ISSN 0026-2617, Microbiology, 2010, Vol. 79, No. 5, pp. 672–678. © Pleiades Publishing, Ltd., 2010.

EXPERIMENTAL ARTICLES

Biodiversity and Monitoring of Colorless Filamentous Bacteitrtn Sulfide Aquatic Systems of North Caucasus Region[1]

E. Yu. Chernousova[a, b, 2], V. N. Akimov[b], E. V. Gridneva[a], G. A. Dubinina[c], and M. Yu. Grabovich[a]

[a] *Voronezh State University, Voronezh, Russia*

[b] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

[c] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 1173 Russia*

Received November 9, 2009

Abstract—Bacterial mats in sulfide aquatic systems of North Caucasus are basically composed by the species of genera *Thiothrix* and *Sphaerotilus*. Additionally, several non-filamentous sulfur-oxidizing bacteria were isolated from the mats and several minor 16S rRNA phylotypes were found in clone libraries from these mats. The minor components were affiliated with *Proteobacteria, Chlorobia, Cyanobacteria* and *Firmicutes*. Even in an individual mat population heterogeneity of *Thiothrix* spp. was revealed by analysis of 16S rRNA gene and RAPD-PCR. Five *Thiothrix* isolates were described as new species *Thiothrix caldifontis* sp. nov. and *Thiothrix lacustris* sp. nov. In the *Thiothrix-Sphaerotilus* type of bacterial mat the proportion of dominant organisms might be influenced by sulfide concentration in the spring water. The higher sulfide concentration (more than 10 mg/1) in the spring water is more favorable for the development of bacterial mats with dominant *Thiothrix* organisms than for *Thiothrix-Sphaerotilus* type of sulfur mat.

Key words: colorless sulfur bacteria, sulfide springs, *Thiothrix, Sphaerotilus*, 16S rRNA, RAPD-PCR

DOI: 10.1134/S0026261710050127

Filamentous sulfur-oxidizing bacteria are widespread in aquatic environments, where they are effective biogeochemical agents [1, 2]. Sulfur - oxidizing filamentous bacteria like *Thiothrix* and *Sphaerotilus* are capable of form microbial mats and frequently dominate in them. *Thiothrix* mats are found in sulfide caves [3], in zone of marine hydrotherms and volcanic activity [4], in sulfide mineral springs [5], and in anthropogenic ecosystems [6]. It is known the symbiotic relationship of *Thiothrix* spp. with invertebrates from sulfide-rich habitats [7]. *Sphaerotilus* mats are often associated with activated sludge systems [8], where they as well as *Thiothrix* mats are known to cause serious technological problems, such as pipe clogging and bulking of activated sludge in wastewater treatment plants [9]. Microbial mats formed by simultaneously *Thiothrix* and *Sphaerotilus* are usually observed in activated sludge of treatment facilities and in the surface layers of fresh water heavily contaminated with agricultural waste [10–12]. The preference of both organisms for low ambient oxygen content and their ability to utilize a broad range of organic carbon sources explain their mass development in the above anthropogenic ecosystems [11, 12].

In the previous studies we have described several new species of colorless sulfur bacteria isolated from sulfide springs and sulfide lakes of the North Caucasus region [13]. We have explored in situ and ex situ the taxonomic diversity of colorless sulfur bacterial mat from mineral sulfide spring and showed that the colorless sulfur bacterial mat, in which *Thiothrix* and *Sphaerotilus* are predominant organisms, can be found not only in activated sludge systems but also in natural aquatic environment [14]. This study summarizes biodiversity findings in sulfide springs and sulfide lakes of the North Caucasus region and provides data on population dynamics of filamentous sulfur bacteria.

