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PCR-screening of bacterial strains isolated from the microbiome of the *Lubomirskia baicalensis* sponge for the presence of secondary metabolite synthesis genes

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ABSTRACT. The microbial communities of sponges (Porifera) are often a source of natural bioactive metabolites. From the microbiome of the endemic *Lubomirskia baicalensis* sponge, 35 bacterial strains were isolated and identified using molecular methods. The strains belonged to the phyla Actinobacteria, Firmicutes, and Proteobacteria (classes Alpha- and Betapriteobacteria). To analyze the strains for the presence of genes in the synthesis of secondary metabolites, polyketide synthases (PKS), PCR screening was applied using degenerate primers. Overall, 15 out of 35 strains contained PCR products corresponding in size to a fragment of the ketosynthase domain of the PKS gene cluster. Thus, the proposed method is applicable for rapid screening of the potential ability of microorganisms of different taxonomic groups to produce secondary metabolites. The work contributes to the study of the taxonomic diversity of cultured microorganisms, potential producers of biologically active substances, isolated from the microbiomes of Baikal sponges.

Keywords: Lake Baikal, sponges, *Lubomirskia baicalensis*, bacterial strains, genes of bioactive metabolite synthesis, polyketide synthase, PCR-screening

1. Introduction

Symbiotic associations between sponges and microorganisms have existed for over 600 million years (Wilkinson et al., 1984; Love et al., 2009). Sponges are an important component of the benthic fauna of the world's oceans, as well as many freshwater habitats (Hooper and van Soest, 2002; Bell, 2007). These animals are effective filter feeders: the volume of water that a sponge pumps per day can 10,000 times exceed its body volume (Weisz et al., 2008). Sponge microorganisms average about 35% of the animal biomass, reaching 70% in some species (Taylor et al., 2007; Ribes et al., 2012). Many metabolites of bacterial origin (such as antibiotics, toxins or statins) are known to be polyketides and synthesized by multienzyme complexes, polyketide synthases (PKS). Such multi-enzyme complexes use acyl-coenzyme-A monomers as a substrate and consist of several proteins,"building blocks" (Ehrenreich et al., 2005; Barrios-Llerena et al., 2007). Each protein has a domain structure and, accordingly, several active centers. A group of domains responsible for one condensation cycle forms a "module" consisting of at least three domains: ketosynthase (KS), acyltransferase (AT) and acyl-carrying protein (ACP) (Jenke-Kodama and Dittmann, 2009). Since the sequences of modules in PKS systems correspond to gene clusters in the genomes of microorganisms, it is possible to detect the ability of communities of microorganisms and their individual strains to produce bioactive components using PCR detection of these genes. Notably, bacterial strains obtained from unusual and unexplored communities are often the source of new bioactive metabolites (Jenke-Kodama and Dittmann, 2009). The large species richness of sponges inhabiting Lake Baikal (18 species, 14 of which are endemic) is associated with a variety of ecological niches and habitat conditions (Kozhov, 1962). Therefore, an important scientific direction is a research aimed at identifying the ability of microorganisms of the Baikal sponges to produce bioactive metabolites. In this work, we identified 35 bacterial strains isolated from the endemic Baikal sponge, *Lubomirskia baicalensis*, and also performed PCR screening of these strains for the presence of fragments of the gene from the PKS ketosynthase domain in their genomes.

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2. Material and methods

Samples of *L. baicalensis* were collected in August 2011 in the area of the Listvyanka settlement (southwest coast of Lake Baikal) from a depth of 15 m using diving equipment. Bacterial strains from the sponge were isolated out on the R2A agar medium (Becton Dickinson, United States) according to the previously published method (Parfenova et al., 2008). DNA from bacterial cultures was isolated using the RiboSorb kit according to the manufacturer's instructions (AmpliSens, Russia). The strains were identified by analyzing the sequences of 16S rRNA genes amplified using the 9F and 1093R eubacterial primers, as described in our previous article (Kaluzhnaya et al., 2012). The primers for PCR screening of polyketide synthase (PKS) genes were selected based on literature data (Ehrenreich et al., 2005). Comparison with databases of nucleotide sequences was carried out using the BLASTX program of the NCBI server.

