

EXPERIMENTAL
ARTICLES

Analysis of Bacterial Communities of Two Lake Baikal Endemic Sponge Species

A. S. Gladkikh^{a, 1}, Ok. V. Kalyuzhnaya^a, O. I. Belykh^a, T. S. Ahn^b, and V. V. Parfenova^a

^a Limnological Institute, Siberian Branch, Russian Academy of Sciences, Irkutsk, Russia

^b Kangwon National University, Chuncheon, Republic of Korea

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Abstract—Bacterial diversity of two Lake Baikal endemic sponges characterized by different life forms, branching *Lubomirskia baicalensis* and encrusting *Baikalospongia* sp., was studied using 454 pyrosequencing of the 16S rRNA gene fragments. In the communities associated with *L. baicalensis* and *Baikalospongia* sp., 426 and 428 OTUs, respectively, were identified. In microbial associations of these sponges, 24 bacterial phyla with predominance of *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* were identified. Analysis of the taxonomic composition of bacterial communities of the sponges was carried out by searching the dominant phylotypes within the clusters of phylum level. Comparison of bacterial associations of the sponges with Lake Baikal bacterioplankton revealed both the shared OTUs and the unique ones characteristic of the studied species.

Keywords: Lake Baikal, pyrosequencing, 16S rRNA, endemic sponges, *Lubomirskia baicalensis*, *Baikalospongia* sp.

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Sponges (*Porifera*) attract researchers' interest due to their ability to accumulate a wide variety of microorganisms, such as heterotrophic bacteria, cyanobacteria, microscopic algae, archaea, dinoflagellates, fungi, etc., within their body. These sessile animals are attached to benthos substrates and filter up to 24000 L water per 1 kg body weight per day [1]. Some planktonic microorganisms floating with water along the sponge channel system are used as a food source, while others are accumulated in the body and form a symbiotic community with the sponge. In some sponge species, microorganisms make up to 35% of the biomass of the community and are involved in photosynthesis, carbon fixation, nitrogen transformation, and anaerobic metabolism; they also perform protective functions [2]. Many secondary metabolites produced by sponge-associated microorganisms are used in medicine and biotechnology [3]. It is not surprising that sponge microbial communities have been a subject of research for over 30 years worldwide. However, until recently most studies focused on marine sponges reaching up to 8000 of species diversity [1, 4]. Associations of microorganisms within freshwater sponges (*Porifera*, *Haplosclerida*, *Spongillina*) remain extremely poorly studied. Meanwhile, freshwater sponge fauna includes over 150 species inhabiting various ecological sites: lakes, rivers, brooks, bays, reservoirs, etc. [5].

To date, pyrosequencing was used for analysis of the symbionts associated with 32 marine sponge species. Phylogenetic diversity of the microorganisms in studied communities was represented by 28 bacterial phyla, two archaeal phyla, and several groups of microalgae and fungi [4]. Analysis of marine sponge samples revealed that 70–96% of the sequences belonged to bacteria, while this ratio in water was as high as 99% [6]. Currently, there are few reports on the diversity of microbial communities associated with freshwater sponges [7–10]. In the earlier studies, microbial associations were investigated by generation of libraries of the 16S rRNA genes, which were further sequenced by the classical Sanger method. In total, nine bacterial phyla were detected in the microbiomes of freshwater sponges: *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Planctomycetes*, *Cyanobacteria*, *Chloroflexi*, and *Nitrospirae*, as well as the TM7 phylum represented by uncultured bacteria.

In present work, we applied the 16S rRNA fragment pyrosequencing to study the diversity of bacterial communities of two sponge species belonging to the endemic family *Lubomirskiidae* inhabiting Lake Baikal, the oldest and the deepest lake on the planet. Sponge species used in the work belonged to two different genera and were characterized by different life forms; they are the branching *Lubomirskia baicalensis* and the encrusting *Baikalospongia* sp. Communities associated with the sponges were compared with the bacterioplankton community of Lake Baikal.

¹ Corresponding author; e-mail: angladkikh@gmail.com

MATERIALS AND METHODS

Sample collection. Samples of *Lubomirskia baicalensis* and *Baikalospongia* sp. sponges were collected during the expedition in June 2010, in the neighborhood of the Bolshie Koty settlement (southeastern coast of Baikal) at a depth of 10–15 m using SCUBA diving equipment. The samples of bacterioplankton were collected at the same time [11].

Pyrosequencing. Total DNA was extracted from the samples of sponges and bacterioplankton immediately after collection using a DNKSorb B kit (InterLab-Servis, Russia). Amplification was performed using the eubacterial primers 9F and 541R flanking the V1–V3 regions of the 16S rRNA gene [12]. Apart from the bacterial 16S rRNA, this pair of primers amplifies the 16S rRNA genes from the chloroplasts of algae [15]. Metagenomic sequencing of the 16S rRNA gene fragments was performed on a FLX Titanium Roche/454 Genome Sequencer by ChunLab Inc. (Seoul National University, Republic of Korea). The maximum length of the obtained sequences was 510 nucleotides; chimeric sequences and sequences shorter than 300 nucleotides were excluded from the analysis. Thus, each sequence contained at least two out of three (V1, V2, or V3) hypervariable 16S rRNA gene sites.

Analysis of diversity and taxonomic composition of the communities. Each of the sequences derived was taxonomically identified by comparison with sequences available in the EzTaxon-e database [13] by using the BLASTIn search algorithms and pairwise comparison. The thresholds of similarity (x) were used to identify species ($x \geq 97\%$), genera ($97 > x \geq 94\%$), families ($94 > x \geq 90\%$), orders ($90 > x \geq 85\%$), classes ($85 > x \geq 80\%$), and phyla ($80 > x \geq 75\%$).