1. MATERIALS AND METHODS

Environmental Samples and Isolates. Samples of colorless sulfur bacterial mats were obtained from mineral sulfide springs and sulfide lakes of the North Caucasus region. The samples were taken from the sites in August 2004, August 2006 and August 2007. For molecular biological studies, the samples of mats were immediately fixed in 25% ethanol (final concentration) and stored at –20°C. All filamentous sulfur-oxidizing isolates analyzed in the study were recovered from these sulfur bacterial mats using Williams and Ambruster media [15]. Isolation of sulfur-oxidizing unicellular bacteria was carried out on semi-liquid PSS medium [16] containing FeS, which was buffered at a pH of 7.5.

[1] The article is published in the original.

[2] Corresponding author; e-mail: ru-elen@yandex.ru

DNA Extraction. DNA was isolated from bacterial strains and environmental samples by the protocol of Ausubel et al. [17], with slight modifications, and by the freeze-thaw method [18].

Amplification and cloning of the 16S rRNA genes. 16S rRNA genes were amplified using primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). Amplification was performed with a GeneAmp PCR System 2700 (Applied Biosystems, USA). The amplicons were purified with a MiniPrep kit (Promega, USA) and subsequently cloned into the pGEM-T vector cloning system (Promega, USA) as described in the manufacturer's instructions. For screening of the clone library, the cloned 16S rRNA genes were amplified with primers T7f (5'-TAATACGACTCACTATA-3') and Sp6r (5'-TATTTAGGTGACACTATAG-3') specific for appropriate sites of the plasmid; the inserts thus obtained were reamplified with eubacterial primers 27f and 1492r. To reveal the clone groups with similar DNA fragments, restriction analysis (ARDRA) with *Hae*III and *Hha*I endonucleases was used. For the representatives of each clone group revealed in the clone library, 16S rDNA fragments (500 to 1300 bp) were sequenced. Clones with 16S rRNA gene sequence similarity more than 99.5% were assigned to the same phylotype.

Nucleotide sequence of 16S rRNA genes was determined in a CEQ2000 XL automatic sequencer (Beckman Coulter, USA) according to the manufacturer's recommendations.

Phylogenetic analysis. The obtained 16S rDNA sequences were subjected to BLAST search (http://www.ncbi.nlm.gov/BLAST/) for closely related sequences in the GenBank database. The retrieved sequences were aligned using the CLUSTAL X program [19]. The phylogenetic trees were constructed with the TREECON program [20]. Bootstrap analysis was performed with 1000 replicates.

RAPD - PCR. Genomic RAPD fingerprints were obtained as described in de Bruijn [21] using the primer M13 (5'-TTATGTAAAACGACGGCCAGT-3') with subsequent analysis by electrophoresis in 1.5% (w/v) agarose gel. RAPD fingerprint patterns were converted into binary data matrix by scoring the presence of a band as 1 and its absence as 0. Bands that were not reproducible were excluded from the analyses. Faint and visually indistinguishable bands were also ignored. The binary matrix was subjected to the FreeTree software [22], using the Nei and Li coefficient [23] to generate a similarity matrix.

RESULTS AND DISCUSSION

Our investigations of colorless sulfur bacterial mats from the mineral sulfide springs and lakes of the North Caucasus region have been carried out during 3 years (2004, 2006, 2007). The community composition of sulfur bacterial mats was explored by means of both cultivation and molecular biological techniques.

There are seven species in the genus *Thiothrix* and only organisms closely related to *T. unzii* were found in the mats of sulfide springs and lakes of the North Caucasus region. All other *Thiothrix* isolates were finally described as new species *Thiothrix caldifontis* sp. nov. and *Thiothrix lacustris* sp. nov. (Table 1). *Thiothrix unzii* was previously found in sulfide-rich waters [6] and our studies confirm its wide biogeography in natural aquatic ecosystems. Up to date, there is no information about occurrence of *Thiothrix caldifontis* and *Thiothrix lacustris* in other regions.

