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EXPERIMENTAL ARTICLES

Phylogenetic in situ/ex situ Analysis of a Sulfur Mat Microbial Community from a Thermal Sulfide Spring in the North Caucasus

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Abstract—A phylogenetic in situ/ex situ analysis of a sulfur mat formed by colorless filamentous sulfur bacteria in a thermal sulfide spring (northern spur of the main Caucasian ridge) was carried out. Nine phylotypes were revealed in the mat. *Thiothrix* sp. and *Sphaerotilus* sp. were the dominant phylotypes (66.3% and 26.3%, respectively). The 16S rRNA gene nucleotide sequence of *Sphaerotilus* sp. phylotype from the clone library was identical to the sequences of the seven *Sphaerotilus* strains isolated from the same source. A very high degree of similarity of *Sphaerotilus* strains revealed by ERIC-PCR fingerprints indicated little or no population diversity of this species in the mat. *Thiothrix* phylotype from the clone library and two *Thiothrix* strains isolated from the same mat sample differed in one to three nucleotides of 16S rRNA genes; this is an indication of this organism's population variability in the mat. 16S rRNA genes of the strains and clones of *Thiothrix* sp. exhibited the highest similarity (ca. 99%) with *Thiothrix unzii*; the strains and clones of *Sphaerotilus* had 99% similarity with the type species *Sphaerotilus natans* (the only species of this genus) and therefore can be assigned to this species. The minor seven components belong to the phylotypes from the *Proteobacteria* (3%), as well as the *Chlorobia, Cyanobacteria, Clostridia*, and *Bacteroidetes* phylogenetic groups, each of them constituting not more than 1%. Intracellular accumulation of elemental sulfur by *Sphaerotilus* similar to other filamentous sulfur bacteria was demonstrated for the first time (both in the population of the sulfur spring and in cultures with sulfide). Although mass growth of *Sphaerotilus* and *Thiothrix* is typical of bacterial populations of anthropogenic ecosystems (the activated sludge of treatment facilities), stable communities of these bacteria have not been previously found in the sulfur mats or "threads" of natural sulfide springs.

Key words: phylogeny, 16S rRNA, biodiversity of colorless sulfur bacteria, *Sphaerotilus, Thiothrix*, microbial communities of sulfide springs.

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Colorless sulfur bacteria are a group of morphologically distinctive prokaryotes which is evolutionally, taxonomically, and physiologically heterogeneous [1]. Its members are characterized by considerable cell size (exceeding the cell size of most unicellular microorganisms by several orders of magnitude) and, most importantly, by their ability to accumulate intracellular inclusions of elemental sulfur. Colorless sulfur bacteria are widespread in various aquatic environments, both marine and freshwater, in the zones of marine hydrotherms and volcanic activity, in sulfide mineral springs, and in anthropogenic ecosystems. The ecological niches of these bacteria are usually located at the oxygen–sulfide interface. Their mass growth results in formation of dense benthic communities (sulfur mats), which sometimes cover large areas of the bottom sediments. In such communities, one or two species of filamentous or unicellular sulfur bacteria usually predominate. Due to the difficulties of isolation and maintaining pure cultures of sulfur bacteria, few members of this group have been studied from the physiological and taxonomic point of view. Most of the known morphotypes belong to uncultured organisms.

Recently, application of molecular methods resulted in sufficient progress in the study of colorless sulfur bacteria, especially in deep-water marine habitats. Tax-

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onomy and the evolutional position of sulfur bacteria are reviewed in [1, 2].

Taxonomic characterization of microbial communities which include colorless filamentous sulfur bacteria can be carried out directly in environmental samples (in situ identification) at the level of genus and species, primarily by 16S rRNA gene analysis. Identification at the species and intraspecies level in most cases still requires individual cultures (ex situ identification). Due to the problems of microbial "culturability" and the limitations of direct molecular methods, assessment of microbial diversity in a community both in situ and ex situ is currently not common.

In the present work, the results of an in situ/ex situ investigation of the community of filamentous colorless sulfur bacteria from a sulfur mat of one of the thermal sulfide springs in the foothills of Northern Caucasus are presented. The taxonomic composition of the community, the quantitative ratio of its major bacterial components, and the population heterogeneity of the dominant species (members of the genera *Sphaerotilus* and *Thiothrix*) were determined.

MATERIALS AND METHODS

Source of isolation. The sulfur mat sample was collected in August 2004 at the outflow of the Petushok slightly mineralized sulfide spring. The spring is located in the discharge region of the deep underground waters of the Psekups mineral water deposit in the northern spur of the Central Caucasus ridge, in Goryachii Klyuch, Krasnodar krai, Russia.