3. Results

Thirty-five bacterial strains isolated from the *L. baicalensis* freshwater sponge in August 2011 were identified by the 16S rRNA gene sequences. The collection contains representatives of 16 genera belonging to three bacterial phyla (Table): Firmicutes, Actinobacteria, and Proteobacteria (classes Alphaproteobacteria and Betaproteobacteria). The table shows the homology of the obtained 16S rRNA sequences with those published in the NCBI database (http://www.ncbi.nlm.nih.gov/). Most of the sequences demonstrated 97-100% nucleotide sequence identity with known bacterial strains.

Overall, 17 strains were assigned to the phylum **Actinobacteria** identified as *Janibacter* sp. 04-Lb11/2, *Promicrospora* sp. 05-Lb11/2, *Arthrobacter* sp. 06-Lb11/2, 07-Lb11/2, 13-Lb11/2, 20-Lb11/2, 32-Lb11/2, *Rathayibacter* sp. 08-Lb11/2, 11-Lb11/2, *Kocuria* sp. 12-Lb11/2, 31-Lb11/2, *Rhodococcus* sp. 14-Lb11/2, 33-Lb11/2, *Microbacterium* sp. 15-Lb11/2, 19-Lb11/2, 38-Lb11/2, and *Flexivirga* sp. 21-Lb11/2; 8 strains were assigned to the phylum **Proteobacteria**: *Methylobacterium* sp. 03-Lb11/2, *Massilia* sp. 17-Lb11/2, *Rhodopseudomonas* sp., 22-Lb11/2, 23-Lb11/2, 42-Lb11/2, *Heminiimonas* sp. 30-Lb11/2, and *Tardiphaga* sp. 36-Lb11/2 and 40-Lb11/2; and 10 strains - to the phylum **Firmicutes:** *Staphylococcus* sp. 10-Lb11/2, *Bacillus* sp. 16-Lb11/2, 18-Lb11/2, 24-Lb11/2, 35-Lb11/2, 37-Lb11/2, *Paenibacillus* sp. 26-Lb11/2, 27-Lb11/2, 28-Lb11/2, and 29-Lb11/2 (Table).

According to the results of PCR screening, a product corresponding in size (700 bp) to a fragment of the ketosynthase domain of the polyketide synthase gene was found in 15 out of 35 strains. Of these, 7 strains belonged to the phylum Actinobacteria; 5 strains - to the phylum Firmicutes, and 3 strains - to the phylum Proteobacteria (Table). These bacterial cultures are interesting for further research because they can produce secondary metabolites of a polyketide nature that are important for medicine and biotechnology.

The nucleotide sequences were deposited in GenBank under the accession numbers MZ646072-MZ646106.

4. Discussion

The members of the phylum **Actinobacteria** were the most numerous and diverse group among the isolated cultures. These microorganisms are widespread in both marine and freshwater communities and are one of the most studied groups of bacteria due to their importance in biotechnology, medicine, and ecology. They are efficient producers of new secondary metabolites that show a range of biological activities, including antibacterial, antifungal, anticancer, antitumor, cytotoxic, cytostatic, anti-inflammatory, anti-parasitic, anti-malaria, antiviral, antioxidant, anti-angiogenesis, etc. (Manivasagan et al., 2014; Lee et al., 2020). Actinobacteria were found in all previously studied communities of freshwater sponges, constituting a significant part of the bacterial 16S rRNA sequences in them (Kaluzhnaya et al., 2011; 2012; Kaluzhnaya and Itskovich, 2014; Gladkikh et al., 2014; Seo et al., 2016; Kulakova et al., 2018).

In our study, the PCR-signal was detected in the following actinobacterial strains: 04-Lb11/2 (*Janibacter* sp.), 08-Lb11/2 (*Rathayibacter* sp.), 11-Lb11/2 (*Rathayibacter* sp.) 14-Lb11/2 (*Rhodococcus* sp.), 15-Lb11/2, 38-Lb11/2 (*Microbacterium* sp.), and 20-Lb11/2 (*Arthrobacter* sp.). These bacterial cultures are interesting for further research because some related strains have shown the ability to produce secondary metabolites that are important for medicine and biotechnology. In the scientific literature, we can find many similar investigations.