Intraspecific heterogeneity of *Thiothrix unzii*-like organisms and *Thiothrix caldifontis* was shown as in different sulfide springs and lakes so in the same one. Strains T4 and T19 were isolated from the mat of the Petushok spring; their 16S rRNA genes had 99% similarity with the *T. unzii* type strain. The difference of two nucleotides in 16S rRNA gene was revealed between two strains. This difference can not be assigned to multicopy of 16S rRNA gene in the same organism because *Thiothrix unzii*-like strains T4 and T19 had also the difference in some cultural and even morphological characteristics.

Four strains of *Thiothrix caldifontis* were isolated from bacterial mats of two different sulfide springs and lake, which are located in the northern spurs of the Central Caucasus Ridge, in Krasnodar and Stavropol Krai. Strains G1 and G2 were isolated from the bacterial mat of Petushok spring in 2004 and 2006, correspondingly; strain P was from bacterial mat of Proval lake, and strain K2 was from mat of Kabardinskii spring. Strains G1, G2, K2 and P were compared by RAPD-PCR (randomly amplified polymorphic DNA-PCR) (Fig. 1). A high genetic similarity was observed between those strains, above 75%. Nevertheless, RAPD fingerprint patterns of all four strains were slightly different from each other indicating population heterogeneity of *Thiothrix caldifontis* between the springs and the lake as well as within the spring.

Sphaerotilus natans—like organisms (the only species in this genus) was found in various sulfide springs and geographical regions of North Caucasus (Table 1). All isolates accumulated elemental sulfur when grown in the media with sulfide. The accumulation of elemental sulfur in cells of *S. natans* during growth on medium with sulfide has already been noted by Skerman [24]. In contrast to the known *S. natans* strains, new strains D-501, D-502, D-504, D-505, and D-507 from the bacterial mat of sulfur spring Petushok were capable of both organoheterotrophic growth and lithotrophic growth with reduced sulfur compounds as electron donor for energy conservation. All isolates were found to be identical based on ERIC-PCR [14] and RAPD-PCR (Fig. 2). But the existence of the metabolic heterogeneity within the cluster was demonstrated [25]. Strains BV-1 and BV-2 obtained from the bacterial mats of Besstyzhiy Vanny spring were

Table 1. Species and 16S rRNA phylotypes found in bacterial mats of sulfide springs and lakes of North Caucasus. ND, no data

Place	Isolated cultures	Retrieved phylotypes	Reference
	2004 year		
Sulfide spring Petushok	*Thiothrix* sp. strain T4 *Thiothrix* sp. strain T19 *Thiothrix caldifontis* strain G1 *Sphaerotilus* sp. strains D-501–D-507	*Thiothrix* sp. (CK9) *Sphaerotilus* sp. (CK8) *Polaribacter* sp. (CK3) *Rhodobacter* sp. (CK5) *Burkholderia* sp.(CK6) *Allochromatium* sp. (CK7) *Acidaminobacter* sp. (CK1) *Chlorobium* sp. (CK2) *Cyanobacteria* (CK4)	13, 14, 25
Sulfide spring Yama	ND	*Sphaerotilus* sp. (CK8) *Thiothrix* sp. (CK9) *Chlorobium* sp. (CK2)	This study
Sulfide spring Ruchey	ND	*Thiothrix* sp.(CK9) Uncultured gammaproteobacteria (CK10)	This study
	2006 year		
Sulfide spring Petushok	*Thiothrix caldifontis* strain G2	*Thiothrix* sp. (CK9) Uncultured betaproteobacteria (CK11)	This study
Sulfide spring Ruchey	ND	*Thiothrix* sp. (CK9)	This study
	2007 year		
Sulfide lake Proval	*Thiothrix caldifontis* strain P	ND	13
Sulfide spring Kabardinskii	*Thiothrix caldifontis* strain K2	ND	13
Sulfide lake Lower	*Thiothrix lacustris* strain BL	ND	13
Sulfide spring Besstyzhiy Vanny	*Sphaerotilus* sp. strains BV-1 and BV-2 *Azospirillum* sp. strain BV-S *Aquaspirillum* sp. strain Spb *Pseudomonas* sp. strain BV-el	ND	This study and 25

