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ORIGINAL RESEARCH ARTICLE

Cyanobacteria *Nostoc Punctiforme* from Abyssal Benthos of Lake Baikal: Unique Ecology and Metabolic Potential

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Abstract A strain of *Nostoc punctiforme* was isolated from the bottom sediments of the oil seep at Gorevoy Utes (Central Baikal) at a depth of 890 m. The Baikal strain is highly similar (98–99%) to the *N. punctiforme* CCAP 1453/9 strain and the typical *N. punctiforme* PCC 73103 strain isolated from soil ecotopes. Based on the analysis of functional genes and mass spectrometry data, we determined that the strain can produce bioactive peptides and polyketides, but does not produce known cyanobacterial toxins, saxitoxin or its analogs, or microcystins. The peptides aeruginosinamide, aeruginosin 606, aeruginosin 98-A, kasumigamide C, and microginin 91-D were recorded in the metabolic profile of the strain. The major ion found in the MALDI mass spectrum is most likely to be an ion of a polyketide substance with unknown function.

Keywords *Nostoc punctiforme* · Lake Baikal · Oil seep · Bioactive peptides · Polyketides

Introduction

The metabolism of cyanobacteria has a rare plasticity allowing them to inhabit different environments. As important primary producers, they are an obligatory component of the photic layer in aquatic environments. At the same time, cyanobacteria can change the nutrition type from autotrophic to heterotrophic, when they lack light energy [1]. Mixotrophy is a mixed type of metabolism, due to which a cell simultaneously supports all structures and enzymes of photosynthesis as well as transporters and lytic enzymes for assimilation of exogenous nutrients [2]. Experiments have demonstrated the mixotrophic ability in cyanobacteria of the genus *Nostoc* (including *N. punctiforme*) [3, 4].

In Lake Baikal, areas of natural oil seepages have been known since the 18th century. In 2005, in the central basin of Lake Baikal near Cape Gorevoy Utes, a new area was discovered, where oil (up to 4 tonnes) and methane are discharged at a depth 855 m annually [5]. There, bitumen structures are formed on the bottom surface under high pressure and low temperature (from 2.8 to 3.2 °C), and gas hydrates are found in near-surface layers of bottom sediments, from which oil seeps and methane are discharged [6, 7]. Sunlight cannot reach such depths, and it is thus an aphotic zone in Lake Baikal. A special microbial community capable of utilising aromatic hydrocarbons and n-alkanes has formed under these specific geochemical conditions [8, 9]. Cyanobacteria recorded in the bacterial community from these ecotopes were considered as deposited from the water column. However, the record of cyanobacteria, similar to *Gloeotrichia* (*Nostocales*) whose number reached 715 colonies per m^2 [10], and *Nostoc* sp. colonies was very unusual for this area. Seven species of the genus *Nostoc* have been registered in the Lake Baikal benthos for long-term investigations of algae, but the species *N. punctiforme* has not previously been described [11]. Because of the absence of *Nostoc* cyanobacteria in the lake plankton and its record at greater depths, excluding an accidental appearance of such a number of colonies in the

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sediment samples, we suggested that the cyanobacteria found inhabit this ecotope. Cyanobacteria of the genus *Nostoc* are able to colonise the most inaccessible habitats due to metabolic strategies, such as diazotrophy, formation of protective polysaccharides, synthesis of biologically active peptides and polyketides providing communicative functions, and abilities for photoheterotrophic metabolism [12, 13]. In addition, cyanobacteria of this genus have been well studied in terms of biotechnology, and numerous investigations have confirmed the value of their metabolites [14–19].

This study presents new data on the abyssal benthos of Lake Baikal. The cyanobacterium *N. punctiforme* is a member of the benthos, which demonstrates an unusual metabolic profile.

Materials and Methods

Strain Isolation and Identification Procedure

Samples of bottom sediments were collected with a gravity corer from aboard the RV "Vereshchagin" in the central basin of Lake Baikal at a depth of 890 m, in the area of an oil seep at Gorevoy Utes (eastern shore of the lake, 53.30° N, 108.39° E) in June 2013. The upper layer of the oil core (0–5 cm) was divided into 50 µL aliquots and placed into sterile liquid medium Z-8 without nitrogen. Cultivation was carried out at an illumination of 700 lx and in the day/night regime. After 3 weeks, the enrichment culture was transferred onto agar medium Z-8 to obtain single colonies. Morphological analysis of the cyanobacteria was performed on an Axio Imager light microscope (Carl Zeiss, Germany) equipped with AxioCam MRm and MRc5 cameras. The cyanobacteria species were identified based on their morphology according to the available guide [20].

