8 | 0.95–1.8 | 1.6–2.5 |
| Resistance to NaCl (%) | <0.5 | <0.5 | <0.4 |
| pH range for growth | 4–7.5 | 4–7.5 | 4.2–7.2 |
| $pH_{optimum}$ | 6.0–6.5 | 6.0–6.5 | 5.1–6.2 |
| Temperature range for growth (°C) | 4–30 | 4–30 | 4–33 |
| Carbon sources: | | | |
| lactate | + | + | + |
| pyruvate | – | – | + |
| xylan | + | + | + |
| cellulose | + | + | – |
| starch | – | + | – |
| pectin | – | – | + |
| dextrin | – | – | N/D |
| inulin | – | – | N/D |
| gelatin | N/D | N/D | + |
| Nitrogen sources: | | | |
| leucine | + | + | – |
| serine | + | + | – |
| tryptophan | – | – | + |
| tyrosine | + | + | – |
| Requirement for yeast extract | + | + | – |
| Sensitivity to antibiotics: | | | |
| kanamycin | + | + | – |
| ampicillin | + | + | – |
| streptomycin | + | + | – |
| chloramphenicol | + | + | – |
| lincomycin | + | + | – |
| novobiocin | – | – | – |
| neomycin | + | + | + |
| gentamycin | + | + | + |
| DNA G + C base content, mol % | 51.2 | 51.7 | 59.9 |

**Table 2.** Fatty acid composition of strains Z-0071$^T$, Z-0072, and *Singulisphaera acidiphila* MOB10$^T$ (percent of total fatty acids)

| Fatty acid | Designation | 1 | 2 | 3 |
|---|---|---|---|---|
| | $C_{14:0}$ | 8.60 | 10.69 | 1.06 |
| | $C_{15:0}$ | 0.89 | 0.828 | 0.23 |
| | *iso*-$C_{16:0}$ | 0.79 | 0.996 | – |
| 9-hexadecenoic | $C_{16:1w7ñ}$ | 0.72 | 0.583 | 1.65 |
| Hexadecanoic | $C_{16:0}$ | 24.58 | 27.059 | 31.12 |
| | $C_{17:1}$ | 0.90 | 0.623 | – |
| Heptadecanoic | $C_{17:0}$ | 0.56 | 0.378 | 0.13 |
| | cyclo-$C_{17:0}$ | – | – | 0.43 |
| Octadecadienoic | $C_{18:2}$ | 15.21 | 11.31 | 13.79 |
| 9-octadecenoic | $C_{18:1w9}$ | 40.23 | 38.288 | 46.73 |
| 11-octadecenoic | $C_{18:1w7ñ}$ | 1.29 | 1.078 | 0.79 |
| Octadecanoic | $C_{18:0}$ | 5.33 | 5.058 | 3.51 |
| 10-hydroxy-octadecanoic | 10-h-$C_{18:0}$ | 0.90 | 3.109 | – |

Note: Designations: 1, strain Z-0071$^T$; 2, strain Z-0072; 3, strain *S. acidiphila* MOB10$^T$ [4]; –, not detected.

able to utilize $C_1$ compounds and is not capable of chemolithoautotrophic growth. The bacterium hydrolyzes polysaccharides (xylan and cellulose), but not pectin or dextrin. Yeast extract is required for growth.

The organism is catalase- and oxidase-positive.

The main fatty acids are represented by $C_{16:0}$, $C_{18:1\omega9c}$, and $C_{18:2\omega6c,\ 12c}$.

Menaquinone MQ-6 is the main quinone.

The DNA G + C base content is 51.2 mol %. The type strain is resistant to novobiocin and sensitive to kanamycin, streptomycin, neomycin, chloramphenicol, lincomycin, ampicillin, and gentamycin.

The type strain is Z-0071$^T$ (VKM B-2626).

The organism was isolated from acidic (pH 4.3), low-mineral, low-mineral waters formed by the xylotrophic fungal community grown on decaying spruce wood.

