

## RESEARCH ARTICLE

## Bacteria associated with planktonic diatoms from Lake Baikal

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Algal-bacterial associations were studied in unialgal xenic cultures of *Synedra acus* subsp. *radians*, *Asterionella formosa* and *Fragillaria crotonensis* planktonic diatoms from Lake Baikal, using epifluorescent and scanning electron microscopy. It was found that rod- and ovoid-shaped bacteria colonized cell walls of diatoms. Cloning and sequencing of fragments of 16S rRNA gene in diatom cultures revealed members of Gammaproteobacteria (*Pseudomonas* sp.), Betaproteobacteria (*Janthinobacterium* sp., *Hydrogenophaga* sp., *Methylophilus* sp.), Bacteroidetes (*Flavobacterium* sp., *Pedobacter* sp.), and Actinobacteria (*Nocardioideis* sp.).

**Key words:** diatoms; bacteria; 16S rRNA sequencing; Lake Baikal

### Introduction

Phytoplankton and bacteria play a key role in biogeochemical cycles in aquatic ecosystems (Cole, 1982; Azam, Malfatti, 2007). Diatoms are common microeukaryotes responsible for 20% of the photosynthesis on Earth (Armbrust, 2009). Diatom cells are surrounded by phycosphere containing extracellular metabolites that stimulate growth of heterotrophic bacteria (Amin et al., 2012; Seymour et al., 2017). Relationships between diatoms and bacteria vary from symbiotic to commensal to parasitic (Cole, 1982; Amin et al., 2012). Heterotrophic bacteria associated with phytoplankton consume organic matter produced by live algae or generated during their lysis (Riemann et al., 2000; Azam, Malfatti, 2007). Bacteria facilitate the build-up of aggregates from diatom cells and other compounds, thereby, increasing settlement of organic compounds in the water column (Gärdes et al., 2011). Determining the composition of bacterial communities associated with certain species of algae helps to determine the relationship between phytoplankton and bacteria. Composition and physiological characteristics of diatom-associated bacteria are typically studied using lab cultures (Schäfer et al., 2002; Grossart et al., 2005; Sison-Mangus et al., 2014; Mila et al., 2018). Members of Proteobacteria, Bacteroidetes and Actinobacteria were detected earlier in marine (Schäfer et al., 2002; Grossart et al., 2005; Sapp et al., 2007a; Sapp et al., 2007b; Guannel et al., 2011) and freshwater diatom cultures (Bruckner et al., 2008; Zakharova et al., 2010; Mila et al., 2018). When co-cultured with bacteria, diatoms increase production of polysaccharides (Bruckner et al., 2008). Bacteria produce vitamins necessary for microalgae growth (Croft et al., 2005).

In the spring, a massive bloom of phytoplankton dominated by diatoms (13-98% of phytoplankton biomass) takes place in Lake Baikal (Popovskaya et al. 2015). Although the composition of dominant phytoplankton species is different in different areas of the lake, the composition of bacterial communities is similar (Mikhailov et al., 2015). Earlier, culturable heterotrophic Proteobacteria and Actinobacteria were found in culture of *Synedra acus* subsp. *radians* (Kütz.) Skabitsch (*Fragillaria radians* (Kützing) D.M. Williams & Round) isolated from the Listvenichny Bay (Lake Baikal) (Zakharova et al., 2010). Since nonculturable bacteria account for a large share of bacterial communities, the analysis of fragments of 16S rRNA gene from the total DNA allows to better identify the composition of bacterial communities (Schäfer et al., 2002; Grossart et al., 2005; Mila et al., 2018).

The goal of this work was to study algal-bacterial associations using microscopy and to determine the bacterial composition in cultures of planktonic diatoms by means of cloning and sequencing of the 16S rRNA gene.

