Toxicon 150 (2018) 66–73

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

A new microcystin producing *Nostoc* strain discovered in broad toxicological screening of non-planktic Nostocaceae (cyanobacteria)

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ARTICLE INFO

Keywords:
Anabaena
Cyanotoxins
Heterocytous cyanobacteria
Microcystin
Non-planktic Nostocaceae
Nostoc

ABSTRACT

Benthic cyanobacteria recognized as producers of natural products, including cyanotoxins, have been neglected for systematic toxicological studies. Thus, we have performed a broad study investigating cyanotoxin potential of 311 non-planktic nostocacean representatives combining molecular and chemical analyses. Out of these, a single strain *Nostoc* sp. Treb K1/5, was identified as a new microcystin producer. Microcystins [Asp^3]MC-YR, [Asp^3]MC-FR, [Asp^3]MC-HtyR and Ala-Leu/Ile-Asp-Arg-Adda-Glu-Mdha are reported for the first time from the genus *Nostoc*. All the studied strains were also analyzed for the occurrence of nodularins, cylindrospermopsin and (homo)anatoxin-a, yet no novel producer has been discovered. Our findings indicate rare occurrence of the common cyanotoxins in non-planktic nostocaceae which is in contrast with frequent reports of cyanotoxin producers among phylogenetically closely related planktic cyanobacteria.

1. Introduction

Production of all known major cyanotoxin types has been found in non-planktic cyanobacteria: hepatotoxic microcystins, nodularins, cylindrospermopsins, and the neurotoxic, anatoxin-a, and homoanatoxin-a (e.g., Gugger et al., 2005; Izaguirre et al., 2007; Wood et al., 2007, 2010a,b; Řeháková et al., 2014; Bohunická et al., 2015). However, previous studies were performed on a restricted number of strains, resulting in an incomplete understanding of the actual extent of cyanotoxin production.

The cyanobacterial order Nostocales (heterocytous cyanobacteria) and the family Nostocaceae are extremely active in terms of general secondary metabolite production. They are responsible for production of 26% of the total known metabolites produced by filamentous cyanobacteria (Jones et al., 2010). However, no systematic toxicological studies have been carried out on any non-planktic Nostocacean representatives, despite the fact that particular strains have been repeatedly recognized as cyanotoxin producers (e.g., Genuário et al., 2010; Kurmayer, 2011).

For example, while a number of planktic strains of the nostocacean genera *Nodularia* and *Dolichospermum* (previously *Anabaena*) (Laamanen et al., 2001; Li et al., 2016) are well known cyanotoxins producers, their benthic counterparts have evaded scrutiny. Systematic data on cyanotoxin production are also missing for genera frequently occurring in soil and benthic habitats, such as *Nostoc, Trichormus*, and *Cylindrospermum*. An exception to this was provided by a broad study performed on *Nostoc* lichen cyanobionts (Kaasalainen et al., 2012), which reported high incidence of microcystin biosynthetic genes and microcystins production pointing out the toxic potential of *Nostoc*.

The aim of this study was therefore to systematically investigate non-planktic Nostocaceae and their genetic potential and actual production of several frequently reported cyanotoxins using a combination of molecular and chemical methods.

2. Materials and methods

2.1. Strains under study

Soil and benthic samples were collected from different habitats across a wide range of localities on different continents (for details see Table S1). Monoclonal strains representing eight genera (*Anabaena, Cylindrospermum, Desmonostoc*, *Mojavia*, *Nodularia, Nostoc, Trichormus,*

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https://doi.org/10.1016/j.toxicon.2018.05.007
Received 20 December 2017; Received in revised form 10 May 2018; Accepted 13 May 2018

and *Wollea*) were isolated and maintained in the culture collection of the Institute of Hydrobiology and Institute of Soil Biology, Biology Centre of the Czech Academy of Sciences (CAS), and Centre Algatech, Institute of Microbiology of the CAS. Strains were grown in liquid and agar-solidified (1.5% agar) WC or BG11 media (Guillard and Lorenzen, 1972; Rippka et al., 1979), depending on growth preference. Conditions in the culture collection room were set at 21 °C with a light intensity of 50 μmol $m^{-2}s^{-1}$ (16:8 light/dark cycle). Strain identification was performed based on morphological characteristics (Komárek, 2013) and phylogenetic position in the 16S rRNA tree, where available. A list of the strains and their origin is shown in Table S1.

