



Molecular identification of filamentous cyanobacteria overgrowing the endemic sponge *Lubomirskia baicalensis*

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ABSTRACT

In recent years, the surfaces of endemic Baikalian sponges have exhibited signs of damage and disease such as tissue bleaching, atypical microbial films, and necrotic areas. One of the most common sources of damage on branching sponges *Lubomirskia baicalensis* is a reddish-brown mucous film fully or partially covering the body of the sponge. Sequencing of the 16S rDNA gene revealed that the film on the surfaces of the sponge consists of Oscillatoriales cyanobacteria belonging to the following genera: *Tychonema*, *Phormidium*, and *Leptolyngbya*, with 44 of the 86 identified sequences belonging to *Tychonema*. Cyanobacterial fouling of the Baikalian sponge may be caused by changes in environmental conditions of the sponge habitat, in particular, temperature increases and eutrophication of the lakes.

KEYWORDS

Endemic sponges; filamentous cyanobacteria; fouling; Lake Baikal; *Leptolyngbya*; *Lubomirskia baicalensis*; *Phormidium*; *Tychonema*

Introduction

Water filtering organisms are bioindicators of the ecological state of aquatic environments (Gadzała-Kopciuch et al. 2004, Volkmer-Ribeiro and de Souza Machado 2007). Sponges (phylum Porifera) in particular are sessile, filter-feeding, multicellular animals able to pump up to 24 000 L of water per 1 kg of body weight per day through a system of channels in their bodies (Taylor et al. 2007). Changes in environmental conditions such as anthropogenic pollution, temperature increase, and eutrophication can lead to diseases in sponges and subsequent death (Webster et al. 2008a, 2008b, Lyepez-Legentil et al. 2010). In Lake Baikal, the world's largest freshwater reservoir, sponges inhabit all depths (Efremova 2004, Kaluzhnaya and Itskovich 2014). The largest biomass of Baikalian sponges is at the depth where the level of light penetration causes them to be the dominant organisms of benthic communities; here they have a green color due to the development of photosymbiotic microorganisms in their tissues (Kozhov 1962, Pile et al. 1997, Kulikova et al. 2013). Of the 18 species of sponges that live in the lake, 14 are endemic (Efremova 2004), and *Lubomirskia baicalensis* is the only species with cylindrical “branches” that allow some specimens of *L. baicalensis* to reach a length of 1 m (Rezvoi 1936).

In recent years, it has been observed that multiple areas of the lake with numerous exemplars of *L. baicalensis* have

included sponges with tissue damage (bleaching, microbial fouling, and necrotic areas), leading eventually to the necrosis of the individual “branches” or death of the whole organism. Various studies of marine sponges and corals have shown that changes in environmental conditions affect the composition of their symbiotic microflora and lead to diseases and tissue damage (Webster et al. 2008a, 2008b, Lyepez-Legentil et al. 2010). In our recent study of the microbial composition of bleached tissue of *L. baicalensis* (Kaluzhnaya and Itskovich 2015), we also showed an increase in the number of cyanobacterial 16S rDNA sequences and an unusually low number of representatives of the phyla Bacteroidetes and Betaproteobacteria. Furthermore, some bacterial phylotypes related to microorganisms specific to substrates with a high content of organic compounds and heavy metals were detected in the microbiome (Kaluzhnaya and Itskovich 2015).

One of the most common signs of damage on Baikalian sponges is a reddish-brown mucous or brown film that partially or completely fouls the branches of *L. baicalensis* (Fig. 1). The aim of this study was to use molecular methods (16S rDNA sequencing) to determine the taxonomic affiliation of the microorganisms that overgrow the sponge and form mucous films and to explore whether this contributes to the damage of death of the endemic sponges in Lake Baikal.