Primary analysis of the pyrosequencing data, removal of short and chimeric sequences, clustering into operational taxonomic units (OTUs), biodiversity assessment by calculation of the ACE, Chao1, and Shannon indexes, and construction of Venn diagram were carried out with the Mothur v. 1.32.1 software package (<http://mothur.org>). To determine species diversity and taxonomic composition and to compare the communities, the Pyrosequencing pipeline software was used (<http://pyro.cme.msu.edu>). The sequences were aligned and cluster analysis was undertaken with the use of the Complete Linkage Clustering package (a component of the Pyrosequencing pipeline). Clustering was performed at various levels; distance between the clusters was varied from 0 to 0.1 with an increment of 0.01. Phylotypes (OTUs) were recognized at the cluster distance of 0.03; taxonomic complexity of communities was evaluated using the Rarefaction package (a component of the Pyrosequencing pipeline) at various distance values corresponding to the taxa as follows: species, 0.03; genus, 0.05; and family, 0.1. To characterize the taxonomic composition of the communities, cluster analysis was

performed at the cluster distance of 0.3, corresponding to the phylum level. Then, for each cluster a representative nucleotide sequence corresponding to the cluster center (i.e., the sequence having a minimal sum of squared distances to other sequences in the cluster) was found using the Dereplicate Request package (Pyrosequencing pipeline). Species were classified on the basis of genotypic approach in accordance with the International Code of Nomenclature for Bacteria (ICNB). Based on the percent similarity with the sequence of a validated microorganism, a cluster was referred to the relevant taxon (from species to phylum) in accordance with the similarity limits presented above.

The sequence data obtained in the work are deposited in the NCBI database under record number SRP020221.

RESULTS AND DISCUSSION

General characteristics of species diversity in Lake Baikal sponge-associated bacterial communities. Pyrosequencing revealed 7071 sequences of the 16S rRNA gene over 300 nucleotides long in the microbial community of the branching sponge *L. baicalensis*; 6873 sequences belonged to the *Bacteria* domain (2935 of them unique) and 298 belonged to the *Eukarya* domain. Total length of the analyzed bacterial sequences was 3000660 nucleotides, and the average length was 470 nucleotides. The number of identified phylotypes was 426.

In the microbial community of the encrusting sponge *Baikalospongia* sp., 7042 sequences of the 16S rRNA gene over 300 nucleotides long were revealed with pyrosequencing; 6817 sequences belonged to the *Bacteria* domain (2601 of them unique) and 225 to the *Eukarya* domain. Total length of the analyzed bacterial sequences was 2931465 nucleotides, and the average length was 470 nucleotides. The number of identified OTUs was 428.

Among the eukaryotes, genotypes of the chloroplasts of green and diatomic algae, dominated by the *Chlorella* genotype, were noticed.

Taxonomic complexity of communities was estimated by plotting rarefaction curves, demonstrating the number of detected OTUs depending on the number of sequences analyzed for different similarity levels of the nucleotide sequences. In communities associated with both *L. baicalensis* and *Baikalospongia* sp., curves of species accumulation upon increase in the number of analyzed sequences did not reach the plateau and the number of OTUs revealed increased linearly (Fig. 1). The curves demonstrate that the sequencing coverage achieved in the work is insufficient for complete characterization of biodiversity of the communities, and additional phylotypes may be revealed.

The rank abundance curves reflected unevenness in species distribution and species richness of the micro-

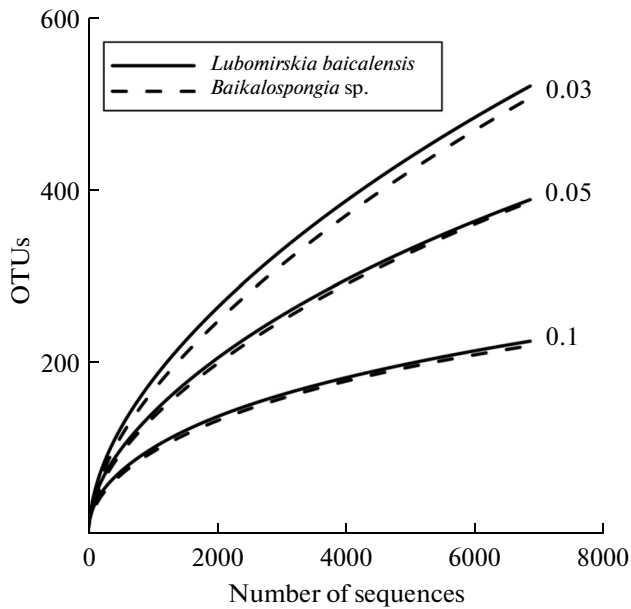


Fig. 1. Rarefaction curves at different cluster distance values.