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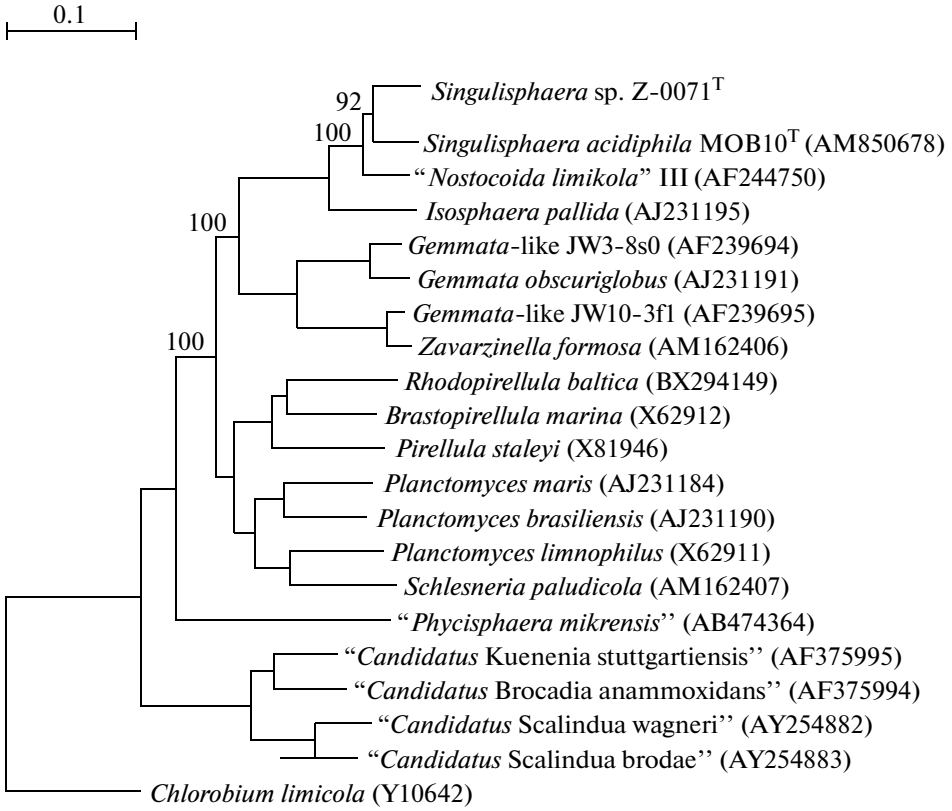


**Fig. 4.** Phylogenetic position of strain Z-0071$^T$ among the type and reference strains and nonculturable members of the family *Planctomycetaceae*. The numerals show the results of bootstrap analysis.

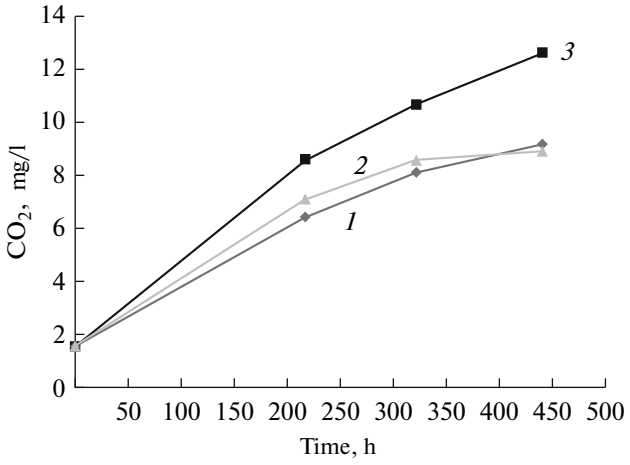


**Fig. 5.** Dynamics of the $CO_2$ concentration in the gas phase of the experimental vials during growth of strains Z-0071$^T$ and Z-0072 on cellulose: *1*, control; *2*, Z-0072; *3*, Z-0071$^T$.

Personnel of Innovative Russia" (state contract with Rosnauka no. 02.740.11.0023).

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