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EXPERIMENTAL ARTICLES

Two New Species of Microaerophilic Sulfur Spirilla, *Spirillum winogradskii* sp. nov. and *Spirillum kriegii* sp. nov.

D. A. Podkopaeva[a], M. Yu. Grabovich[b], G. A. Dubinina[a,1], A. M. Lysenko[a], T. P. Tourova[a], and T. V. Kolganova[c]

[a] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*
[b] *Voronezh State University, Universitetskaya pl. 1, Voronezh, 394893 Russia*
[c] *Bioengineering Center, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*

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Abstract—New microaerophilic sulfur-oxidizing spirilla were isolated from hydrogen sulfide sludge of wastewater treatment plants. Strains D-427 and D-430 have spiral cells that are highly motile due to bipolar flagellum bundles covered with mucous sheaths. Under a phase-contrast microscope, these bundles are visible as single polar flagella. Spheroplasts are formed in the stationary growth phase. Both strains are obligate organotrophs able to oxidize a number of reduced sulfur compounds. The oxidation of sulfide and polysulfide leads to the formation of intracellular globules of elemental sulfur; thiosulfate oxidation results in tetrathionate accumulation in the medium. The cells are unable to utilize reduced sulfur compounds in the energy metabolism; their oxidation is caused by a chemical interaction with H_2O_2 and O_2^-, synthesized in the electron transport chain. Both strains are obligate microaerophiles with an optimal oxygen concentration in the gas phase of 2 and 0.8% for strains D-427 and D-430, respectively. The strains utilize a limited number of organic acids as growth substrates, mainly tricarboxylic-acid-cycle intermediates. The DNA G+C content is 38.0 mol % (T_m) for strain D-427 and 38.9 mol % for strain D-430. Phylogenetic analysis, based on the comparison of 16S rRNA gene sequences, revealed that the new isolates of sulfur spirilla are the most closely related to *Spirillum volutans*, the type species of the genus (97.4% similarity). They were assigned to the genus *Spirillum* within the class *Betaproteobacteria* as two new species, *S. winogradskii* sp. nov. (D-427^T = DSM 12756^T) and *S. kriegii* sp. nov. (B-430^T = VKM B-2372^T). The emended description of the genus *Spirillum* is provided.

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Key words: sulfur spirilla, microaerophily, genus *Spirillum.*

Sulfur spirilla are widespread in natural, technogenic, and anthropogenic water bodies and form a relatively big subgroup within the morphologically peculiar group of colorless sulfur bacteria. They are present in the bacterial communities of water and sediments and often form mats or dense populations on the upper boundary of the redox zone [1–4]. According to the ecological observations, sulfur spirilla exist in environments where there are low concentrations of both oxygen and hydrogen sulfide; microaerophilic organisms can thrive in this microaerobic niche. However, there is no data that concerns microaerophilic representatives of sulfur spirilla.

Several species of sulfur spirilla belonging to the genus *Thiospira* (Visloukh 1914) and capable of forming intracellular globules of elemental sulfur were described based on a morphological approach in the beginning of the previous century [3]. A number of pure cultures of sulfur spirilla were obtained for the first time only in the 1980s [5, 6]. Pheno- and genotypic research of a number of strains of aerobic sulfur spirilla of the genus *Thiospira* led to their reclassification; they were assigned to the genus *Aquaspirillum* as new species [6]. One more genus of sulfur spirilla, *Titanospirillum*, was described based on electron microscopy of the material from marine sulfur mats and from enrichment cultures [4]. The authors, however, did not obtain the representatives of this genus in pure cultures.

The goal of the present work was characterization of two new microaerophilic representatives of the group of colorless sulfur spirilla.

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

MATERIALS AND METHODS

Source of isolation. The two strains of large, colorless, sulfur spirilla, which were used in the present work, D-427 and D-430, were isolated from samples of hydrogen sulfide sludge from municipal wastewater. Strain D-427 was isolated and partially characterized earlier [6].