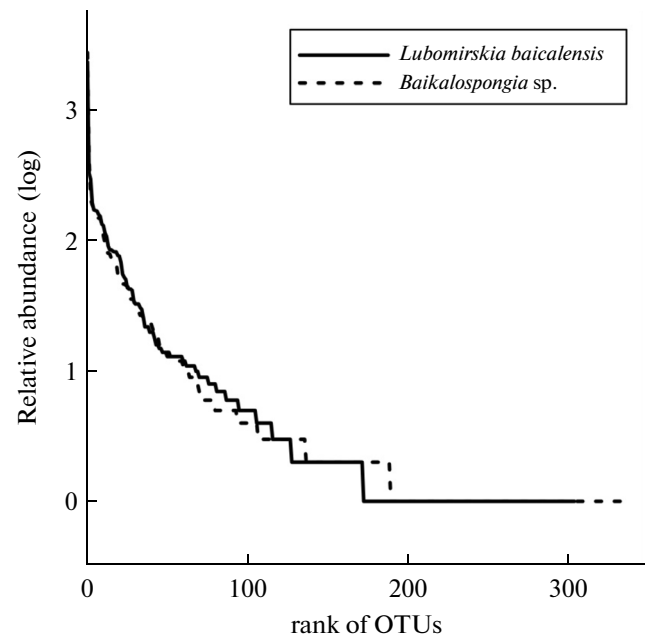


Fig. 2. Rank abundance curves at cluster distance of 0.03.

bial communities (Fig. 2). Rather low-sloped curves of the plots indicated the presence of more than one dominant species in the communities, with its considerable fraction comprised by the taxa represented by several sequences. Approximately half of the phylotypes belonged to rare organisms represented by a single sequence each.

Real species richness in the communities was determined using the Chao1 and ACE non-parametric criteria. The Chao1 diversity index at the level of species (cluster distance of 0.03) for *L. baicalensis* and *Baikalospongia* sp. was 948 and 1111, respectively. The values of the ACE index were 1660 for *L. baicalensis* and 2005 for *Baikalospongia* sp. (Table 1). Species richness of the *Baikalospongia* sp. community predicted on the basis of Chao1 estimation was 2.6 times higher than that derived from the pyrosequencing data; for *L. baicalensis*, it was 2.2 times higher than the

number of OTUs. Meanwhile, the shape of the species abundance curve (Fig. 2) evidenced that the most abundant taxa determining the structure of the community have already been identified and further pyrosequencing could only result in detection of rare species. The values of the Shannon index of species diversity for the endosymbiotic communities of *L. baicalensis* and *Baikalospongia* sp. were 3.45 and 3.14, respectively. The level of diversity observed in the bacterial communities of Lake Baikal freshwater sponges was comparable to the estimated complexity of marine sponge-associated bacterial communities [6, 14].

In the community of the branching sponge *L. baicalensis*, eight bacterial phyla (*Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Nitrospirae*, OD1, and *Chloroflexi*) were identified by cluster analysis. Similar bacterial phyla (except for *Nitrospirae*) were identified in the commu-

Table 1. Indices of species richness and diversity at different cluster distances

Sample	Cluster distance	Number of sequences	Number of OTUs	ACE	Chao1	Shannon
<i>Lubomirskia baicalensis</i>	0	6873	2935	25578	11290	6.48
	0.03		426	1660	948	3.45
	0.05		321	935	655	3.25
	0.1		175	360	285	2.68
<i>Baikalospongia</i> sp.	0	6817	2601	21642	9817	6.29
	0.03		428	2005	1111	3.14
	0.05		321	1072	691	2.97
	0.1		166	339	242	2.49

Table 2. Composition of the symbiotic bacterial community associated with *Lubomirskia baicalensis**

Phylum	Number of sequences	Share in the cluster, %	Representative sequence characterizing the cluster; accession number of the closest homologue in GenBank	Class	Similarity to the closest homologue, %
<i>Bacteroidetes</i>	2778	40.4	<i>Sediminibacterium</i> sp.; JN381502	<i>Sphingobacteria</i>	95.3
	431	6.3	<i>Flavobacterium</i> sp. HME6144; HQ000017	<i>Flavobacteria</i>	97.3
	72	1.0	Class <i>Flavobacteria</i> ; JN712178	<i>Flavobacteria</i>	83.2
	19	0.3	Order <i>Sphingobacteriales</i> ; AB682145	<i>Sphingobacteria</i>	87.4
<i>Proteobacteria</i>	1563	22.7	<i>Polynucleobacter necessarius</i> ; AB607317	<i>Betaproteobacteria</i>	100
	336	4.9	Family <i>Hyphomicrobiaceae</i> ; <i>Pedomicrobium australicum</i> FM886902	<i>Alphaproteobacteria</i>	92.8
	19	0.3	Class <i>Alphaproteobacteria</i> ; DQ003193	<i>Alphaproteobacteria</i>	84.1
<i>Actinobacteria</i>	1009	14.7	<i>Planktophila limnetica</i> ; FJ428831	<i>Actinobacteria</i>	98.9
<i>Planctomycetes</i>	474	6.9	Order <i>Phycisphaerales</i> ; <i>Phycisphaera mikurensis</i> NBRC 102666; AP012338	<i>Phycisphaerae</i>	87.1
	26	0.4	Order <i>Planctomycetales</i> ; GQ889476	<i>Planctomycetacia</i>	89.0
<i>Verrucomicrobia</i>	66	1.0	Order <i>Opitutales</i> ; AY695840	<i>Opitutae</i>	88.0
<i>Nitrospirae</i>	29	0.4	<i>Nitrospira moscoviensis</i> ; NR029287	<i>Nitrospira</i>	97.4
OD1	37	0.5	Phylum OD1; AY168743		87.8
<i>Chloroflexi</i>	6	0.1	Phylum <i>Chloroflexi</i> ; JF922925		98.0
Bacteria_unc	7	0.1	AJ306894		71.9

* The table presents only the clusters with the share in the community exceeding 0.1%; more than 97% homology indicated in bold.

nity of the encrusting sponge *Baikalospongia* sp. Several sequences corresponded to uncultured bacteria and did not belong to any known phylum.