For example, helquinoline that exhibits high antibacterial and antifungal activity was isolated by a group of German scientists from the *Janibacter limosus* strain Hel 1 ethyl acetate extract (Asolkar et al., 2004). Actinobacteria belonging to the genus *Rathayibacter* were capable of producing toxigenic glycoprotein, the tunicaminyluracil antibiotics (Tancos et al., 2019). Strains of the phytopathogenic *Rhodococcus fascians* bacteria are able to produce phytohormones, leading to the development of so-called leafy galls on a wide range of host plants (Nacoulma et al., 2013). It has been also shown that various *Rhodococcus* strains are involved in the synthesis of bioactive steroids (Haroune et al., 2004) as well as in the biodegradation of a wide range of organic components, including environmentally hazardous toxins, herbicides, naphthalene, toluene, biphenyl, etc. (Zhao et al., 2011).

Sponges are often a source of bacterial strains-producers of bioactive substances. For example, strains of the genus *Microbacterium* isolated from a marine sponge, *Halichondria panacea,* produced glucosylmannosyl-glycerolipid that inhibits the growth of tumor cells (Lang et al., 2004).

Analysis of 70 genomes belonging to 20 species of *Microbacterium* revealed that most of them contain gene clusters encoding pathways for the production of terpenoids, type III polyketide synthases and non-

Table. Bacterial strains isolated from sponge *L. baicalensis* (collection LB11/2), sampled in August 2011

Strain	Acc. No.	Closest homologues (Acc. No.)	Per. Ident, %	Phylum	PCR-sygnal (PKS)
03-Lb11/2	MZ646072	*Methylobacterium variabile* (AB900978)	100.0	Alphaproteobacteria	+
04-Lb11/2	MZ646073	*Janibacter limosus* (MN826598)	99.0	Actinobacteria	+
05-Lb11/2	MZ646074	*Promicromonospora iranensis* (MN187291)	100.0	Actinobacteria	-
06-Lb11/2	MZ646075	*Arthrobacter* sp. (KY476520)	100.0	Actinobacteria	-
07-Lb11/2	MZ646076	*Arthrobacter agilis* (JN934384)	99.9	Actinobacteria	-
08-Lb11/2	MZ646077	*Rathayibacter caricis* (LN774722)	100.0	Actinobacteria	+
10-Lb11/2	MZ646078	*Staphylococcus hominis* (MT487620)	100.0	Firmicutes	-
11-Lb11/2	MZ646079	*Rathayibacter tritici* (KR085826)	99.8	Actinobacteria	+
12-Lb11/2	MZ646080	*Kocuria palustris* (MT534060)	100.0	Actinobacteria	-
13-Lb11/2	MZ646081	*Arthrobacter agilis* (KF924209)	99.9	Actinobacteria	-
14-Lb11/2	MZ646082	*Rhodococcus cercidiphylli* (KY056167)	100.0	Actinobacteria	+
15-Lb11/2	MZ646083	*Microbacterium* sp. (MN889292)	100.0	Actinobacteria	+
16-Lb11/2	MZ646084	*Bacillus aryabhattai* (MH041178)	100.0	Firmicutes	-
17-Lb11/2	MZ646085	*Massilia aurea* (LN880088)	99.8	Betaproteobacteria	+
18-Lb11/2	MZ646086	*Bacillus* sp. (MG470665)	100.0	Firmicutes	+
19-Lb11/2	MZ646087	*Microbacterium* sp. (KM187178)	99.8	Actinobacteria	-
20-Lb11/2	MZ646088	Arthrobacter sp. (KC019196)	99.9	Actinobacteria	+
21-Lb11/2	MZ646089	*Flexivirga alba* (NR_113034)	100.0	Actinobacteria	-
22-Lb11/2	MZ646090	*Rhodopseudomonas* sp. (KF974286)	99.9	Alphaproteobacteria	-
23-Lb11/2	MZ646091	*Rhodopseudomonas palustris* (CP000463)	99.5	Alphaproteobacteria	+
24-Lb11/2	MZ646092	*Bacillus* sp. (KF582892)	100.0	Firmicutes	-
26-Lb11/2	MZ646093	*Paenibacillus* sp. (MW578439)	97.0	Firmicutes	-
27-Lb11/2	MZ646094	*Paenibacillus* sp. (KX881397)	97.0	Firmicutes	+
28-Lb11/2	MZ646095	*Paenibacillus* sp. (MW578439)	98.4	Firmicutes	+
29-Lb11/2	MZ646096	*Paenibacillus* sp. (MW578439)	95.6	Firmicutes	+
30-Lb11/2	MZ646097	*Heminiimonas* sp. (GU932947)	98.9	Betaproteobacteria	-
31-Lb11/2	MZ646098	*Kocuria palustris* (LR215141)	100.0	Actinobacteria	-
32-Lb11/2	MZ646099	*Arthrobacter agilis* (KC019195)	99.9	Actinobacteria	-
33-Lb11/2	MZ646100	*Rhodococcus cercidyphylli* (KY056167)	100.0	Actinobacteria	-
35-Lb11/2	MZ646101	*Bacillus megaterium* (MK474949)	100.0	Firmicutes	-
36-Lb11/2	MZ646102	*Tardiphaga robiniae* (MW960262)	99.4	Alphaproteobacteria	-
37-Lb11/2	MZ646103	*Bacillus subtilis* (KU904288)	99.6	Firmicutes	+
38-Lb11/2	MZ646104	*Microbacterium* sp. (KM187178)	100.0	Actinobacteria	+
40-Lb11/2	MZ646105	*Tardiphaga robiniae* (KY319041)	99.4	Alphaproteobacteria	-
42-Lb11/2	MZ646106	*Rhodopseudomonas palustris* (KT873846)	98.6	Alphaproteobacteria	-