Sulfide content in the spring water determined by iodometric titration was 5.5 mg/l. Due to the turbulent mixture of water flows contacting with air, oxygen content (as determined by the Winkler method for reducers-containing water [3]) varied from 0.1 to 0.5 mg/l. The water temperature was 40°C. Total water mineralization did not exceed 0.6 g/l. Immediately after sampling, mat samples for molecular analysis were placed in 30% ethanol and stored on ice prior to analysis.

Isolation of pure cultures of sulfur bacteria was carried out by the serial dilutions method in semiliquid medium in petri dishes; the colonies were then transferred into liquid medium of the same composition. The medium contained the following (g/l): $(NH_4)_2SO_4$, 0.2; $CaCl_2 \cdot 6H_2O$, 0.02; $MgSO_4 \cdot 7H_2O$, 0.05; $Na_2S_2O_3$, 2.0; sodium succinate, 0.1; $NaHCO_3$, 0.4; KH_2PO_4, 0.02; agarose, 4.0 (or 1.5 for test tubes); 10% HEPES buffer, 30 ml; distilled water, 800 ml; water from well 102 e with S/Na_2S content of 100 mg/l, 200 ml; vitamins and trace elements (Pfennig, Lippert) [3].

DNA isolation. The cells were disrupted by three cycles of freezing to –70°C and thawing at 50°C with subsequent 5-min heating of the cell suspension at 85°C [4]. DNA was extracted by the phenol method [5].

16S rRNA gene amplification was carried out with bacterial primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') or with archaeal primers A8f (5'-TCCGGTTGATCCTGCCGG-3'), A800r (5'-GTTTAC(R)GCC(R)GGACTAC-3'), and A1041r (5'-GGCCATGCACC(W)CCTCTC-3'). PCR was carried out in a GeneAmp PCR System 2700 device (Applied Biosystems, United States).

Cloning. The obtained PCR fragments were cloned in pGEM-T vector (Promega, United States). 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 [6]. The clone groups differing according to the results of restriction analysis are further designated as phylotypes. For the representatives of each phylotype revealed in the clone library, 16S rDNA fragments (500 to 1300 bp) were sequenced.

REP-PCR. Genomic ERIC fingerprints were obtained as described in [7]. ERIC fingerprints were obtained using the primers ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') with subsequent analysis in 1.5% (wt/vol) agarose gel.

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

Phylogenetic analysis. In order to determine closely related organisms, 16S rRNA gene sequences were compared with the sequences of type strains in the ribosomal database (RDPII, http://rdp.cme.msu.edu) and the National Center for Biotechnology Information gene bank (NCBI, http://www.ncbi.nlm.nih.gov). Aligning of 16S rRNA gene sequences was carried out using the CLUSTALX software package [8]. Phylogenetic trees were constructed using the TREECON software package [9].

RESULTS AND DISCUSSION

Ecological and taxonomic study of sulfide springs of the Psekups mineral water deposit (Goryachii Klyuch) revealed mass development of sulfur mats formed by various colorless filamentous sulfur bacteria.

Microscopy of the sulfur mat samples from the Petushok sulfide spring flowing in the Psekups river revealed the predominance of filamentous sulfur bacteria morphologically resembling *Thiothrix* [10–13].

Inoculation of the sulfur mat material into semiliquid medium with low content of organic matter and reduced sulfur compounds resulted in the isolation of two cultures of filamentous sulfur bacteria. Their characteristic morphology (sheaths around the nonmotile filaments,