Representatives of the *Bacteroidetes* phylum constituted the greatest proportion of both communities: 48% of the bacterial 16S rRNA sequences in the *L. baicalensis* community and 52%, in the *Baikalospongia* sp. community (see Tables 2 and 3 and Fig. 3). The phyla *Proteobacteria* (28 and 29% sequences), *Actinobacteria* (14.7 and 9.5%), and *Planctomycetes* (7.3 and 7.7%) were relatively abundant in the studied communities. Altogether, these four phyla accounted for approximately 98% of the total number of bacterial sequences in both sponge communities.

Earlier, Kalyuzhnaya et al. used direct sequencing to study the composition of the *L. baicalensis* community and revealed 54 OTUs belonging to six bacterial phyla: *Actinobacteria*, *Proteobacteria*, *Verrucomi-*

crobia, *Bacteroidetes*, *Cyanobacteria*, and *Nitrospirae*. *Actinobacteria* and *Proteobacteria* were the dominant phyla, while *Bacteroidetes* accounted for 7.5% of all sequences [8]. The differences in the composition of the dominant groups of the bacterial community associated with *L. baicalensis* were probably due to the difference in the season of sample collection: in the work of Kalyuzhnaya and co-authors the samples were collected in February and April, and in the current work they were collected in June. As was previously shown for a marine sponge *Axinella corrugata*, microbial community composition is subject to seasonal changes with maintenance of a stable composition of most of the taxonomic groups [15].

Dominant phylotypes in the bacterial communities associated with the Lake Baikal sponges. In the bacterial community associated with *L. baicalensis*, the *Bacteroidetes* phylum contained two classes: *Sphingo-*

Table 3. Composition of the symbiotic bacterial community associated with *Baikalospongia* sp.*

Phylum	Number of sequences	Share in the cluster, %	Representative sequence characterizing the cluster; access number of the closest homologue in GenBank	Class	Similarity to the closest homologue, %
<i>Bacteroidetes</i>	3202	47.0	<i>Sediminibacterium</i> sp.; JN381502	<i>Sphingobacteria</i>	95.4
	291	4.3	<i>Flavobacterium glaciei</i> 0499; NR043891	<i>Flavobacteria</i>	98.7
	67	1.0	<i>Arcicella</i> sp. HME7208, JN408290	<i>Cytophagia</i>	98.3
<i>Proteobacteria</i>	827	12.0	Family <i>Burkholderiaceae</i> ; AB470420	<i>Betaproteobacteria</i>	91.8
	641	9.4	Family <i>Hyphomicrobiaceae</i> ; FM886902	<i>Alphaproteobacteria</i>	82.0
	457	6.7	Family <i>Oxalobacteraceae</i> ; AB252072	<i>Betaproteobacteria</i>	91.3
	18	0.2	Phylum <i>Proteobacteria</i> ; D13945		78.6
	28	0.4	Class <i>Alphaproteobacteria</i> ; AY792295	<i>Alphaproteobacteria</i>	82.0
<i>Actinobacteria</i>	652	9.5	<i>Planktophila limnetica</i> MWH-EgelM2-3; FJ428831	<i>Actinobacteria</i>	99.6
<i>Planctomycetes</i>	13	0.2	Order <i>Brocadiales</i> ; AF375994	<i>Planctomycetia</i>	85.3
	490	7.2	Family <i>Phycisphaeraceae</i> ; <i>Phycisphaera mikurensis</i> NBRC 102666; AP012338	<i>Phycisphaerae</i>	91.7
	21	0.3	<i>Schlesneria</i> sp.; NR042466	<i>Planctomycetacia</i>	95.3
<i>Verrucomicrobia</i>	54	0.8	Family <i>Opitutaceae</i> ; AY695840	<i>Opitutae</i>	88.9
OD1	41	0.6	Phylum OD1; AY168743		88.0
<i>Chloroflexi</i>	5	0.07	Order <i>Anaerolineales</i> ; NR041354	<i>Anaerolineae</i>	86.7
	3	0.04	Phylum <i>Chloroflexi</i> ; JQ793474		83.0
Bacteria_unc	5	0.07	Bacteria_unc; FJ612418		99.7

* The table presents only the clusters with the share in the community exceeding 0.1%; more than 97% homology indicated in bold.

bacteria and *Flavobacteria* (Table 2). The class *Sphingobacteria* comprised a cluster represented by a cultured genus *Sediminibacterium*, accounting for 40.4% of the total community population, and a cluster with a representative phylotype belonging to the order *Sphingobacteriales*. Representatives of this order have been previously noted in freshwater sponge communities [8, 10]. Two clusters, an uncultured phylotype, and the genus *Flavobacterium* represented members of the class *Flavobacteria* in the *L. baikalensis* community.

In the bacterial community associated with *Baikalospongia* sp., the phylum *Bacteroidetes* was represented by three classes: *Sphingobacteria*, *Flavobacteria*, and *Cytophagia*, each comprising a single cluster. Here, as in the case of the *L. baikalensis*-associated community, the *Sediminibacterium* sp. cluster was the most abundant (47% of the community). Two other

clusters were represented by the *Flavobacterium glaciei* species (class *Flavobacteria*) and the *Arcicella* genus (class *Cytophagia*), with over 97% similarity of the relevant 16S rRNA gene fragments (Table 2).