Cultivation media and growth conditions. For enrichment cultures, gradient media were used with FeS precipitate added to modified semiliquid MPSS medium (g/l): $(NH_4)_2SO_4$, 1.0; $MgSO_4$, 1.0; $CaCl_2 \cdot \cdot 2H_2O$, 0.03; $FeCl_3 \cdot 6H_2O$, 0.002; $MnSO_4$, 0.002; casein hydrolysate, 1.0; agar (Difco), 1.0; distilled water, 1 l; pH 7.0 [5]. Sterile solutions of sodium succinate (to 1 g/l), trace elements and vitamins [6], and freshly prepared FeS suspension [7] (0.2 ml per 10 ml of medium) were added prior to the inoculation. Incubation was performed in test tubes. For the isolation of sulfur spirilla in pure cultures, an MPSS medium of the same composition with 15 g/l of agar (Difco) was used. To study the effect of aeration conditions on the growth of spirilla, rubber-stoppered 50-ml vials with 10 ml of medium were flushed with filter-sterilized (0.2-μm ultrafilter) argon, and a calculated volume of sterile air was then injected to create the final oxygen concentrations in the gas phase of 0.4, 0.8, 1, 2, 5, 10, and 20%. Cultivation was performed in vials with agitation on a shaker at 28°C.

Cell morphology and ultrastructure. Cell morphology was investigated with the use of an NU-2 phase-contrast microscope (Zeiss, Jena, Germany). Polarized light microscopy was used to identify elemental sulfur by its specific light refraction. The ultrastructure was studied using a JEM-100 (Jeol, Japan) transmission electron microscope at an accelerating voltage of 80 kV. Mucous polysaccharide capsules were revealed by Luft staining with ruthenium red [9]. Whole cells were contrasted with 1% ammonium molybdate. For ultrathin sectioning, cells were fixed with 1% OsO_4; the sections were stained with lead citrate and uranyl acetate.

Physiologo-biochemical tests. The ability to utilize various carbon sources was tested on an MPSS mineral medium [5] of the following composition (g/l): $(NH_4)_2SO_4$, 1.0; $MgSO_4$, 1.0; $CaCl_2 \cdot 2H_2O$, 0.03; and 10% phosphate buffer, 1 ml. The medium was supplemented with carbon sources (1 g/l or, in the case of amino acids, 0.25 g/l). A medium of the same composition without $(NH_4)_2SO_4$ and supplemented with sodium succinate was used to test the utilization of different nitrogen sources, inorganic (1 g/l) or organic (0.25 g/l). The capability to utilize carbon and nitrogen sources was also tested in the same medium with 0.1 g/l yeast extract as a growth factor source. The capacity for anaerobic growth was tested in a semiliquid medium with nitrate, fumarate, sulfate, thiosulfate, or elemental sulfur as alternative electron acceptors. Fermentative growth was assayed under anaerobic conditions on the MPSS mineral medium with 1 g/l of glucose, fructose, sucrose, arabinose, or galactose.

Bacterial growth was determined nephelometrically after seven days on an SF-26 spectrophotometer at $\lambda = 500$ nm.

The catalase activity in cell suspensions was determined according to [8] and spectrophotometrically [10]. The oxidase activity was determined according to [10]. Protein was determined by the Lowry method.

Molecular-genetic techniques. Extraction and purification of DNA from a bacterial biomass were performed according to Marmur [11]. The DNA G+C content was determined using thermal denaturation curves on a Pye Unicam SP 1800 spectrophotometer. The DNA–DNA homology was studied by the optical reassociation method [12].