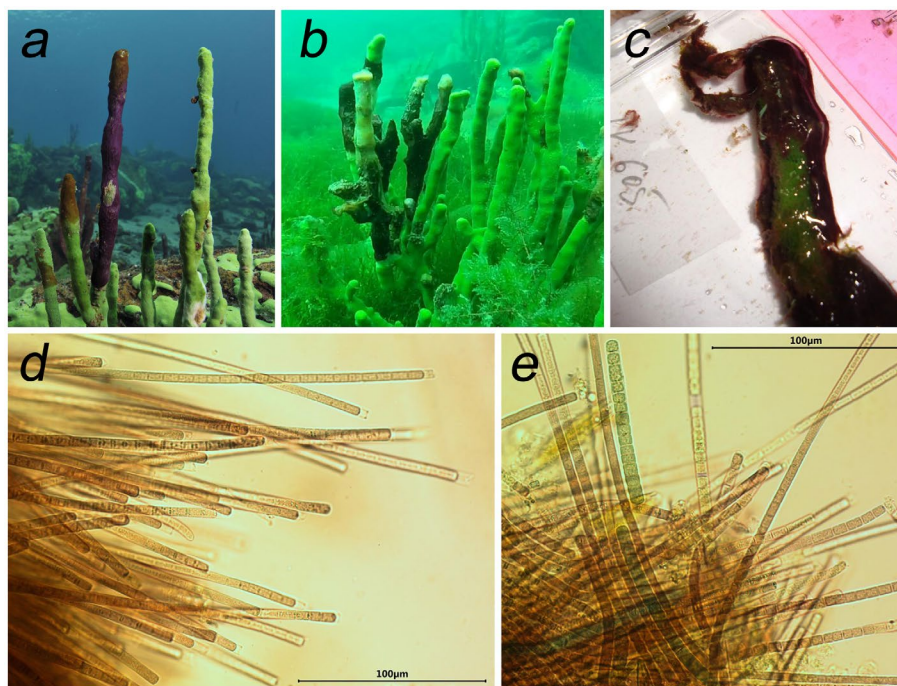


Figure 1. Cyanobacterial films on the surface of the endemic sponge *Lubomirskia baicalensis*: (a and b) underwater pictures of branching sponges, partially affected by fouling; (c) mucous film on the surface of the *L. baicalensis*, sample IK605; (d and e) microphotograph of filamentous cyanobacteria, forming mucous film.

Methods

Sponge tissue samples of *L. baicalensis* (IK605) covered with mucous film were collected at 10 m depth by SCUBA divers during expedition work in September 2014, in the area of Olkhon Island (west coast of Lake Baikal; 053°00.962N, 106°55.788W).

Light microscopy was performed using a microscope Olympus CX22 (Japan). Microscopic analysis suggested all samples contained similar cyanobacteria, and one sample was selected for molecular characterization. Fouling biofilms were separated from sponge tissue using a sterile scalpel and gloves, placed in liquid nitrogen in cryogenic microtubes, and stored at -70°C . The total DNA from the selected representative was isolated using the kit RiboSorb (Russia). For the molecular identification of microorganisms from the mucous biofilm, a partial region of the 16S rDNA gene (V2–V8) was amplified using cyanobacteria-specific primers:

PLG1.1 (109–128): 5-ACGGGTGAGTAACGCGTRA-3

PLG2.1 (1260–1282): 5-CTTATGCAGGCGAGTTGC-AGC-3 (Urbach et al. 1992).

The polymerase chain reaction (PCR) conditions were as follows: initial activation of Taq-DNA-polymerase for 5 min at 94°C , followed by 35 cycles of 30 s denaturation at 94°C , primer annealing for 60 s at 56°C , and elongation for 90 s at 72°C . The program ended with 10 min of final elongation at 72°C . The amplification products (~ 1200 bp)

were cloned into the vector pTZ57A/T (Fermentas), followed by transformation of chemically competent cells of *Escherichia coli* XL1BL. In total, 108 clones were PCR amplified from 16S rDNA clone library using M13 primers (Eurogen) following the cycling conditions: initial activation of Taq-DNA-polymerase for 5 min at 94°C , followed by 35 cycles of 30 s denaturation at 94°C , primer annealing for 60 s at 58°C , and elongation for 60 s at 72°C . Determination of nucleotide sequences of the 86 recombinant clones that were PCR positive was carried out on an automatic ABI sequencer 3130XL (Applied Biosystems). Sequences were aligned and percentages of their identity were defined using ClustalW software module of BioEdit 7.0 package. These sequences were compared to those published in genetic databases using the BlastN program. The nucleotide sequences were deposited in GenBank with the accession numbers: KU168738–KU168741.