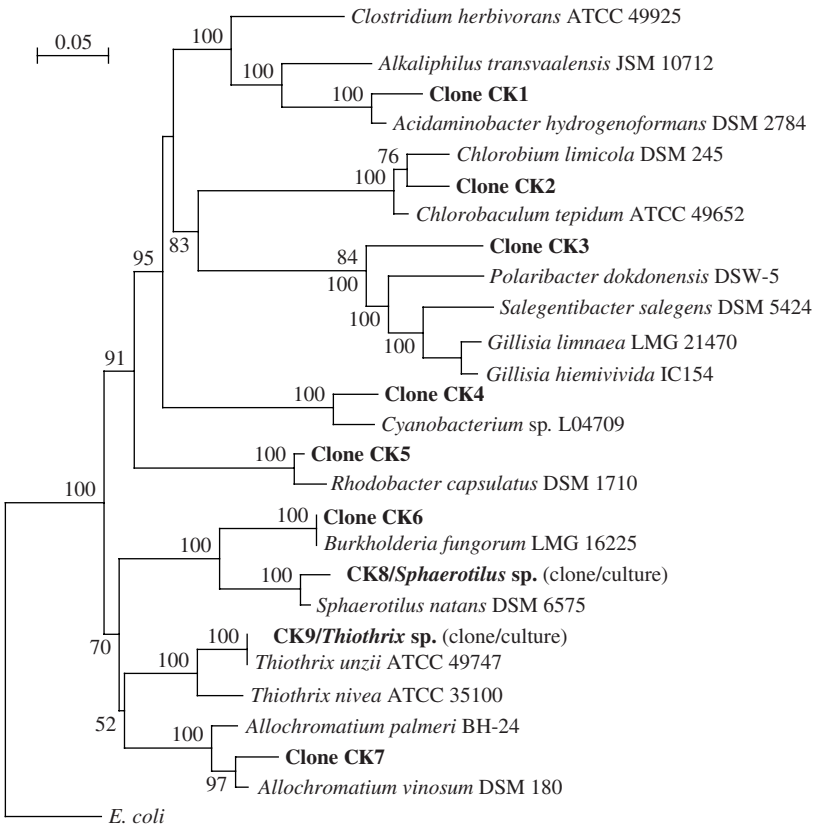

Fig. 1. Phylogenetic tree inferred from 16S rRNA gene sequences, demonstrating the positions of clones and cultures obtained from the sulfur mat of the Petushok thermal sulfide spring within the *Bacteria* domain. The scale corresponds to five nucleotide substitutions per 100 nucleotides. The *E. coli* 16S rRNA gene sequence is used as the external outgroup.

gliding gonidia, and formation of rosettes), together with the results of molecular genetic analyses supported assigning them to the genus *Thiothrix.* Apart from these, other filamentous bacteria with abundant inclusions of elemental sulfur were revealed both in the sulfur mat and among the isolates obtained on agarized media; a number of their characteristics was different from those of *Thiothrix* species. Their morphotype, i.e., the presence of motile swarm cells and the filaments consisting of cell chains within a common sheath (unlike *Thiothrix* trichomes) suggested their assignment to the genus *Sphaerotilus*; this was subsequently confirmed by research of the pheno- and genotypic characteristics of seven isolates in pure culture. Further investigation of *Sphaerotilus* pure cultures demonstrated that all the strains were capable of sulfide oxidation resulting in intracellular sulfur accumulation; the mechanisms of oxidative reactions were elucidated [14].

Determination of the taxonomic composition of the sulfur mat community by molecular analytic methods was the major research goal.

The DNA isolation procedure applied in the present work has been used in numerous molecular ecological investigations and is suitable for a wide range of bacterial and archaeal cells, apart from endospores and possibly cystlike cells. The primers used for amplification (27f and 1492r) can be applied for 16S rRNA gene amplification in the case of most bacteria, though not for archaea. Several universal archaeal 16S rRNA primers were tested; no archaea were revealed in the mat samples.

A clone library of amplicons obtained from the mat total DNA with 27f and 1492r primers was used for the taxonomic characterization of the mat. The library was screened for 95 clones. In order to determine the taxonomic position of the phylotypes obtained by screening of the clone library, the nucleotide sequences of 16S rRNA fragments (500 to 1300 bp) were analyzed.

Screening of the clone library of 16S rRNA PCR products obtained from the Petushok bacterial mat revealed the domination of *Thiothrix* sp. (66.3%) and *Sphaerotilus* sp. (26.3%). The minor components included *Proteobacteria* (3%), *Chlorobia, Cyanobacteria, Clostridia*, and *Flavobacteria* (1% each) (Fig. 1, Table).

The 16S rRNA clones representing the *Thiothrix* sp. phylotype in the Petushok mat gene library exhibited 99% similarity to *T. unzii* type strain. Two *Thiothrix* sp. strains were isolated from the Petushok mat; their 16S rRNA genes also had 99% similarity with the *T. unzii* type strain. However, the difference of one to three

Bacteria most closely related to the phylotypes from the Petushok sulfide stream clone library

Phylotype	Number of clones	Closest relative (% of 16S rRNA similarity)	Phylum or class
CK1	1	*Acidaminobacter hydrogenoformans* (95%)	*Firmicutes*
CK2	1	*Chlorobium limicola* (97%)	*Chlorobia*
CK3	1	*Polaribacter dokdonensis* (95%)	*Bacterioidetes*
CK4	1	*Cyanobacteria* (93%)	*Cyanobacteria*
CK5	1	*Rhodobacter capsulatus* (97.6%)	*Alphaproteobacteria*
CK6	1	*Burkholderia fungorum* (100%)	*Betaproteobacteria*
CK7	1	*Allochromatium vinosum* (97%)	*Gammaproteobacteria*
CK8	25	*Sphaerotilus natans* (99.9%)	*Betaproteobacteria*
CK9	63	*Thiothrix unzii* (99%)	*Gammaproteobacteria*

nucleotide substitutions in a 16S rRNA gene fragment of ca. 500 nucleotides were revealed both between the clones and the isolates of *Thiothrix* sp and between two strains. These differences indicate a certain diversity of the population of these organisms within the mat. Some morphological and cultural characteristics of *Thiothrix* sp. strains do not confirm with the *T. unzii* species description; moreover, the differences between the strains are significant. Thus, in spite of the high similarity of the 16S rRNA gene sequences, the data on DNA–DNA hybridization are required for unequivocal assignment of the two *Thiothrix* sp. isolates to *T. unzii.*