## Materials and Methods

**Objects of study.** Phytoplankton samples collected with a Jedy net in the photic 0-25 m layer from the RV 'G.Yu. Vereschagin' in late May – early June 2011 in the southern basin of the lake at the Listvyanka-Tankhoy and Peschanaya Bay stations (1,425 m and 966 m depths, respectively); and in the middle basin in the Chuvyrkuy Bay (10 m depth) (Fig. 1). Using micromanipulation, single diatom cells were transferred to a well of a 96-well microplate (Linbro Biomedicals, INC, Denmark) containing 200  $\mu$ l of sterile diatom medium (DM) (Zakharova et al., 2010). Cells were grown in a microincubator at 8°C and continuous light at 16  $\mu$ mol of photons  $m^{-2} s^{-1}$  on a 12-hour alternating day/night cycle (Safonova et al., 2007). As the number of diatom cells reached  $10^3$ , unialgal cultures were transferred to a 1 L Erlenmeyer flask for further growth. Diatoms were cultivated for 8 months as described above.

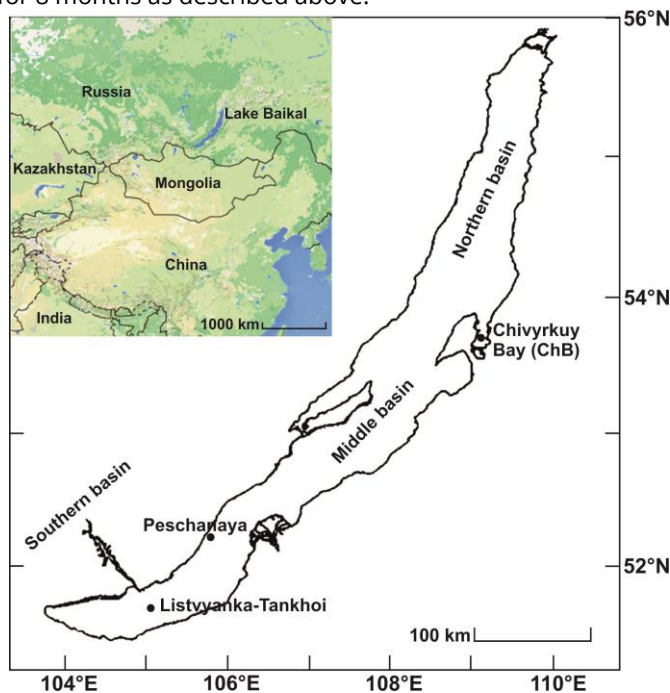


Fig. 1. Map of sampling stations at Lake Baikal

**Epifluorescent microscopy.** Total bacterial abundance was determined in a 1 ml sample fixed with glutaraldehyde and stained with 4,6 diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA) with a final concentration of 1  $\mu$ g/ml during 15 minutes (Porter, Feig, 1980). A stained sample was filtered through a polycarbonate membrane (0.2  $\mu$ m) (Whatman GE Healthcare Life Science, UK) using a filter unit (Sartorius, Germany). Bacterial cells were enumerated using an inverted microscope Axiovert 200 (Carl Zeiss, Germany) with an ultraviolet lamp HBO 50W/AC ASRAM with an excitation spectrum of 365 nm. For total bacterial abundance counts were taken at least 20 fields of view per sample.

**Scanning electron microscopy.** Scanning electron microscopy was used to analyze diatom-bacteria associations. A diatom culture sample was fixed with a 1% glutaraldehyde solution, placed on 0.2  $\mu$ m polycarbonate filters (Millipore, Ireland), and air dried. The sample was dehydrated in ethanol solutions of 30%, 50%, 70% and 96% (during 10 min each time) and then dried. A filter holding the sample was then fixed at a SEM screen with a double-sided scotch tape and coated with gold in a vacuum unit SDC 004 (BALZERS). Samples were examined under a scanning electron microscope FEI Quanta 200 (USA).