ribosomal peptide synthetases, potentially responsible for the synthesis of siderophore-like compounds. Many *Microbacterium* strains, as shown by *in vivo* test, produce siderophores, ACC deaminase, and auxins (IAA) and can solubilize phosphate (Corretto et al., 2020). The analysis of the genome of the *Pseudarthrobacter phenanthrenivorans* strain MHSD revealed gene clusters of biosynthetic pathways for various phytohormones such as auxin, salicylic acid, ethylene, cytokinin, jasmonic acid, abscisic acid, and gibberellins (Tshishonga and Serepa-Dlamini, 2020).

Three strains of **Proteobacteria** showed an amplification product corresponding to the expected size of the PKS KS domain fragment: 03-Lb11/2 (*Methylobacterium* sp.), 23-Lb11/2 (*Rhodopseudomonas* sp.), and 17-Lb11/2 (*Massilia* sp.). Proteobacteria is a very heterogeneous group, which includes both symbionts of eukaryotes and a large number of pathogenic and opportunistic microorganisms, photo- and chemotrophic species of bacteria, autotrophs and heterotrophs. Alphaproteobacteria, as a rule, dominate proteobacteria in freshwater sponge microbiomes

(Gernert et al., 2005; Costa et al., 2013; Kaluzhnaya et al., 2011; 2012; Kaluzhnaya and Itskovich, 2014). Among Alphaproteobacteria, some phylotypes specific for communities of freshwater sponges have been identified, which indicates the possible co-evolution of sponges and some representatives of symbiotic bacteria as well as the existence of vertical transfer of symbiotic microorganisms (Taylor et al., 2007). This group of bacteria is also rather abundant in strains producing natural bioactive compounds. As an example, Alphaproteobateria of the genus *Methylobacterium* are pink pigmented, strictly aerobic, and facultative methylotrophic bacteria, commonly found in various environments; extracts obtained from these microorganisms exhibited antibacterial, cytotoxic, anticancer, and antioxidant properties (Balachandran et al., 2012; Photolo et al., 2020). Also, *Methylobacteria* have been described as beneficial bacteria owing to their function in toxic pollutant biodegradation, the stimulation of germination, and plant development (Xu et al., 2014). Another representative of Alphaproteobateria, *Rhodopseudomonas palustris*, is a photosynthetic purple non-sulfur bacteria (Austin et al., 2015) exhibiting diverse biological activities. Su et al. (2015) found an antiviral protein showing significant inhibitory activity against tobacco mosaic virus (TMV) *in vivo* and *in vitro* in the JSC-3b bacterial strain. Nookongbuta et al. (2020) demonstrated that exopolymeric substances (EPS), lipopeptides and photo-pigments extracted from the KTSSR54 strain showed antifungal activity against three rice fungal pathogens. Other representatives of this species demonstrated the ability to degrade aromatic compounds and utilize short-chain organic acids during photoheterotrophic cultivation in an anaerobic environment (Austin et al., 2015).