Thus, in the communities of both the encrusting and branching sponges the dominating cluster was represented by the genus *Sediminibacterium* of the family *Chitinophagaceae*. The closest strain, *Sediminibacterium* sp. (JN381502), was isolated from a freshwater spring in South Korea. Representatives of the family *Chitinophagaceae* are capable of chitin degradation. Chitin is a widespread complex polymer; it is a structural component of the skeleton of both marine and freshwater sponges [16].

Many of the representatives of the class *Flavobacteria* are typical planktonic species [17]; the class was bet-

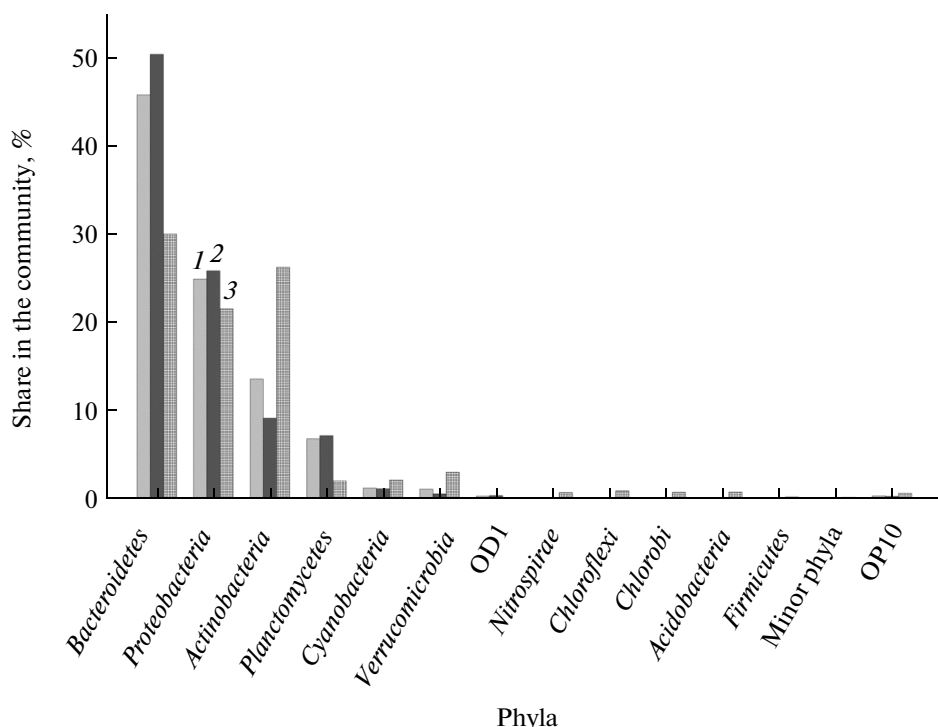


Fig. 3. Composition of bacterial communities associated with endemic sponges from Lake Baikal based on BLASTn search. *L. baicalensis* (1), *Baikalospongia* sp. (2) and plankton (3).

ter presented in the community of *L. baicalensis* (7.3%) than in that of *Baikalospongia* sp. (4.3%). The class *Cytophagia* was revealed only in the community of encrusting sponge; it was represented by the genus *Arcicella*, comprising the cosmopolitan freshwater species which were detected in the neuston biofilms [18].

The phylum *Bacteroidetes* is a phylogenetically and metabolically diverse group of chemoorganotrophic bacteria [17]. The presence of members of this phylum in the sediments and water column of the oligotrophic Lake Baikal has been demonstrated in a number of previous works [19, 20]. Moreover, our latest work on the analysis of planktonic and biofilm communities from Lake Baikal demonstrated domination by the representatives of *Bacteroidetes* in water samples collected in June 2010 [11]. *Bacteroidetes* is a typical phylum of freshwater microbiomes, together with the *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* [21]. High abundance of *Bacteroidetes* in aquatic environments is due to their capacity for enzymatic hydrolysis of polymers, such as cellulose, chitin, proteins, and other high-molecular weight fractions of organic substances [22]. The size of the *Bacteroidetes* population was found to correlate positively with the growth of plankton and blooming of cyanobacteria, both in marine and freshwater ecosystems, owing to the ability of members of the phylum to utilize organic metabolites of algae, including cyanobacterial toxins [23].

As a rule, representatives of the *Bacteroidetes* are present in communities associated with marine

sponges, where the relative amount of the *Bacteroidetes* varies within a wide range depending on the sponge species and environmental conditions [6, 14]. Among the three studied freshwater sponge-associated communities, the phylum *Bacteroidetes* was detected in two cases: within a cosmopolitan sponge *Ephydatia fluviatilis* [10] and in an endemic Baikalian sponge *L. baicalensis* [8]. The phylum accounted for 9.8 and 7.5% of the total number of clones analyzed, respectively.

The phylum *Proteobacteria* was represented in the communities associated with *L. baicalensis* and *Baikalospongia* sp. by two classes, *Alpha-* and *Betaproteobacteria*, the latter one being predominant in both cases (Tables 2 and 3).

In the *L. baicalensis*-associated community, the *Betaproteobacteria* constituted 22.7% and were represented by the *Polynucleobacter necessarius* (family *Burkholderiaceae*) cluster. Among the *Proteobacteria*, *Polynucleobacter* sp. sequences comprised the most important share of the libraries of *L. baicalensis* and *E. fluviatilis* clones [8, 10]. The *Alphaproteobacteria* were represented by bacteria related to *Pedomicrobium australicum* (family *Hyphomicrobiaceae*) (4.9%), while an insignificant number of sequences (0.3%) were related to the uncultured DQ003193 phylotype (Table 2).