A universal primer system was used for the polymerase chain reaction and for the subsequent sequencing of the PCR-amplified fragments of the 16S rRNA genes. The PCR reaction mixture of the following composition was used: primers, 25 pmol each; 10× buffer, 2.5 μl; 2 mM dNTP, 2.5 μl; BioTaq polymerase (Dialat, Russia, 5 U/μl), 0.2 μl; template DNA, 50 ng, and H_2O, to 25 μl. The PCR reaction included 30 cycles each of 94°C for 0.5 min, 45°C for 1 min, and 72°C for 1 min and a final polymerization for 7 min. The PCR products were analyzed by electrophoresis in 2% agarose gel at 6 V/cm. The sequencing of PCR products was performed with a Silver Sequencing kit (Promega, United States), according to the recommendations of the manufacturer with slight modifications. Both internal and external primers were used, and sequencing was performed in two directions.

16S rRNA gene sequences were aligned with the corresponding sequences of the most closely related species, using the CLUSTAL.X software package. Unrooted phylogenetic trees were constructed by methods implemented in the TREECONW software package [13].

Deposition of sequences. The 16S rRNA gene sequences of strains D-427 and D-430 were deposited in GenBank under accession nos. AY845251 and AY845252, respectively.

RESULTS

Isolation of pure cultures. The H_2S-containing sediments from the aeration tanks for municipal wastewater treatment were used as inocula for the isolation of sulfur spirilla. Sulfide content in the overlaying liquid of the samples varied within the range of 1–2 mg/l; the oxygen content was 2–3.5 mg/l. To obtain enrichment cultures, liquid MPSS medium supplemented with a FeS suspension was inoculated with the wastewater sediment. The incubation was performed at pH 7.0 and 28°C. On the second or third day of incubation, white films appeared in the medium 0.3–0.5 cm below the surface; the cells of large motile spirilla with intracellu-