Results

BlastN-analysis of 16S rDNA sequences showed that the composition of the mucous biofilm overgrowing the branching sponge *L. baicalensis* contained filamentous cyanobacteria (order Oscillatoriales) belonging to the genera *Tychonema*, *Phormidium*, and *Leptolyngbya*. Of the 86 sequences analyzed, 44 were similar to each other by more than 97%, and the consensus sequence showed a high identity to the species of the genus *Tychonema*:

Table 1. Taxonomic identification of cyanobacteria fouling the surface of *L. baicalensis*, based on the BlastN comparison of the 16S rDNA gene sequences obtained from the National Center for Biotechnology Information (NCBI).

Consensus sequences	NCBI accession number	Closest homologues with NCBI accession number	Identity
01T_LB (n = 44)	KU168738	<i>Tychonema</i> sp. K27 (GQ324965)	99%
		<i>Tychonema borneyi</i> NIVA-CYA (LM651414);	99%
		<i>Tychonema bourrellyi</i> NIVA-CYA 96/3 (LM651417)	99%
02L_LB (n = 19)	KU168739	<i>Leptolyngbya</i> sp. ATA11-WF-KO3 (KJ939030)	99%
		<i>Leptolyngbya</i> sp. SAG 2411 (KF417652)	97%
03P1_LB (n = 14)	KU168740	Uncultured <i>Phormidium</i> sp. MED-28 (JN382236)	96%
04P2_LB (n = 8)	KU168741	Uncultured Antarctic cyanobacterium Fr048 (AY151726)	98%
		<i>Phormidium autumnale</i> CYN79 (JQ687337)	97%
		<i>Phormidium</i> cf. <i>uncinatum</i> CAW-BG523 (JX088098)	97%

T. borneyi, *T. bourrellyi*, and *T. sp.* K27 from biofilms of European and Antarctic water reservoirs (Table 1). The sequences of 19 clones were united in one phylotype with 97% to 99% similarity to the cyanobacterial strains *Leptolyngbya* sp.; 22 clones were closely related to species of the genus *Phormidium* and formed 2 phylotypes, one of which showed 96% identity to the uncultivated clone *P. sp.* MED-28 from microbial mats of a freshwater stream. The other phylotype had 98% similarity to the cyanobacterial clone of Antarctic microbial mats and the Antarctic strain *P. autumnale* CYN79 (97%; Table 1; Martineau et al. 2013).

Discussion

The cyanobacterial order Oscillatoriales includes filamentous genera that can form biofilms or mats (Jungblut et al. 2010, Loza et al. 2013). Species of the genera *Phormidium* and *Leptolyngbya* are widespread in various marine and freshwater ecosystems, including lakes (Marquardt and Palinska 2007, Stoyanov et al. 2014). Representatives of the genus *Tychonema* are stenothermal psychrophilic microorganisms that mainly inhabit the northern latitudes (Shams et al. 2015), so the presence of this genus in the Siberian oligotrophic reservoir (Lake Baikal) is natural. Note that, based on the number of 16S rDNA sequences, the *Tychonema* species are dominant (about 50% of the analyzed clones) in the fouling cyanobacterial community of *L. baicalensis*. Previously, multiple populations of *T. bourrellyi* were observed in aquatic communities in Northern Europe and Canada, South Norway, and Northern Ireland, as well as in Western Europe (Marquardt and Palinska 2007, Stoyanov et al. 2014). The dominance of *Tychonema* species in cyanobacterial mats

in the creeks on the coast of Antarctica was shown in Callejas et al. (2011). The authors found that 83% of the 16S rDNA clones in the investigated community belonged to *Tychonema* (*T. bourrellyi* and *T. borneyi*). In addition, some species of the genus *Tychonema* are capable of producing the neurotoxin anatoxin-a (Lopez-Legentil et al. 2010, Quiblier et al. 2013).

Intensive development of cyanobacterial mats has long been a threat to coral reef communities. In geographically distant areas of the world's oceans, filamentous cyanobacteria form slimy mats on the surface of marine corals. The cyanobacterial mats of various species of coral are dominated by representatives of a wide range of genera such as *Hydrocoleum*, *Spirulina*, *Symploca*, *Phormidium*, *Nodularia*, *Lyngbya*, *Microcoleus*, and *Oscillatoria* (Charpy et al. 2012). Note that one of the common diseases of corals caused by cyanobacterial fouling is "black band disease," in which the laminated microbial mats migrate horizontally on the surface of the coral and completely destroy its tissues (Sato et al. 2010). Filamentous cyanobacteria are dominant in the composition of such laminated films as well as in mucous films of Baikal sponges. They are also notably present on the coral reefs of the Hawaiian Islands as the species *Phormidium coraliticum* and *Pseudoscillatoria coralii* (Aeby et al. 2015) and on the reefs near the island of Okinawa (Japan) as *Moorea bouillonii* (Yamashiro et al. 2014).