In the community of *Baikalospongia* sp., the phylum *Proteobacteria* was represented by five clusters: *Burkholderiaceae* (12% sequences), *Oxalobacteraceae*

(6.7%), *Hyphomicrobiaceae* (9.4%), an uncultured bacterium related to the *Alphaproteobacteria* AY792295 phylotype (isolate of a humic lake, United States) (0.4%), and an uncultured phyloptype D13945 of the phylum *Proteobacteria* (0.2%). No representative phylotypes in the clusters had over a 92% degree of homology with any of known cultured strains, which prevented sequence identification at a level below the family. Therefore, the proportion of the *Betaproteobacteria* in the community of *Baikalospongia* sp. (18.7%) was lower than in that of *L. baicalensis*. On the contrary, proportion of the *Alphaproteobacteria* in the *Baikalospongia* sp. community (10.2%) was twice as high as in that of the *L. baicalensis*-associated community (5.2%).

The family *Burkholderiaceae*, with the representative species *P. necessarius*, formed the most numerous cluster among the proteobacteria associated with the Baikal sponges (Tables 2 and 3). The family dominated among the Lake Baikal planktonic proteobacteria, where its share was as high as 15.8%; however, *Albidiferax ferrireducens* was the dominant species [11]. In freshwater ecosystems, *P. necessarius* is known to be one of the dominant phylogenetic clusters of bacterioplankton; its total amount in the sea may reach 70% of the total bacterial cell population [17]. It is important to note that *P. necessarius* sequences were also detected among the symbionts of freshwater sponges *S. lacustris* [9] and *E. fluviatilis* [10].

Most representatives of the *Alphaproteobacteria* in the communities belonged to the family *Hyphomicrobiaceae* (see Tables 2 and 3). These bacteria are often found in oligotrophic lakes and wet soil and include species able to accumulate iron and manganese and to consume methanol as a main carbon source [24]. According to Parfenova et al. [11], *Alphaproteobacteria* accounted for 3.3% of the Baikal planktonic community, with *Reyranella massiliensis* being the dominant species. *Alphaproteobacteria* formed rather numerous groups in the previously studied communities associated with freshwater sponges: 22, 24, and 51% in the communities of *L. baicalensis* [8], *E. fluviatilis* [10], and *S. lacustris* [9], respectively. Importantly, common bacterial phylotypes were detected in the above-mentioned freshwater sponge communities, evidencing the possible co-evolution of a number of sponge-associated bacteria [8, 10].

Actinobacteria was the third most abundant phylum in Lake Baikal sponge-associated communities. It was represented by a cluster containing a common planktonic species *Planctophila limnetica*. *Actinobacteria* are common in both marine and freshwater communities and are among the best studied taxa due to their importance for biotechnology, medicine, and ecology. Application of molecular methods demonstrated that freshwater actinobacteria formed separate phylogenetic lineages and were distinctly differentiated from the sequences of other habitats, thus representing an autochthonous component of freshwater basins [25]. In various lakes, actinobacteria are often

a predominant fraction of the heterotrophic bacterioplankton, comprising up to 50% of the DAPI-stained prokaryotes. Their active proliferation in surface water layers is attributed to the presence of actinorhodopsin in their cells and, consequently, their resistance to UV irradiation [17]. The most numerous taxa of freshwater actinobacteria are grouped into the acI clade which, as a rule, predominates in bacterioplankton in water basins of various climate zones with versatile trophic status. *P. limnetica* is one of the few cultured species of the clade [25]. In Lake Baikal, the genus *Planctophila* is typical for plankton, in which the *Actinobacteria* is the second most numerous phylum (28%) according to the results of metagenomic analysis [11].

Actinobacteria were detected in all previously studied communities associated with freshwater sponges, where they constituted a considerable part (14–37%) of bacterial sequences [9, 10]. While the presence of the phylum in microbial associates of Baikal sponges was to be expected, their share in the community of the encrusting sponge *Baikalospongia* sp. was somewhat lower (9.5%) than in the community of the branching sponge *L. baicalensis* (14.7%), which may be a consequence of abundance of actinobacteria decreasing with depth.

The phylum *Planctomycetes* encompassed two clusters of the *L. baicalensis*-associated community, i.e. the orders *Phycisphaerales* (AP012338) and *Planctomycetales* (GQ889476); *Baikalospongia* sp.-associated community comprised three clusters: family *Phycisphaeraceae* (AP012338), *Schlesneria* sp. (NR042466), and the order *Brocadia* (AF375994). The representation of members of the phylum in communities of the branching and encrusting sponges was approximately the same, 7.3 and 7.7%, respectively. The *Phycisphaeraceae* cluster (6.9%)—with the closest homologous species being *Phycisphaera mikurensis* NBRC 102666 isolated from a marine alga *Porphyra* sp.—was found to be the most abundant one in both sponges. However, due to the low homology rate (87–91%), sequences of the sponge-associated bacterial community were only identified at the level of family.

Representatives of the *Planctomycetes* are characterized by a wide metabolic adaptation range: they may be found in ocean sediments, biofilms, freshwater ecosystems, wastewater bioreactors, and soil, as well as in associations with organisms like sponges, corals, macroalgae, crustaceans, etc. [26–28]. These are chemoheterotrophs and chemoautotrophs able to degrade complex hydrocarbons produced by phytoplankton [29]. These microorganisms probably play an important role in the carbon and nitrogen cycles and in anaerobic oxidation of ammonium [17, 28].