Molecular Genetic Study

Genomic DNA from the unialgal culture of *Nostoc* was extracted by a standard phenol–chloroform method and used as template for PCR [21]. Molecular genetic strain identification by the 16S rRNA gene was performed using the primers 359F and 23S30R [22–24]. Genome mining for genes encoding bioactive components was performed with several primer sets (Table 1).

Detection of Bioactive Metabolites with LS–MS Analysis

Cyanobacteria were grown in liquid Z-8 medium to obtain sufficient biomass. Biomass was separated from the medium by centrifugation. The pellet was dried and put into plastic 50 mL test tubes; a twofold excess of distilled water was added to the pellet volume and repeatedly (at least 20 times) subjected to a freeze/thaw procedure. The release of intracellular content was controlled under a microscope. Methanol was added to a concentration of 75% (v), and then the mixture was extracted in an ultrasonic bath for 1 h. The supernatant was separated by centrifugation, and the pellet was re-extracted. The extracts were combined, evaporated to dryness on a rotary evaporator and dissolved in 75% methanol (400 µL/100 mg sample). To carry out the MALDI-TOF/TOF (Bruker UltrafleXtreme) analysis, the target was consequently coated with 0.5 µL of a test sample and 0.5 µL of α-cyano-4-hydroxycoric acid solution (10 mg/mL in MeCN/H_2O/AcOH 70:29.9:0.1 v/v/v). The detection was performed in positive ions registration mode. The mass range was 100–3500 Da. MS2-spectra were registered in the LIFT mode.kl.

Results

Cyanobacteria of the genus *Nostoc* were isolated from the abyssal benthos collected in the aphotic zone of the lake at a depth of 890 m. The strain had the following morphological characteristics: small colonies of irregular globular shape consisting of closely intertwined trichomes, globose heterocysts and trichomes of 3 µm wide (Supplementary Fig. S1). Cyanobacteria were attributed to the species *N. punctiforme*, and the strain was assigned a number GU2015. The 16S rRNA gene sequence with a length of 1246 base pairs was deposited in GenBank (KX267828). Comparative analysis of the obtained 16S rRNA gene nucleotide sequence showed high similarity (99%) of our strain with the uncultured *N. punctiforme* from soil ecotopes and the lichen *Peltigera dolichorrhiza*, and homology with the type strain *N. punctiforme* PCC 73102 was 98.1%. Using specific primers, we recorded the synthesis genes of polyketides (KY681686–KY681697) and aeruginosin (KY681685) in the genome of the Baikal strain. However, no genes of microcystin or saxitoxin synthesis were revealed. Analysis of the methanol extract indicated aeruginosins in several variants, with masses of 561.3 (aeruginosinamide), 607.3 (aeruginosin 606), and 609.3 (aeruginosin 98-A) (Fig. 1). Additionally, we identified microginin 91-D with a mass of 738.4 and a tetrapeptide kasumigamide C. Traces of unknown peptides with masses of 545.4, 625.4, 631.3, 661.3, 736.4 and 782.4 were also found. The major ion with m/z 593.3 was a polyketide substance.

Table 1 Primers used for studying genes responsible for bioactive compound synthesis

Target gene	Size	Primer and nucleotide motif	References
Fragment of *sxt*A gene (polyketide synthase of saxitoxin)	555 bp	sxtAf GCGTACATCCAAGCTGGACTCG sxtAr GTAGTCCAGCTAAGGCACTTGC	[25]
Fragment of *mcy*E gene (microcystin nonribosomal peptide synthetase)	470 bp	hepF TTTGGGGTTAACTTTTTTGGGCATAGTC hepR AATTCTTGAGGCTGTAAATCGGGTTT	[26]
Fragment of *aer*A gene (aeruginosin nonribosomal peptide synthetase)	470 bp	AerAF GATAGCACCCAGAACGGAAGC AerR* TGGGAGCAACCGCTTACATAC	[27]
Fragment of KS gene (polyketide synthases)	700 bp	DKF GTGCCGGTNCCRTGNGYYTC DKR GCGATGGAYCCNCARCARYG	[28]