Global climate change and other anthropogenic impacts close to coral reef communities can promote mass coral fouling by cyanobacterial proliferations. Development of cyanobacterial mats can be stimulated by an increase in annual solar radiation, average temperature, and concentration of phosphates, nitrates, and nitrites in the water (Charpy et al. 2012, Brocke et al. 2015, Sato et al. 2015). Researchers have noted that increased anthropogenic nutrient loading (from the development of industrial enterprises, agricultural lands, and tourist infrastructure) leads to eutrophication of the coral reef habitat. Additionally, organic matter from the urban areas is transported by surface runoff and settles in areas with low hydrodynamics. This organic matter can decompose and increase levels of nitrogen and phosphorus, further stimulating the development of cyanobacterial mats. For example, on the coast of Australia, proliferation of benthic cyanobacteria *Lyngbya majuscula* in reef communities was preceded by rain showers and a period of high solar radiation.

Based on the research of coral reef communities, it can be assumed that the formation of cyanobacterial biofilms on surfaces of branched Baikalian sponges was caused by similar changes in habitat. Because branching sponges most actively develop at shallow depths (upper littoral zone) and are exposed to climatic and anthropogenic

impacts, they are likely to respond rapidly to changing environmental conditions. This hypothesis is supported by studies of climate change in the area of the Lake Baikal and data from the Federal Service for Hydrometeorology and Environmental Monitoring (Miach et al. 2011, Climate Changes 2015, Review of Pollution 2015), which in the last decade in the Baikal region show an increase in average annual temperatures near certain settlements (Miach et al. 2011). In addition, the presence of harmful substances in the atmosphere was observed as well as higher levels of organic phosphorus and nitrogen, sulfate ions, chloride ions, and polycyclic aromatic hydrocarbons in the sediment (Climate Changes 2015).

As shown by studies of sponge and coral communities, adverse ecological impacts can change the composition of the natural microflora, resulting in the appearance of microorganisms atypical for the symbiotic associations (Webster et al. 2008a, 2008b, Angermeier et al. 2010, Lypetz-Legentil et al. 2010). Our previous work (Kaluzhnaya and Itskovich 2015) showed that, in the microbiome of Baikalian sponge tissue that had undergone bleaching processes, some phylotypes of bacteria-decomposers of organic matter were present as well as microorganisms, which is typical of ecosystems contaminated by human waste (Kaluzhnaya and Itskovich 2015).

We suggest that products of organic matter decomposition originating from anthropogenic effluents and other substances entering the lake contribute to a favorable environment for the development of cyanobacteria on the surface of sponges. Microbial communities inhabiting sponges are the complex consortia performing various metabolic functions, including protecting sponges from foreign microflora. The sponge-associated bacteria are capable of producing biologically active substances that exhibit antiviral, antimicrobial, antifungal, cytotoxic, and other properties (Thomas et al. 2010). In particular, our previous investigations (Kaluzhnaya et al. 2012, Kaluzhnaya and Itskovich 2016) reported the presence of genes of bioactive metabolites for different types of Baikalian sponge communities. It can be assumed that the intensive development of cyanobacterial films on the sponge surface may cause a shift in these communities, affecting the protective functions of the community as a result of changes in the composition of the natural sponge microbiome. The release of toxins by filamentous cyanobacteria may also affect these communities, although further work is required to confirm this.

Our study showed for the first time that the laminated mucous films on the surface of Baikalian sponges are formed by the filamentous cyanobacteria of genera *Tychonema*, *Phormidium*, and *Leptolyngbya*. Similar processes are well known for coral reef communities in which the intensive

development of cyanobacterial biofilms and “black band disease” lead to the destruction of coral tissues and coral death. The mucous films on the surface of Baikalian sponges, together with diseases, are evidence of adverse ecological conditions prevailing in some areas of the lake, most likely due to the combined effect of climatic and anthropogenic effects on the ecosystem of Lake Baikal. Further research is needed to clarify the causes of Baikalian sponge diseases and relate these processes to climate dynamics and environmental conditions in the Baikal region.

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