**DNA extraction, amplification, cloning and sequencing.** One hundred ml of diatom cultures that were at the exponential growth stage (after 10 days of culturing) were filtered through 0.2  $\mu$ m polycarbonate filters (Millipore, Ireland). Biomass was then washed in sterile flasks containing 5 ml of TE buffer (10mM Tris-HCl containing 1mM EDTA, pH 7.5) and stored at -20°C. DNA was extracted from the biomass with lysozyme, 10% SDS and phenol-chloroform (Marmur, 1961). Fragments of the 16S rRNA gene were amplified using the polymerase chain reaction (PCR) with primers 27L (AGAGTTTGATCMTGGCTCAG), 500L (CGTGCCAGCAGCCGCGGTAA) and 1350R (GACGGGCGGTGTGTACAAG) (Denisova et al., 1999). Obtained PCR products were ligated in pJET using CloneJET™ PCR Cloning Kit. Ligation and transformation were performed according to a methodology (Inoue et al., 1990). Sequencing of fragments of the 16S rRNA gene was performed using BigDye V 3.1 Terminator Cycle sequencing kit and AmpliTaqDNA polymerase FS (Applied Biosystems) on a genetic analyzer ABI 3130XL Genetic Analyser (Applied Biosystems) in Genomics Core Facility (Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk).

**Sequencing data analysis.** Sequences obtained during the sequence analysis were compared to sequences in GenBank, EMBL and DDBL databases via the BLASTN program. MEGA5.1 program and “neighborjoining” algorithm were used to compare sequences and create phylogenetic trees. Statistical accuracy of branching was evaluated with the

“bootstrap analysis” (1,000 replicates). 16S rRNA sequences consisting of 430-694 base pairs were deposited in GenBank with numbers KR870424 – KR870434.

## Results and discussion

Four unialgal xenic cultures of diatoms isolated from the lake’s plankton were used to analyze algal-bacterial associations: two *S. acus* subsp. *radians* cultures (Listvyanka-Tankhoi – Itsa; Peschanaya Bay – psa); *Asterionella formosa* Hassall and *Fragillaria crotonensis* Kitton cultures (Chivyrkuysky Bay – chaf and chfc, respectively). Diatom cells differed in size (Table 1, Fig. 2). In 10-day cultures, the number of diatoms and bacteria was  $2.4\text{--}3.3\times 10^3$  cell/ml and  $0.9\text{--}1.2\times 10^6$  cell/ml, respectively (Table 1). The number of bacteria in diatom cultures (Table 1) is similar to the number of bacteria in plankton of Lake Baikal during spring diatom blooms ( $0.2\text{--}2.2\times 10^6$  cell/ml) (Mikhailov et al., 2015).

Table 1. Diatom and bacteria counts in cultures

Diatom cultures	Length & width of diatom cells	Diatom abundance, cells/ml	Total bacterial abundance, cells/ml
<i>Synedra acus</i> subsp. <i>radians</i> Itsa	160-200 $\mu\text{m}$ , 3 $\mu\text{m}$	$3,3\times 10^3$	$0,9\times 10^6$
<i>Synedra acus</i> subsp. <i>radians</i> psa	160-200 $\mu\text{m}$ , 3 $\mu\text{m}$	$2,4\times 10^3$	$0,9\times 10^6$
<i>Asterionella formosa</i> chaf	40-50 $\mu\text{m}$ , 3 $\mu\text{m}$	$3,0\times 10^3$	$1,0\times 10^6$
<i>Fragillaria crotonensis</i> chfc	30-40 $\mu\text{m}$ , 4 $\mu\text{m}$	$2,9\times 10^3$	$1,2\times 10^6$

Free-living bacteria and diatom-attached bacteria were found in cultures. Scanning electron microscopy revealed that rod- and ovoid-shaped bacteria colonized diatom cell walls (Fig. 2). Bacteria were attached to the diatom cells either individually or in microcolonies (Fig. 2). In some cases, bacteria were located near diatom’s cingulum (Fig. 2e) and combined several diatom cells into aggregates (Fig. 1c). Bacteria in diatom cultures grow without additional sources of carbon, therefore, they use organic matter produced by diatoms. Bacteria associated with phytoplankton blooms have transporters and enzymes to consume low molecular weight molecules and to degrade complex polymer organic compounds (Buchan et al., 2014).