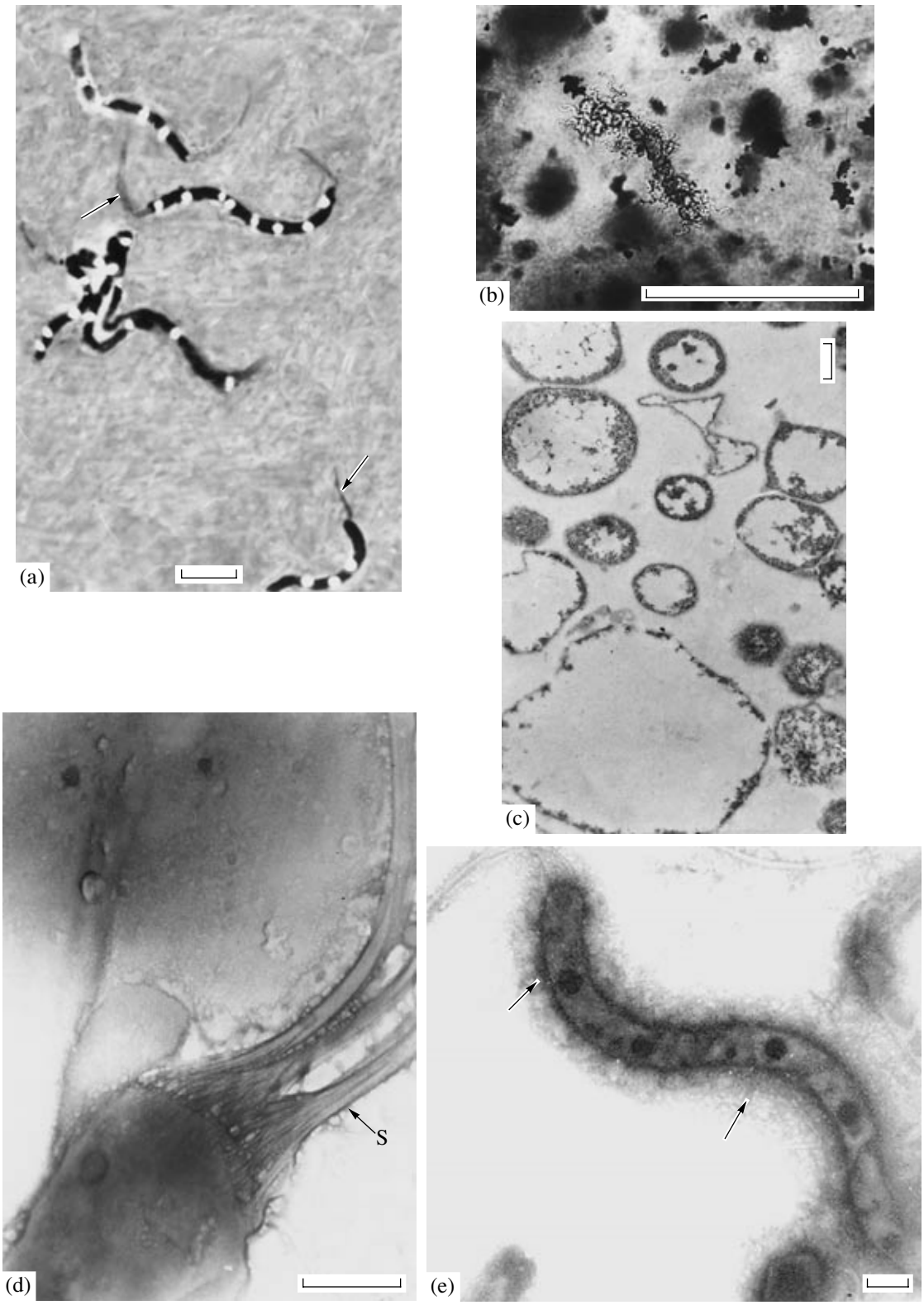

Fig. 1. Morphology and ultrastructure of the cells of strain D-427: (a) exponential-phase cells with intracellular globules of elemental sulfur from MPSS medium with iron sulfide; bar, 5 μm; (b) colonies, phase-contrast microscope; bar, 0.5 mm; (c) ultrastructure of late exponential phase cells; spheroplasts with impaired structure of the cell wall and lacking the peptidoglycan layer are visible; (d) individual flagella are arranged in bundles covered with common sheaths (S); whole-cell preparation stained with 1% ammonium molybdate; (e) mucous capsules of a polysaccharide nature (indicated by arrows); staining with ruthenium red. (c–e) Transmission electron microscope; bar, 1μm.

Comparison of the characteristics of sulfur spirillum strains D-427 and D-430 and of *S.volutans*, the type species of the genus *Spirillum*

Characteristics	*S. volutans* ATCC 19553*	Strain D-427	Strain D-430
Cell size, μm:			
cell width	1.4–1.7	1.7–2.1	0.7–2.1
helix length	14.0–60.0	23.0–49.0	10.5–34.0
wave size	16.0–28.0	15.0–23.0	6.0–14.0
helix diameter	5.0–8.0	6.1–10.3	4.2–8.3
Formation of coccoid bodies****	–	+	+
Utilization of substrates:			
α-ketoglutarate	–	+	+
acetate	–	+	+
Accumulation of S^0			
during sulfide or polysulfide oxidation	ND	+	+
during thiosulfate oxidation	ND	–	–
Oxidation of thiosulfate to tetrathionate	ND	+	+
Resistance to NaCl, %	0.02	0.5	0.3
Range and optimum of O_2 in the gas phase, %	1–12 (6.0)	1–20 (2.0)	0.5–20 (0.8)
Catalase activity	+**	+***	+***
Vitamin requirement	–	+	+
Temperature optimum, °C	30	28	33
DNA G+C content, mol % (T_m)	38	38	38.9

Note: ND stands for "not determined".
* according to [14].
** according to [15].
*** very low activity.
**** spheroplasts.

lar inclusions of S^0 predominated in the films. These cells were collected with a capillary tube and transferred to the MPSS medium with FeS and 1.5% agar. The colonies appeared only below the agar surface. The small semitransparent colonies sized 0.2–0.5 mm consisted of big spiral cells with sulfur inclusions (Fig. 1a). The colonies were flat, with an irregular shape and uneven edges (Fig. 1b). The colonies were then transferred to the semiliquid MPSS medium. The purity of the culture was tested both microscopically and by transfers to a nutrient agar. Two strains of sulfur spirilla were isolated, D-427 and D-430. Although the isolation of strain D-427 was reported previously [6], its physiological, biochemical, and genetic characteristics were not investigated in detail. The summarized results of the study of this strain are presented in this paper.