Betaproteobacteria are known for their morphological diversity; they are often the dominant group in lake ecosystems, but they are not numerous in sponge communities. This bacterial class includes several groups of aerobic or facultative bacteria with various metabolic capabilities (chemolithotrophs and phototrophs) (Newton et al., 2011). Representatives of the Betaproteobacteria, strains of the genus *Massilia*, also showed the ability to produce bioactive substances such as dimethyl disulfide (DMDS) that have the potential to control plant foliar diseases (Feng et al., 2016), degrade chloroacetamide herbicide (Lee et al., 2017), and inhibit pathogenic strains of *Escherichia coli* and *Pseudomonas aeruginosa* (Dahal et al., 2021).

In five of the nine isolated **Firmicutes** strains, there was a positive PCR signal for the presence of genes of biologically active metabolites: 18-Lb11/2, 37-Lb11/2 (*Bacillus* sp.), 27-Lb11/2, 28-Lb11/2, and 29-Lb11/2 (*Paenibacillus* sp.).

Firmicutes as a part of lake communities are usually found among the minor phyla and mainly inhabit the bottom layer and sediments. However, some strains of this phylum (for example, species of the genera *Pseudomonas* and *Bacillus*) are often found among cultured bacteria in aquatic and sponge communities (Newton et al., 2011). Representatives of Firmicutes, along with Actinobacteria, are known as producers of biologically active metabolites (Su, 2014).

The presence of genes for the synthesis of biologically active substances in the strains of the genus *Bacillus* is expectable because many representatives of this genus are known as producers of various bioactive metabolites, including antibiotics and toxins such as bacilysin and fengymycin, iturin and iturin-like substances, surfactin, and bacillomycin (Fickers, 2012; Zeng et al., 2016). For example, the *Bacillus axarquiensis* strain TUBP1 exhibited antifungal activity against *Verticillium dahliae*, a fungus that causes a soil-borne disease of cotton crops (Zeng, et al., 2016). Various strains of *Bacillus tequilensis* exhibited antibacterial activity due to their ability to synthesize alkaline protease (Khan et al., 2019) as well as lipoproteins and biosurfactants (Akinsanya et al., 2019).

Determination of the amplified gene sequences, as well as the identification of the antibiotic activity of the selected strains (in relation to test cultures of opportunistic microorganisms), is the next stage of this study.

5. Conclusions

In this study, we identified 35 bacterial strains isolated from the symbiotic community of the freshwater sponge, *L. baicalensis*. Representatives of 16 genera belonging to three bacterial phyla were detected, which indicates the presence of a significant diversity of cultured microorganisms in the *L. baicalensis* community. Their PCR screening was carried out to select cultures potentially capable of producing secondary metabolites of polyketide nature. PCR product corresponding in size to a fragment of the PKS gene was found in 15 strains. Therefore, the proposed method is convenient for the preliminary analysis of a large number of systematically heterogeneous strains. In future research, the biological activity of strains showing a positive PCR signal can be investigated using microbiological, biochemical, and analytical methods. This work contributes to the study of the diversity and biotechnological potential of the cultured microorganisms of the Baikal sponges.

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Conflicts of interest

The authors declare no conflicts of interest.

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