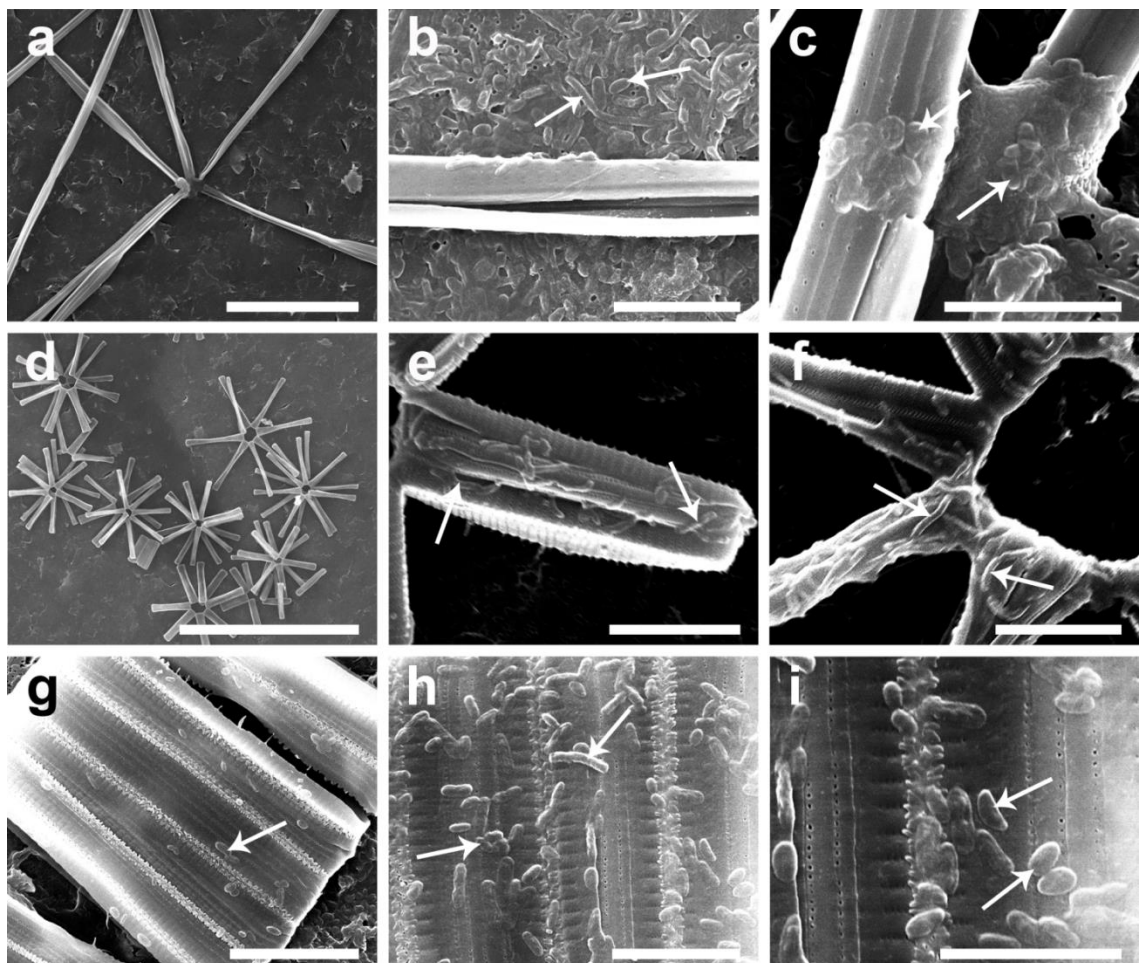


Fig. 2. Associations of diatoms and bacteria in unialgal xenic cultures. a, b, c – *S. acus* subsp. *radians*; d, e, f – *A. formosa*; g, h, i – *F. crotonensis*. Arrows point to bacteria. SEM. Scale: a – 50  $\mu\text{m}$ ; b, e, f, h – 5  $\mu\text{m}$ ; c, i – 4  $\mu\text{m}$ ; d – 100  $\mu\text{m}$ ; g – 10  $\mu\text{m}$ .