Cell morphology and ultrastructure. The cells of both strains are big and of a spiral shape (table). The so-called coccoid bodies, considered a species-diagnostic feature for the spiral-shaped bacteria [14], appear in the cultures from the exponential growth phase. The electron micrographs of thin sections of these coccoid cells show a damaged peptidoglycan layer and an impaired cell wall with an intact cytoplasmic membrane (Fig. 1c); they can thus be considered spheroplasts. The size of the spheroplasts can increase up to 7–10 μm; these cells rapidly lyse from the end of the exponential growth phase on. The cells reproduce by binary division. Gram reaction is negative. The spirilla are motile, with polar bundles of flagella that are visible as single flagella under a phase-contrast microscope. Electron micrographs revealed that the flagella formed several bundles covered with sheaths (Fig. 1d). The cells are surrounded by polysaccharide capsules (Fig. 1e). The capsule size varies with the oxygen regime of incubation; they are minimal under optimal growth conditions at low oxygen partial pressure. Inclusions of polyhydroxybutyrate, volutin (not shown), and elemental sulfur are accumulated intracellularly (the last ones, in the presence of sulfides or polysulfide).

Cultural and physiological properties. The temperature ranges for both strains vary from 4 to 39°C, with the optima at 28°C for strain D-427 and at 33°C for strain D-430. Both strains grow in a pH range from 6.5 to 8.5, with the optima at pH 7.5–7.8 for strain D-427 and at pH 7.0–7.5 for strain D-430 (table).

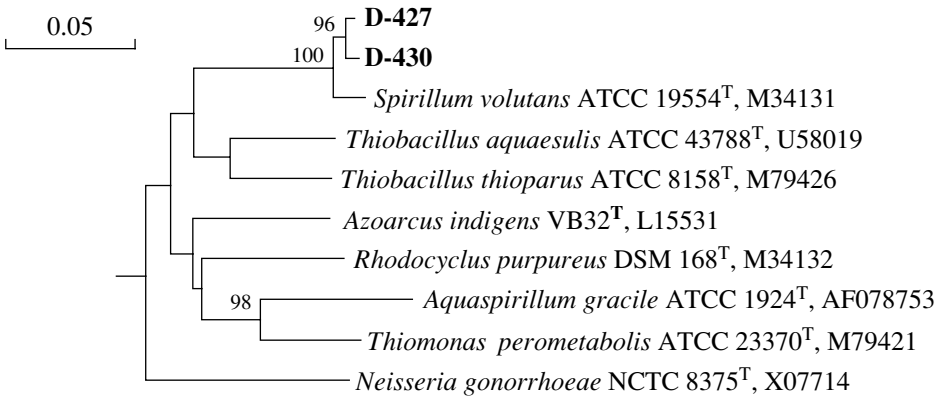

Fig. 2. 16S rDNA-based phylogenetic tree showing the evolutionary position of the new strains of sulfur spirilla D-427 and D-430. Bar 5 nucleotide substitutions per 100 bases. The numerals indicate the statistical significance of the branching order.