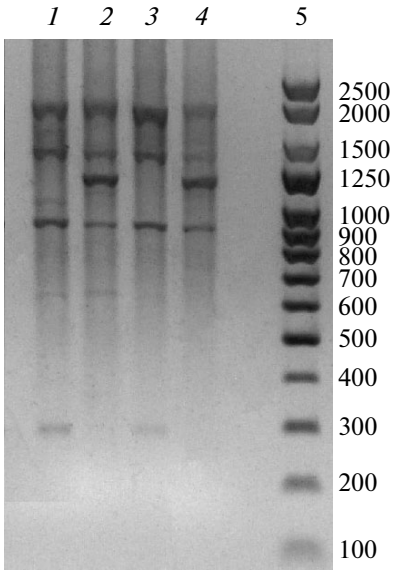

Fig. 1. RAPD patterns of isolated *Thiothrix caldifontis* strains. Lanes: *1*, K2; *2*, Gl; *3*, P; *4*, G2; 5, marker.

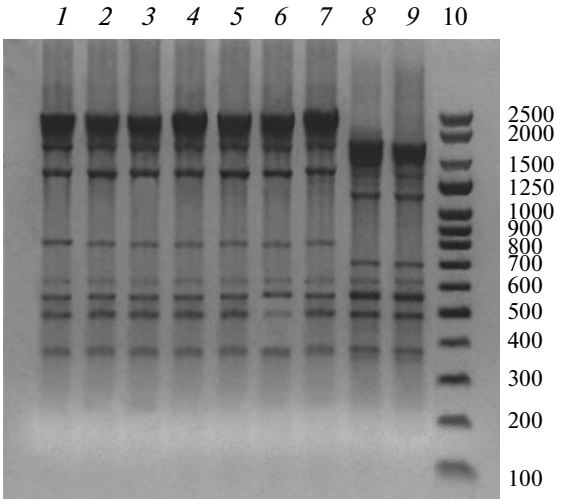

Fig. 2. RAPD patterns of isolated *Sphaerotilus* strains. Lanes: *1*, D-501; *2*, D-502; *3*, D-503; *4*, D-504; *5*, D-505; *6*, D-506; *7*, D-507; *8*, BV-1; *9*, BV-2; 10, marker.

capable of organoheterotrophic growth and litoheterotrophic growth with reduced sulfur compounds as electron donor for energy conservation. Strains BV-1 and BV-2 showed identical RAPD patterns (Fig. 2). However, RAPD patterns of two *Sphaerotilus natans*—like strains of Besstyzhiy Vanny mat were slightly different from the seven *Sphaerotilus natans* - like strains of Petushok mat indicating population heterogeneity of *Sphaerotilus natans*—like organisms between the springs. The Nei and Li genetic similarity index among the *Sphaerotilus* strains isolated from various sulfide springs were 74%.

Several non-filamentous bacteria involved in oxidation of inorganic sulfur compouds were isolated from North Caucasus aquatic systems. Only the strains originated from bacterial mats formed by colorless filamentous bacteria are listed here.

The strain BV-el was identified as the member of genus *Pseudomonas*, the class *Gammaproteobacteria* and has 100% of 16S rRNA gene sequence similarity to *Pseudomonas stutzeri*. Strain BV-el was capable for oxidation of sulfur compounds but was not studied thoroughly. The organisms belonging to Pseudomonas stutzeri group were shown to play a much more significant role in the biogeochemical cycles than was previously recognized [26].

Strain Spb was affiliated with genus *Aquaspirillum*, the class *Betaproteobacteria*, and was closely related to *Aquaspirillum serpens* (16S rRNA gene sequence similarity of 98.9%). The members of the genus *Aquaspirillum* are capable for oxidation of sulfur compounds [27].