Sequences of 16S rRNA genes in diatom cultures were found to be 99-100% similar to sequences of bacteria from GenBank belonging to Proteobacteria, Bacteroidetes, and Actinobacteria (Fig. 3). Only sequences of *Pseudomonas* sp. were found in *S. acus* subsp. *radians* cultures (Itsa, psa) (Fig. 3). Earlier, heterotrophic bacteria of *Sphingomonas* sp., *Caulobacter vibrioides*, *Brevundimonas vesicularis* (Alphaproteobacteria), *Variovorax paradoxus* (Betaproteobacteria), *Pseudomonas fluorescens* (Gammaproteobacteria) и *Microbacterium trichotecenolyticum*, *Rhodococcus* sp. (Actinobacteria) were found in *S. acus* subsp. *radians* culture incubated in a 100 L photobioreactor (Zakharova et al., 2010). Sequences of *Flavobacterium* sp. (Bacteroidetes), *Pseudomonas* sp. (Gammaproteobacteria), *Hydrogenophaga* sp., *Methylophilus* sp. (Betaproteobacteria), and *Nocardioides* sp. (Actinobacteria) were detected in *A. formosa* culture (chaf) (Fig. 3). In the culture of *A. formosa* isolated from a small Esthwaite Water lake (UK), high-throughput sequencing revealed operational taxonomic units most similar to *Flavobacterium*, *Sediminibacterium*, *Leadbetterella* (Bacteroidetes), *Gemmobacter*, *Rhodopseudomonas*, *Novosphingobium* (Alphaproteobacteria), *Pseudomonas* (Gammaproteobacteria), *Aquabacterium*, *Rhizobacter*, *Variovorax*, *Pelomonas* (Betaproteobacteria) (Mila et al., 2018). Sequences of *Flavobacterium* sp., *Pedobacter* sp., and *Janthinobacterium* sp. were found in *F. crotonensis* culture (chfc) (Fig. 3). Obtained 16S rRNA sequences of bacteria in cultures of different Baikal diatoms are most similar to sequences found in freshwater reservoirs, plant surfaces and biofilms of precipitations and stones.