The community associated with the encrusting sponge was characterized by the presence of two additional clusters. The first one was formed by *Schlesneria* sp. (order *Planctomycetales*) living in acidic *Sphagnum* soils and capable of growth at low temperatures [30],

and the second one was formed by the order *Brocadiales* comprising ammonium-oxidizing bacteria [31]. The only cultured nitrifying species comprising the order is the *Candidatus* 'Brocadia anammoxidans'; the level of homology of sponge sequences with the latter one was 85%. Analysis of 16S rRNA gene sequences demonstrated high variability within the order *Brocadiales* [31].

In communities of the Lake Baikal sponges, the proportion of *Planctomycetes* was three times higher than was revealed by Parfenova et al. [11] for the planktonic community of Lake Baikal (2.4%). A similar situation was noted with respect to the community of a cosmopolitan sponge *E. fluviatilis*, in which the share of the phylum was 14.4%, while in the environment it was 3.1% [10]. In microbial associations of marine sponges, the phylum *Planctomycetes* is a rather rare and small group [1, 6]. However, the authors have noted the special role of the *Planctomycetes* in sponge-associated communities in the oceanic processes of denitrification [32]. Apparently, accumulation of these microorganisms in Baikal sponge-associated communities may also be ascribed to their role in the nitrogen and carbon cycles.

The other phyla revealed in microbiomes of Baikal sponges accounted for less than 2.5%. In the community associated with *L. baicalensis*, these phyla included *Verrucomicrobia*, *Chloroflexi*, the candidate division OD1 phylum, and unidentified phylotypes (Table 2). The *Baikalospongia* sp. sponge was characterized by the presence of the phylum *Nitrospirae* (Table 3). Some of the representatives of the minor phyla have previously been detected in freshwater sponge-associated communities: *L. baicalensis* (*Verrucomicrobia* and *Nitrospirae*) [8]; *E. fluviatilis* (*Verrucomicrobia*) [10]; and *S. lacustris* (*Chloroflexi*) [9]. The phylum *Verrucomicrobia* is common in freshwater habitats, constituting up to 6% of bacterial communities [17]. *Verrucomicrobia* (the family *Opiritaceae*) were detected in plankton of 73 out of 81 European lakes [17] and were rather abundant (3.4%) in Baikal [11]. *Verrucomicrobia* occur in both surface water and the hypolimnion, which evidences the versatile metabolic strategies of the group [27].

The candidate division OD1 has been revealed relatively recently; representatives of OD1 members are numerous in anaerobic habitats in both marine and freshwater communities [33]. Genome analysis demonstrated that OD1 bacteria in the sediments are able to reduce sulfur and participate in decomposition of complex organic compounds [34]; they may also be involved in anaerobic oxidation of methane and in the processes of autotrophic denitrification [35]. The candidate division OD1 was detected in microbial communities of several Red Sea sponges [6]. In freshwater sponge-associated communities, the phylum has never been reported previously.

Chloroflexi is a physiologically diverse and abundant phylum detected in soil, marine, and freshwater

communities, as well as in geothermal springs, benthos, and sediments [17]. The *Chloroflexi* associate with various marine sponges forms specific phylogenetic clusters. In some sponge-associated communities, the phylum was found to be dominating. Within sponges, the *Chloroflexi* may play an important role in anoxygenic photosynthesis [36]. This is the first report on *Chloroflexi* detection in freshwater sponge associations. The rate of similarity with the *Chloroflexi* from marine sponge tissues was less than 85%. In plankton of Lake Baikal, the phylum has previously been revealed as a minor group [11].

A nitrite-oxidizing *Nitrospira moscoviensis* NSP M-1 (NR029287) strain was the representative phylotype of the phylum *Nitrospirae*. It is known that the *Nitrospira* spp. participate in the second stage of nitrification, oxidizing nitrite into nitrate. Nitrite-oxidizing bacteria play an important role in removing nitrite, which is toxic for living organisms, from the environment [37]. The phylum has been detected in a number of marine sponges [4] and has previously been found in the Lake Baikal sponge *L. baicalensis*, comprising 2% of the bacteria [8]. In Baikal plankton community, the phylum was represented by few sequences [11].

Therefore, based on cluster analysis the major systematic groups typical for two microbial communities associated with Lake Baikal sponges were identified. In general, the results of BLASTn analysis (Fig. 3) agreed with cluster analysis in both composition and proportion of the dominating phyla. It should be noted that BLASTn analysis also revealed the phylum of *Cyanobacteria*, with a share in the studied communities of approximately 1.4% (Fig. 3). Sequences of cyanobacteria at the cluster distance of 0.3 were among the most abundant clusters of the *Proteobacteria*. Maximum distance at which cyanobacteria formed an independent cluster was 0.13 for the *L. baicalensis* community and 0.2 for the *Baikalospongia* sp. community. Over 90% of the cyanobacterial sequences revealed belonged to a picoplankton genus *Synechococcus*. Cyanobacteria are known to be an essential part of the Lake Baikal ecosystem, present in both planktonic and benthic microbiomes [11].