The results of phenotypic characterization are presented in the table. Both strains utilize a limited number of organic acids, mainly the intermediates of the tricarboxylic acid cycle: α-ketoglutarate, succinate, fumarate, malate, oxalacetate, pyruvate, and acetate. In the presence of yeast extract, strain D-427 can also utilize citrate, aconitate, and isocitrate; strain D-430 utilizes lactate. The compounds that neither of the strains utilizes as carbon sources include the amino acids histidine, tyrosine, phenylalanine, alanine, glutamate, aspartate, asparagine, glutamine, proline, hydroxy proline, serine, glycine, ornithine, arginine, lysine, methionine, cystine, leucine, valine, threonine, tryptophan, and cysteine, the alcohols propanol, mannitol, glycerol, ethanol, and butanol, the sugars glucose, fructose, maltose, arabinose, galactose, and sucrose, and the polymer compounds peptone, starch, and casein hydrolysate.

Ammonium salts, peptone, and casein hydrolysate but not nitrate, nitrite, or individual amino acids can be used as nitrogen sources.

The strains have respiratory metabolism and do not grow anaerobically with fumarate, nitrate, sulfate, thiosulfate, or elemental sulfur as electron acceptors.

The cells of both strains exhibit oxidase activity and very low-catalase activity. Nitrates are not reduced to nitrites. None of the strains can grow in the presence of more than 0.5% NaCl.

Both strains are able to oxidize sulfide and polysulfide to elemental sulfur and to accumulate it in the cells in the presence of an organic growth substrate. Both strains were previously demonstrated to oxidize quantitatively thiosulfate to tetrathionate, which accumulated in the medium [16]. The enzymes of sulfur oxidation metabolism were not found in the bacteria; the oxidation of inorganic sulfur compounds is not coupled to energy metabolism and occurs via the chemical interaction between sulfur compounds and reactive oxygen species [8, 16].

In spite of the presence of sulfur reductase, the enzymatic reduction of sulfur to H_2S occurs slowly; after cell lysis in the stationary phase, the intracellular sulfur globules usually accumulate in the medium.

Effect of oxygen on growth. When inoculated into a semiliquid medium (0.15% agar), strain D-427 grew 0.3–0.5 cm below the surface; this is an indication of its tendency toward microaerophilic growth. In the presence of reduced sulfur compounds, strain D-427 can also grow on the surface of the medium. Strain D-430, however, grew only below the agar surface on aerobically incubated plates, irrespective of the presence or absence of reduced sulfur compounds; this strain was therefore more microaerophilic than strain D-427. When both strains were grown in a liquid or semiliquid medium under unlimited oxygen supply, massive cell lysis was observed microscopically from the exponential growth phase on; this was caused by the intracellular accumulation of reactive oxygen species [8, 16]. The optimal oxygen regime, as determined by the peak increase in cell biomass, was achieved at 2 and 0.8% oxygen in the gas phase for strains D-427 and D-430, respectively. The effect of the oxygen regime of cultivation on the growth of strain D-427 was investigated previously in more detail [8].

Genotypic and phylogenetic analysis. The strains had similar DNA G+C contents of 38.0 and 38.9 mol % for strains D-427 and D-430, respectively. This is much lower than the values characteristic of other species of sulfur spirilla (62–66 mol %) [6] and is close to the DNA nucleotide composition of the only representative of obligately microaerophilic organotrophic spirilla in the genus *Spirillum, S. volutans* (38 mol %). DNA–DNA hybridization revealed 12% homology between strain D-427 and *S. volutans* and 28% homology between strains D-427 and D-430.

Comparative analysis of almost complete 16S rRNA gene sequences revealed that both strains belonged to the class *Betaproteobacteria* and indicated a high similarity between D-427 and D-430 sequences (98.9%);

they were most closely related to *S. volutans* (98.0–97.8%), clustering with it in phylogenetic tree (Fig. 2).

DISCUSSION

Our new isolates of sulfur spirilla are identical to previously described uncultured species *Thiospira winogradskyi* (Omelianski 1905) [5] in their morphological characteristics and in the ability to accumulate elemental sulfur intracellularly. However, comparative analysis of their geno- and phenotypic characteristics and phylogenetic analysis demonstrated that strains D-427 and D-430 are most closely related to microaerophilic *S. volutans*, the type and only species of the genus *Spirillum* (table). The strains studied, similarly to *S. volutans*, have obligatory organotrophic metabolism and utilize a limited range of organic carbon sources.