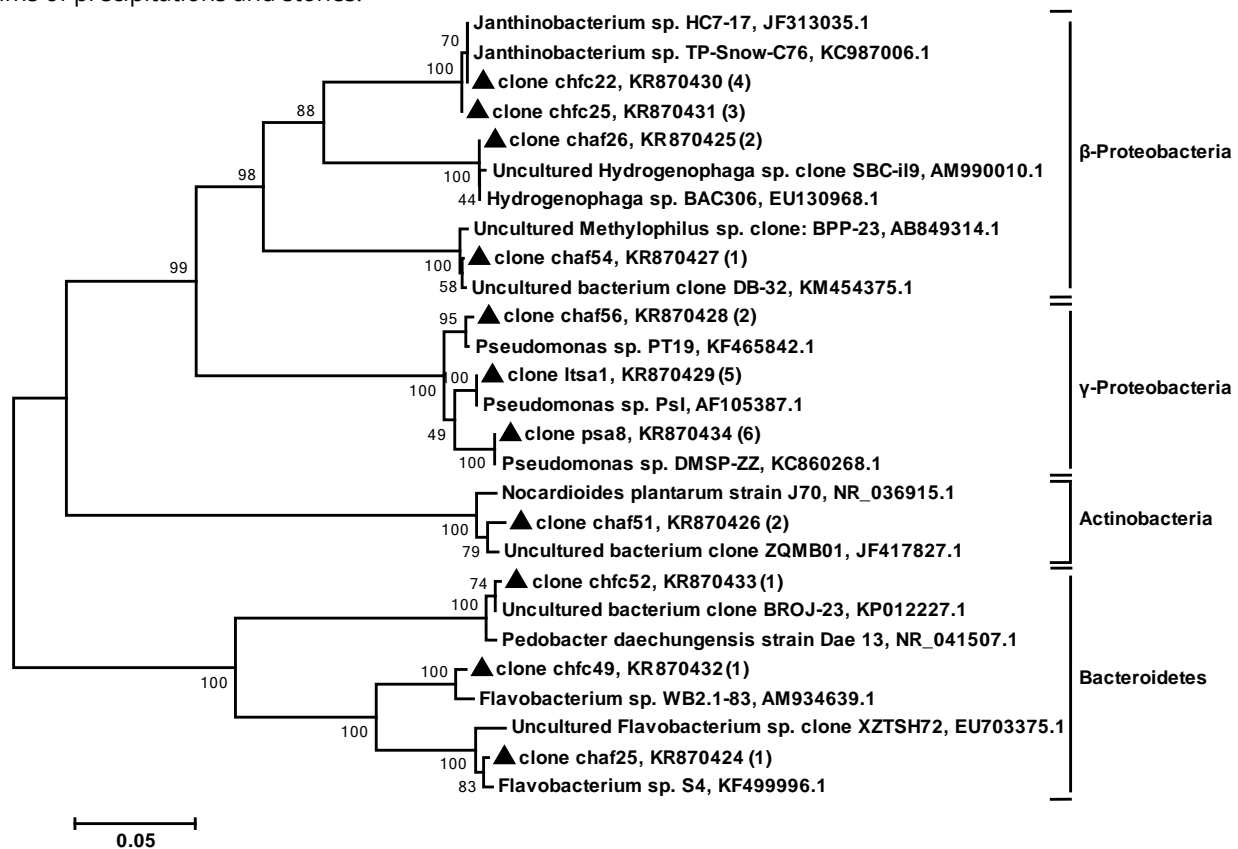


Fig. 3. A phylogenetic tree of 16S rRNA gene fragments for bacteria associated with *S. acus* subsp. *radians* (Itsa, psa), *A. formosa* (chaf) and *F. crotonensis* (chfc), and similar sequences from the GenBank database. Triangles show obtained sequences. The number of sequences are shown in parentheses.

The number of bacterial phylotypes is significantly lower in diatom cultures (Fig. 3) than in the lake's plankton during phytoplankton bloom (Mikhailov et al., 2015). The diversity of bacteria is lower in diatom cultures than in natural phytoplankton samples. Bacteria that are dominant in natural conditions are typically found in diatom cultures (Schäfer et al., 2002). Gammaproteobacteria (*Pseudomonas* sp.) were found in three diatom cultures (*S. acus* subsp. *radians* Itsa, psa, and *A. formosa* chaf), while Betaproteobacteria and Bacteroidetes were found in two (*A. formosa* chaf and *F. crotonensis* chfc), and Actinobacteria was found in one (*A. formosa* chaf). During spring diatom blooms Actinobacteria, Bacteroidetes, and, sometimes, Verrucomicrobia dominated in Lake Baikal. Betaproteobacteria and Gammaproteobacteria are present in much smaller quantities (Mikhailov et al., 2015). Therefore that diatom cultures were found to have taxa of bacteria that are predominant and minor in the lake. Bacteria phylotypes that are prevalent in diatom cultures can be rare in natural conditions. For example, Actinobacteria dominated in hypereutrophic Barra Bonita Reservoir (Brazil), while Alphaproteobacteria, Betaproteobacteria and Bacteroidetes were represented in small quantities. During co-culturing of axenic culture of *Aulacoseira granulata* (Ehrenberg) Simonsen diatom and natural inoculum, the dominant taxa were Alphaproteobacteria, Betaproteobacteria and Bacteroidetes, while the number of Actinobacteria declined significantly (Bagatini et al., 2014). Proteobacteria and Bacteroidetes are the main phyla of heterotrophic bacteria associated with diatoms. Within these phyla, such species as *Sulfitobacter*, *Roseobacter*, *Alteromonas* and *Flavobacterium* are most

frequently found in associations with diatoms (Grossart et al., 2005; Sapp et al., 2007a; Sapp et al., 2007b; Amin et al., 2012).

Therefore, in cultures Baikal diatoms have associations with bacteria that are found in bacterial communities in the photic layer of the lake. The increase in number of diatoms in cultures indicates that bacteria do not have a negative impact on them. Algal-bacterial associations represent a sustainable form of coexistence benefiting both partners.

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