* Constructed for this study

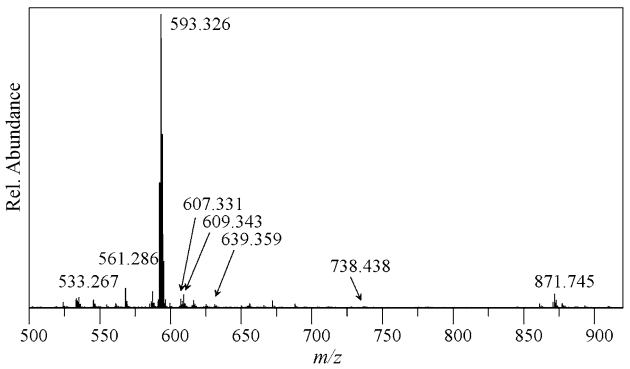

Fig. 1 Mass spectrum of methanol extract of a cyanobacterium *N. punctiforme* GU2015

Discussion

The cyanobacterium *N. punctiforme* GU2015 is able to live under extreme conditions in areas of natural oil discharge in Lake Baikal. This ecotope is characterised by a lack of light due to the depth, pressure over 80 atmospheres, low temperature and a constant supply of oil and gas from the deep zone of sedimentary rocks. The presence of these cyanobacteria under such conditions testifies to their high adaptiveness. Such metabolic strategies as diazotrophy and mixotrophy, as well as the ability to differentiate cells (vegetative cells, akinets, heterocysts and hormogonia) contribute to their survival under extreme ecological conditions [29, 30]. Molecular genetic identification of the strain is consistent with morphological data and also indicates that it belongs to *N. punctiforme*. However, the threshold exceeding 97.5% for *Nostocales* species separation according to 16S rRNA analysis is not the absolute informative value [31]. The uniqueness of our strain is confirmed by its own phylogenetic position among the closely related strains (Supplementary Fig. S2).

Adaptive strategies of cyanobacteria belonging to the genus *Nostoc* are known to include the synthesis of peptides and polyketides, which are regulators of physiological status [32]. For example, polyketides are involved in the formation of glycolipids specialised for heterocysts [33] and regulate symbiotic relationships with fungi [34]. Most polyketides possess properties useful for humans and have long been regarded as a source of natural pesticides for biomedical and agricultural purposes. *Nostoc* extracts have an antibiotic effect on microorganisms and protozoa [35]. The *Nostoc* polyketides inhibit the growth of tumour cell lines and are already undergoing clinical tests as antitumour drugs; for example, dolastatin and cryptophicin [36–38]. The metabolic profile of the *N. punctiforme* GU2015 strain has significant differences from the earlier studied strains in the presence of substances with mass of no more than 900 [39, Supplementary Table S3]. Moreover, the Baikal strain is a producer of a previously unknown polyketide that does not occur in other cyanobacteria. The new polyketide is similar in structure, but is not identical to ergot alkaloids of the endophytic fungi *Neotyphodium lolii* [40]. It is similar in molecular weight to the polyketide discodermolide, a cytostatic agent isolated from a deep marine sponge [41].

Another known family of cyanobacterial polyketides are cyclophanes with antimicrobial and antiproliferative properties [42]. Identified peptides of the aeruginosin family, aeruginosin 98-A, aeruginosin 606 and aeruginosamide, are the members of a promising group of protease inhibitors involved in blood clotting [43]. We also observed a cytotoxic effect of aeruginosins on tumour cell cultures [44]. Aeruginosin 98-A has average efficiency for thrombin inhibition and a high efficiency for trypsin inhibition; for aeruginosin 606 and aeruginosamide, these characteristics have not been studied. Having a stable structure and evident inhibition effect on thrombin and proconvertin, aeruginosins are promising for antithrombotic therapy. The biological function of the tetrapeptide kasumigamide has been poorly investigated

and there is only data for its presence in sponges of the genus *Discodermia* and for antialgal activity [45, 46].

Moreover, *N. punctiforme* GU2015 is a source of microginin 91-D (m/z 736.427), first detected in *Microcystis aeruginosa* [47]. Microginines are linear peptides with characteristic Ahda and Tyr motifs that are able to selectively inhibit an angiotensin-converting enzyme and aminopeptidase M. Although their pharmacological activity was confirmed in vitro, it is necessary to carry out biological studies, which in turn could start clinical trials of these peptides for the treatment of hypertension [48].

Lake Baikal, with its diverse ecotopes, large depths and long history of biota formation, is of great interest in the search for bacterial producers of new biologically active substances. The strain *N. punctiforme* GU2015 is a unique representative of the benthos in Lake Baikal. Further investigations are required to study its specific characteristics that may cause it to inhabit great depths in the absence of light and with a constant discharge of oil and gas. It is also necessary to determine the structure of the polyketide synthesised by *N. punctiforme* GU2015 and evaluate its physiological properties for biotechnological applications.

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