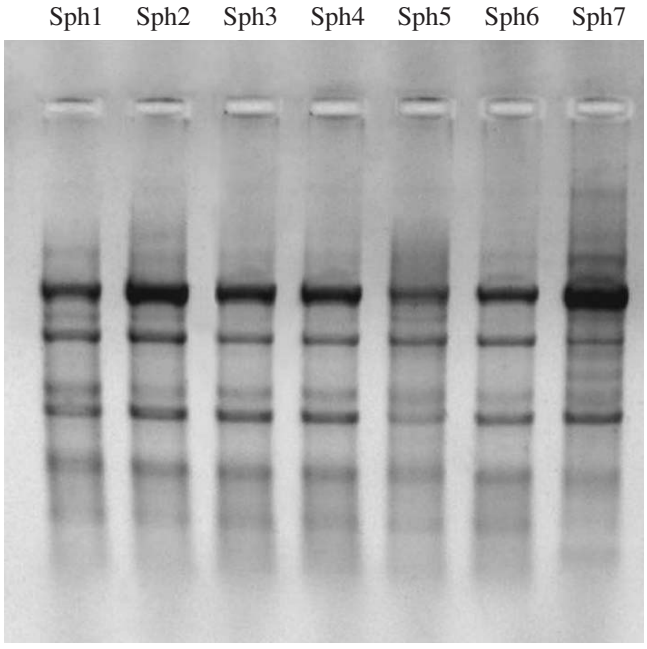

Fig. 2. REP-PCR DNA profiles of seven *Sphaerotilus* sp. isolates from the sulfide spring.

The 16S rRNA clones representing the *Sphaerotilus* sp. phylotype in the Petushok mat gene library exhibited 99.9% similarity to the type strain of *S. natans*, the only species of this genus. In all *Sphaerotilus* sp. clones and cultured isolates, the nucleotide sequences were identical. The high degree of 16S rRNA similarity to the *S. natans* type strain suggests assigning the new *Sphaerotilus* sp. isolates to this species.

Since the 16S rRNA gene sequences of all *Sphaerotilus* sp. isolates from the Petushok sulfur mat were identical, REP-PCR was used to study the population diversity. The REP profiles of all seven *Sphaerotilus* cultures were identical, indicating the absence of population heterogeneity within the mat (Fig. 2).

For molecular identification of the minor components in the mat clone library, 16S rRNA gene fragments of ca. 500 nucleotides were analyzed; this length is usually quite sufficient to determine the taxonomic position at the genus level. The search for the closest relatives for each sequence was performed in the NCBI bank. For only one clone (CK6), a completely identical 16S rRNA sequence of *Burkholderia fungorum* was found in the NCBI bank.

In the Petushok library, the phylum *Proteobacteria* (3%) is represented by the families *Rhodobacteraceae* (α-*Proteobacteria*), *Burkholderiaceae* (β-*Proteobacteria*), and *Chromatiaceae* (γ-*Proteobacteria*); the phylum *Chlorobia* (1%) by *Chlorobium limicola*; the phylum *Clostridia* (1%) by a member of *Clostridiaceae*; and the phylum *Bacteroidetes* (1%) by a representative of the family *Flavobacteriaceae.* Since the systematics of the phylum *Cyanobacteria* is developed by both botanists and microbiologists and is presently in a state of reorganization, the taxonomic position of its representative (1%) is difficult to determine. The data concerning the bacteria phylogenetically related to those revealed in the Petushok library are presented in the table and on Fig. 1.

The simultaneous presence of the members of *Thiothrix* and *Sphaerotilus* genera has usually been reported for the activated sludge of treatment facilities and for the surface layers of fresh water heavily contaminated with agricultural waste [15–17]. 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 [16–18]. It should be mentioned that numerous *Sphaerotilus* isolates, which are still classified with only the species *S. natans*, are known as components of natural iron-containing ecosystems (bogs, chalybeate springs, etc.) and belong to organoheterotrophic iron bacteria [2, 19]. However, their phylogenetic position and relations to the type species of the genus are still not clear.

In the materials presented in this article, the role of *Sphaerotilus* bacteria as one of the major components of sulfur mats in natural sulfide springs and their role in the oxidation of reduced sulfur compounds were demonstrated for the first time.

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