The strain BV-S was belonged to the genus *Azospirillum* within the class *Alphaproteobacteria*. Within the genus *Azospirillum*, strain BV-S is most closely related to *Azospirillum doebereinerae, Azospirillum picis*, and *Azospirillum lipoferum*, with a 16S rRNA gene sequence similarity of 97.7–97.4%. In contrast to the known *Azospirillum* species, the strain BV-S is capable of mixotrophic growth under microaerobic conditions with the simultaneous utilization of organic substrates and thiosulfate as electron donor for energy conservation. Oxidation of sulfide was accompanied by deposition of sulfur globules within the cells.

Phylogenetic *in situ* study showed sufficient biodiversity of microbial communities in the bacterial mats formed in the sulfide springs of North Caucasus. In total, eleven dominant and minor phylotypes were found in five clone libraries based on 16S rRNA gene in 2004, 2006 and 2007 years (Table 1). *Thiothrix unzii*-like and *Sphaerotilus natans*-like organisms were dominant in the clone libraries from the sulfur mats. The strains of closely related to *Thiothrix unzii* and *Sphaerotilus natans* were isolated in parallel with clone library preparation from the same mats and we can suppose that the isolated strains of these species precisely correspond to dominant organisms in the clone libraries. *Thiothrix unzii*-like organisms were detected in five clone libraries (the springs Petushok in 2004 and 2006 years, Yama in 2004 year and Ruchey in 2004 and 2006 years) and *Sphaerotilus natans*-like organisms were detected in two clone libraries (Petushok in 2004 year and Yama in 2004 year). The minor components were affiliated with of different taxa of *Alphaproteobacteria*, *Betaproteobacteria, Gammaproteobacteria, Chlorobia*, *Cyanobacteria* and *Firmicutes.* In the clone libraries the organisms closely related (more than 97% of 16S rRNA similarity) to *Rhodobacter capsulatus,*