Comparative analysis of the bacterial communities of Lake Baikal sponges and of bacterioplankton at the sites of sample collection demonstrated the presence of 110 common OTUs at the level of species (97% homology) and 96 common OTUs at the level of genus (95% homology). Communities of *L. baicalensis* and *Baikalospongia* sp. shared 75 common OTUs at the level of species (97% homology) and 62 common OTUs at the level of genus (95% homology) (Fig. 4). Over 50% of the bacterial phylotypes of each sponge were unique, not having been detected in other communities, and 55% of the planktonic phylotypes have not been detected in associations with sponges. Considerable differences in the microbiomes of sponges have been demonstrated for marine species, where up

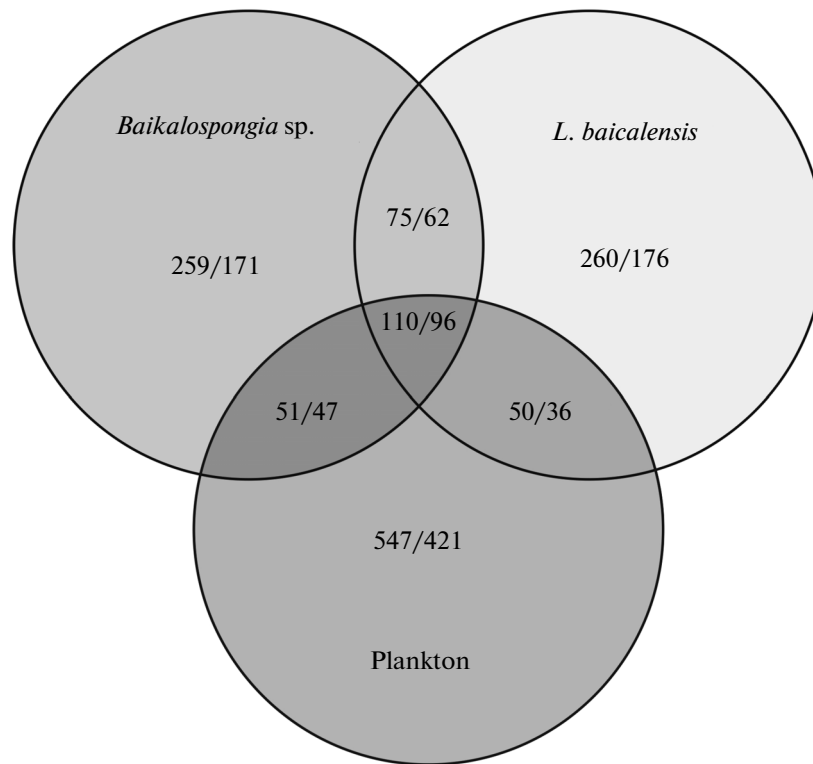


Fig. 4. Venn diagram. The number of common OTUs in bacterial communities of sponges and plankton from Lake Baikal (97/95% sequence homology).

to 70% of bacterial sequences were unique for sponge-associated communities [19]. The library of bacterial sequences from a freshwater sponge *E. fluviatilis* also differed from the water community by species composition [14]. In both marine and freshwater sponges, the existence of specific bacterial clusters found only in sponge tissues has been demonstrated [1, 7, 14].

Sponge species used in this work are endemic for Baikal and differ by life forms they generate: *L. baicalensis* is a 1–1.5 m high branching sponge, while species of the genus *Baikalospongia* generate encrusting forms on stones of the underwater slopes of the lake. This is the first report on application of pyrosequencing to the analysis of freshwater sponge-associated communities of microorganisms. Analysis of the 16S rRNA gene libraries of freshwater sponges *L. baicalensis* and *E. fluviatilis* [12, 14] provided merely a general concept of the composition of the associated bacterial phyla with only occasional clusters identified at the level of genus or family. Pyrosequencing provided for a detailed analysis of taxonomy composition and quantitative proportions of different microorganism groups of the Lake Baikal sponge-associated communities. In total, 24 phyla were revealed, 18 of them were represented by 1–10 sequences. The results of the study evidence that taxonomic diversity of the freshwater sponge-associated communities is comparable to

that of known marine representatives with 28 bacterial phyla identified [7].

Both marine and freshwater sponges are comprised of *Proteobacteria* and *Actinobacteria* as dominating phyla. An important difference of the Baikal sponge-associated communities was predominance of the phylum *Bacteroidetes*, which is typical of the planktonic community of Lake Baikal and a number of other lake communities [11, 38, 39]. The *Bacteroidetes* accounted for up to 50% of the bacteria associated with sponge tissues, while their share in the plankton was only 33%. Among the minor phyla, proportion of *Cyanobacteria* (2.8%) and *Verrucomicrobia* (3.5%) was higher in the plankton, while the content of *Planctomycetes* was several times higher in sponge-associated communities (Fig. 3). In the plankton 28 phyla were identified, versus 23 phyla in *L. baicalensis* and 19 phyla in *Baikalospongia* sp.; total species diversity of the plankton was two times higher than in the sponges.

Apparently, under conditions of the Lake Baikal ecosystem, sponge tissues accumulate certain representatives of bacterioplankton, mostly the representatives of the *Bacteroidetes*, *Proteobacteria*, and *Planctomycetes*, thus forming a symbiotic community. The surface and internal space of sponges are a more favorable substrate for symbionts than the surrounding water since it contains more nutrients [40]. Symbiotic microorganisms help the nutritive process, participat-

ing in intracellular digestion, nitrogen fixation, nitrification, and photosynthesis. Microorganisms also stabilize the sponge skeleton and promote protection from carnivores and encrusting by secretion of chemicals [41].

The results of the 16S rRNA gene pyrosequencing evidence that, despite certain common features of the taxonomic composition, two endemic Lake Baikal sponges develop unique bacterial communities with a set of individual OTUs, although both communities are characterized by the same values of species diversity. Undoubtedly, these data considerably expand our knowledge on the structure and diversity of microorganisms associated with freshwater sponges. For a comprehensive understanding of the principles of symbiotic interactions, functioning, and co-evolution of microbial associations with freshwater sponges, further studies aimed at analysis of interspecies, seasonal, ecological, and other specific features of the freshwater sponge-associated communities are required.

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