At the same time, both new isolates of sulfur spirilla exhibited substantial differences from the type species (see the table). Of these, their capability to form spheroplasts and nonobligatory character of their microaerophily are the most important. The optimal oxygen content in the gas phase is, however, low for these strains; they can be considered as facultatively microaerophilic representatives of the genus. The study of the antioxidant protective system in strain D-427 [8] and of the capability of *S. volutans* ATCC 19554 to grow on the surface of solid media, in the presence of such agents for the removal of the products of oxygen metabolism as catalase or superoxide dismutase [16], have demonstrated that the microaerophily of these bacteria is mainly a result of low catalase activity and absence of cytochrome *c* peroxidase [16, 18].

Production of mucous polysaccharide sheaths and capsules is known as one of the means of protection for the cells inhabiting the redox zone boundary [16, 19, 20]. The ability to produce mucous layers of a polysaccharide nature, which possibly act as barriers preventing direct contact between cells and dissolved oxygen, was noted for strains D-427 and D-430. Similar to other heterotrophic sulfur-oxidizing bacteria, both new isolates of spirilla are capable of the oxidation of sulfur compounds; this process is not coupled to energy metabolism.

The natural occurrence of new strains of sulfur spirilla in the environments where both oxygen and hydrogen sulfide are present simultaneously can be considered as an example of the ecological adaptation of microaerophilic bacteria to the conditions of oxidative stress [16, 17].

Comparative analysis of phenotypic characteristics, together with the results of the phylogenetic analysis, lead us to the conclusion that the strains studied belong to the genus *Spirillum.* However, the low levels of DNA homology both between the new strains (28%) and with the type species *S. volutans* (12%) indicate differences of a species level and enable us to consider them as two new species of the genus *Spirillum.* We suggest the names *Spirillum winogradskii* sp. nov. for strain D-427 and *Spirillum kriegii* sp. nov for strain D-430. These results mean extension of the genus *Spirillum* and necessitate correction of its definition.

The emended diagnosis of the genus *Spirillum* and the diagnoses of the new species are given below.

Emended description of the genus *Spirillum* (Ehrenberg 1832)**.** Spiral cells are 0.7 to 2.3 μm thick; the helix is 4.2 to 10 μm in diameter. Some species form spheroplasts. In the presence of sulfides or polysulfide in the medium, globules of elemental sulfur can accumulate in the cells. Representatives of the genus are obligate or facultative microaerophiles; they are neutrophiles, with a pH growth range from 6.0 to 9.0 and a pH optimum 7.0–7.8. Growth occurs at 4–39°C with an optimum at 28–33°C. The metabolism is obligately chemoorganotrophic; α-ketoglutarate, succinate, fumarate, malate, oxaloacetate, pyruvate, and acetate are utilized as carbon sources. Citrate, aconitate, isocitrate, and lactate can be used in the presence of yeast extract. Ammonium salts, peptone, and casein hydrolysate can be used as nitrogen sources. Some species require growth factors and vitamins. The cells are catalase-negative or have very low catalase activity. Growth occurs at 0.02 to 0.5% NaCl in the medium. DNA G+C content is 36–38.9 mol % (T_m). The similarity of the 16S rRNA gene sequences between the representatives of the species is above 97.4%. Representatives of the genus are widespread in shallow freshwater basins and in municipal and industrial wastewaters containing hydrogen sulfide. The genus is represented by three species. The type species of the genus is *S. volutans* (Ehrenberg 1832) with the type strain ATCC 19554^T.

Description of the species *Spirillum winogradskii* sp. nov. (basonym, *Thiospira winogradskyi*, Omelianski 1905; wi.no.grad'ski.i. M.L. *winogradskii* in honor of the Russian microbiologist S.N. Winogradsky).