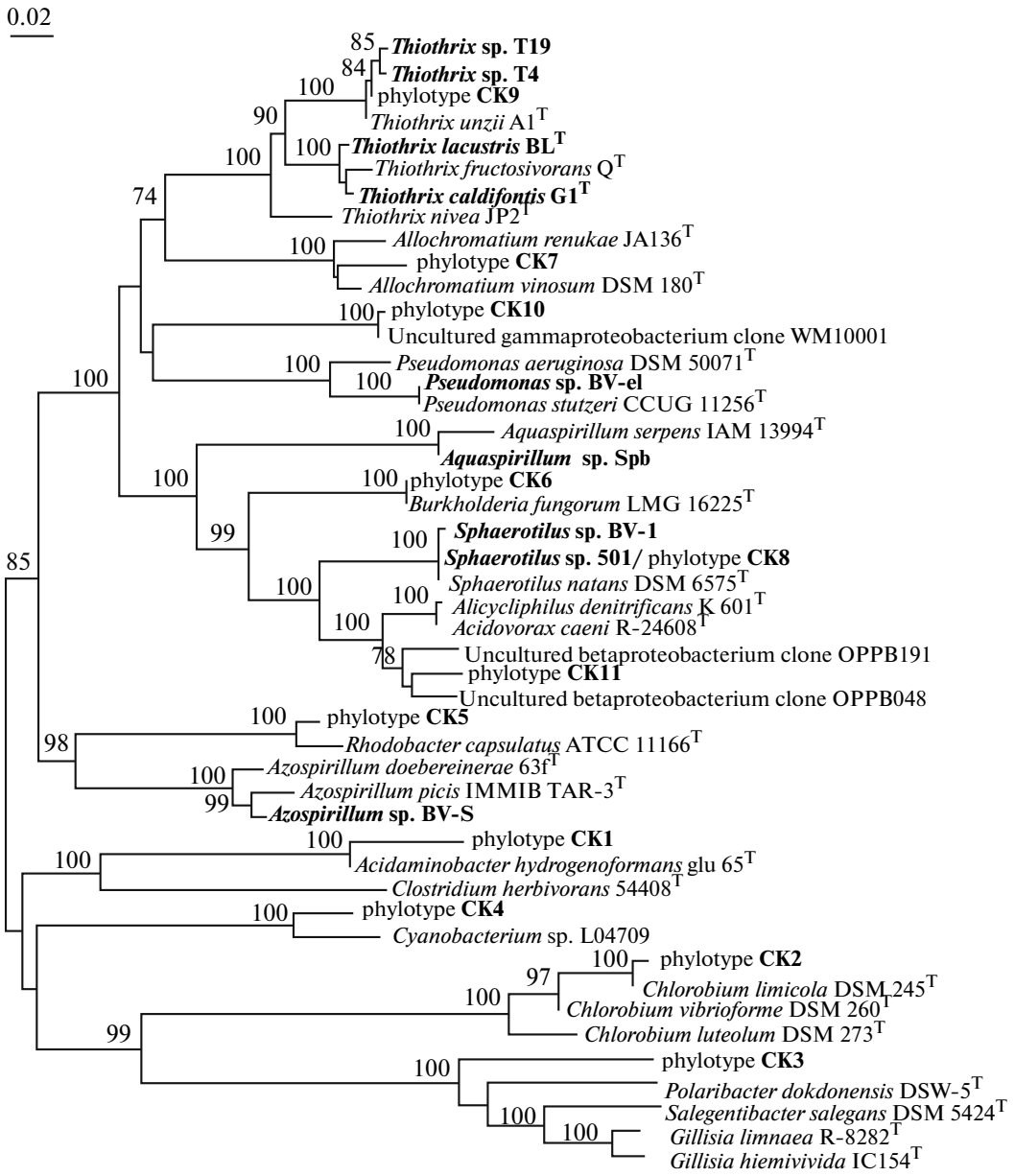

Fig. 3. Phylogenetic tree inferred from 16S rRNA gene sequences, demonstrating the positions of clones and cultures obtained from the sulfur mats of the sulfide springs and sulfide lakes of the North Caucasus within the *Bacteria* domain. The scale corresponds to nucleotide substitutions per 100 nucleotides. The *Rubrobacter xylanophilus* DSM 9941^T 16S rRNA gene sequence is used as the external group.

Allochromatium vinosum and *Chlorobium limicola* are capable to use sulfide and sulfur as electron donors [27]. The data concerning the bacteria phylogenetically related to those revealed in various sulfide springs and sulfide lakes of North Caucasus are presented on Fig. 3.

The proportion of *Thiothrix unzii*-like and *Sphaerotilus natans*-like organisms in bacterial mats was influenced by sulfide concentration in the springs and was reflected in clone libraries of 16S rRNA gene. The data presented in the Table 2 allow to propose that the higher sulfide concentration in the spring water might be more favorable for the development of bacterial mats with dominant *Thiothrix unzii*-like organisms than for *Thiothrix-Sphaerotilus* type of sulfur mat. It is evident from growth characteristics of *Thiothrix* and *Sphaerotilus* isolates. The concentrations of sulfide more than 10 mg/l in the culture media inhibit the growth of *Sphaerotilus* cultures isolated from sulfide

Table 2. Influence of sulfide concentration in spring waters on proportion of dominant 16S rRNA phylotypes found in bacterial mats

Place	Year	Sulfide concentration	Proportion of retrieved dominant phylotypes, %	Reference
Yama	2004	1.5–2 mg/l	*Thiothrix* sp. (50%) and *Sphaerotilus* sp. (45%)	This study
Petushok	2004	2–5 mg/l	*Thiothrix* sp. (66%) and *Sphaerotilus* sp. (26%)	14
Ruchey	2004	10–12 mg/l	*Thiothrix* sp. (95%)	This study
Petushok	2006	10–12 mg/l	*Thiothrix* sp. (95%)	This study
Ruchey	2006	10–12 mg/l	only *Thiothrix* sp.	This study

springs Petushok and Besstyzhiy Vanny but did not inhibit the growth of *Thiothrix* isolates.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (project nos. 09-04-00799).

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