Spiral cells are 1.7–2.3 μm thick; the helix consists of one to three coils and is 6.0–10.0 μm in diameter. The cells are motile by means of bipolar bundles of flagella; each bundle is covered with an individual sheath and looks like a thick polar flagellum under phase-contrast microscope. Cells are gram-negative. Poly-β-hydroxybutyric acid and volutin are accumulated inside the cells. The colonies are very small, flat, of irregular shape, and with fringed edges. Cells are facultatively microaerophilic; the optimal O_2 concentration in the gas phase is 2%. Growth occurs within the pH range 6.5–8.5, with an optimum at pH 7.5–7.8; the temperature optimum is at 28°C. The metabolism is obligately chemoorganoheterotrophic; in its course, chemical interaction with reactive oxygen species generated in the electron transport chain results in the intracellular accumulation of elemental sulfur when sulfide or polysulfide are oxidized; thiosulfate oxidation leads to tetrathionate accumulation in the medium.

Organic acids (α-ketoglutarate, succinate, fumarate, malate, oxaloacetate, pyruvate, and acetate) are used as carbon and energy sources; citrate, aconitate, and isocitrate can be used in the presence of yeast extract. Amino acids, sugars, and alcohols are not utilized. Vitamins are required. Ammonium salts, casein hydrolysate, and peptone can be used as nitrogen sources. Nitrates are not reduced to nitrites. Casein and starch are not hydrolyzed. Nitrate, fumarate, sulfate, thiosulfate, and elemental sulfur are not used as terminal electron acceptors. Activity of oxidase and low activity of catalase are present. Sulfide is produced from cysteine. DNA G+C content is 38.0 mol % (T_m).

The source of isolation is sediments from the aeration tanks for the treatment of municipal wastewater containing hydrogen sulfide.

The type strain is D-427^T = DSM 12756^T. Its properties correspond to the species description.

Description of the species *Spirillum kriegii* sp. nov. (krie'gi.i. M.L. *kriegii* in honor of the American microbiologist N.R. Krieg, who contributed much to the study of heterotrophic spirilla).

Spiral cells are 0.7–2.1-μm thick; the helix consists of one to three coils and is 4.2–8.3-μm in diameter. Cells are motile by means of bipolar bundles of flagella, visible as a thick polar flagellum under a phase-contrast microscope. Each bundle is covered with an individual sheath. Cells are gram-negative. Poly-β-hydroxybutyric acid and volutin are accumulated inside the cells. The colonies are very small, flat, of irregular shape, with uneven edges. Only submerged colonies are formed. Cells are facultatively microaerophilic; the optimal O_2 concentration in the gas phase is 0.8%. Growth occurs within the range pH 6.5–8.5 with an optimum at pH 7.0–7.5; the temperature optimum at 33°C. The metabolism is obligately chemoorganoheterotrophic; the chemical interaction with reactive oxygen species results in the accumulation of elemental sulfur inside the cells when sulfide or polysulfide are oxidized; thiosulfate oxidation leads to tetrathionate accumulation in the medium. Organic acids (citrate, aconitate, isocitrate, α-ketoglutarate, succinate, fumarate, malate, oxaloacetate, pyruvate, and acetate) are used as carbon and energy sources; lactate can be used in the presence of a yeast extract. Amino acids, sugars, and alcohols are not utilized. Vitamins are required. Ammonium salts, casein hydrolysate, or peptone can be used as nitrogen sources. Nitrates are not reduced to nitrites. Casein and starch are not hydrolyzed. Nitrate, fumarate, sulfate, thiosulfate, and elemental sulfur are not used as terminal electron acceptors. Activity of oxidase and low activity of catalase are present. Sulfide is produced from cysteine. DNA G+C content is 38.9 mol % (T_m).

The source of isolation is sediments from the aeration tanks containing municipal and industrial wastewater.

The type strain is D-430^T = VKM B-2372^T. Its properties correspond to the species description.

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