2.2. Phylogenetic study

The biomasses of the strains were harvested in Eppendorf microcentrifuge tubes (2 mL) by centrifugation and dried at room temperature for 48 h in silica gel, and subsequently pulverized in a Retsch MM200 (Retsch GmbH, Haan, Germany) laboratory mill with wolfram carbide beads (3 min, 30 s^{-1}). Total genomic DNA was isolated following a modified xanthogenate-SDS (Sodium Dodecyl Sulfate) buffer extraction protocol with the addition of 3% PVPP (Polyvinyl Polypyrrolidone) and PEG (Polyethylene Glycol)–$MgCl_2$ precipitation (Yilmaz et al., 2009). The quality of isolated DNA was checked on a 1.5% agarose gel. PCR (polymerase chain reaction) amplification of the 16S rRNA gene was performed using Plain PP Master Mix (Top Bio, Czech Republic) with primer 1 and primer 2 (Table 1), as described by Boyer et al. (2001). Amplification was carried out as follows: an initial denaturation step of 5 min at 94 °C, followed by 38 cycles of 45 s at 94 °C, 45 s at 55 °C, and 2 min at 72 °C, followed by a final elongation step of 10 min at 72 °C. Alternatively, a PCR protocol using the primers 16S27F and 23S30R (Taton et al., 2003, Table 1) included an initial denaturation step of 5 min at 94 °C, 10 cycles of 45 s at 94 °C, 45 s at 57 °C, and 2 min at 72 °C, 25 cycles of 45 s at 94 °C, 45 s at 54 °C, and 2 min at 72 °C, and a final elongation step of 10 min at 72 °C. PCR products were sequenced at SEQme s.r.o. (Dobříš, Czech Republic) using the primers listed in Table 1. The SeqMan 5.06 (Burland, 1999) computer program was used for analysis and assembly of the sequences. Out of 267 studied strains used for phylogenetic analyses, 192 were newly sequenced at the time of publication, covering a wide range of phylogenetic clusters across all the studied genera. The sequences of the 16S rRNA gene of strains were deposited in the NCBI GenBank database under the accession numbers KX424390–KX424499, KX442795, and MG596677-MG596761. Additional 177 sequences for phylogenetic analyses were selected from the GenBank online database (http://www.ncbi.nlm.nih.gov) including published 16S rRNA available sequences of Nostocacean taxa previously proven by other authors to contain the synthetase genes for microcystin, nodularin, cylindrospermopsin, or anatoxin-a, and representative strains of the major lineages of Nostocales. MAFFT v. 7 (Katoh and Standley, 2013) was used for sequence alignment. Ambiguous positions and the variable ITS regions were removed prior to phylogenetic analysis. A Maximum Likelihood phylogenetic tree was obtained using RaxML v. 8 (Stamatakis, 2014) and was calculated by the CIPRES supercomputing facility (Miller et al., 2015); 1000 bootstrap pseudo-replications were performed to evaluate the relative support of branches. The GTR + I + G evolutionary substitution model was selected using the AIC criterion in jModelTest-2.1.6 (Guindon and Gascuel, 2003; Darriba et al., 2012). The tree was edited using FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

2.3. Toxic potential and toxin production

Toxic potential of the tested strains was examined by PCR for microcystin (*mcy*E), nodularin (*nda*F), cylindrospermopsin (*cyr*J), and anatoxin-a (*ana*C) synthetase genes using published protocols and primers (Table 1). DNA samples isolated from known toxin-producing strains were used as positive controls. The PCR product obtained in *Nostoc* sp. Treb K1/5 was sequenced commercially at SEQme s.r.o. (Dobříš, Czech Republic) using the same primers and the sequence was deposited in GenBank under accession number KX495361. A BLAST search was performed to confirm the result and identify the most similar *mcy*E sequence in other cyanobacteria.

All strains were simultaneously analyzed for actual toxin production using high-performance liquid chromatography connected to high resolution mass spectrometer with electrospray ionization (HPLC-ESI-HRMS/MS). After centrifugation and lyophilization, 10–20 mg biomass samples were extracted in Eppendorf tubes (500 μL) using sea sand and a pestle; 100–150 μL of 70% MeOH was used for microcystin and nodularin extraction, 100–150 μL of 80% acetonitrile solution acidified with 0.1% formic acid was used for extraction of anatoxin-a and cylindrospermopsin (Dell'Aversano et al., 2004). To detect microcystins, 70% methanol extracts were analyzed on a Dionex UltiMate 3000 UHPLC+ (Thermo Scientific, Sunnyvale, CA, USA) equipped with a diode-array detector (range 220–650 nm). Separation of compounds was performed on reversed-phase C18 column (Phenomenex Kinetex, 150 × 4.6 mm, 2.6 μm, Torrance, CA, USA) using H_2O (A) and acetonitrile (B) (both containing 0.1% HCOOH) as a mobile phase at a flow rate of 0.5 mL min^{-1}. The linear gradient was as follows: A/B 85/15 (0 min), 85/15 (in 1 min), 0/100 (in 19 min), 0/100 (in 25 min), and 85/15 (in 30 min). The acetonitrile extracts were analyzed on the same HPLC-ESI-HRMS/MS system, using a TSK gel Amide-80 column (Tosoh Bioscience, 250 × 4.6 mm, 5 μm, Stuttgart, Germany) with isocratic elution with an A/B ratio of 21/79 and a flow rate of 0.5 mL min^{-1}.

Table 1
Primers used for PCR and sequencing.

Target gene	Primer pair	Sequence (5′-3′)	Reference
*ana*C (anatoxin-a)	anaxgenF	ATG GTC AGA GGT TTT ACA AG	Rantala-Ylinen et al., 2011
	anaxgenR	CGA CTC TTA ATC ATG CGA TC	
*cyr*J (cylindrospermopsin)	cyrJF	TTC TCT CCT TTC CCT ATC TCT TTA TC	Mazmouz et al., 2010
	cyrJR	TGC TAC GGT GCT GTA CCA AGG GGC	
*mcy*E (microcystin)	mcyEF2	GAA ATT TGT GTA GAA GGT GC	Vaitomaa et al., 2003
	mcyER4	AAT TCT AAA GCC CAA AGA CG	Rantala et al., 2004
*nda*F (nodularin)	ndaF8452	GTG ATT GAA TTT CTT GGT CG	Koskenniemi et al., 2007
	ndaF8640	GGA AAT TTC TAT GTC TGA CTC AG	
16S rRNA	primer1	CTC TGT GTG CCT AGG TAT CC	Boyer et al., 2001
	primer2	GGG GAA TTT TCC GCA ATG GG	
	16S27F	AGA GTT TGA TCC TGG CTC AG	Taton et al., 2003
	23S30R	CTT CGC CTC TGT GTG CCT AGG T	
	CYA781F(a)	AAT GGG ATT AGA TAC CCC AGT AGT C	Nübel et al., 1997
	K6	GAC GGG CCG GTG TGT ACA	Wilmotte et al., 1993
	K8	AAG GAG GTG ATC CAG CCA CA	Flechtner et al., 2002
	16S781R(a)[b]	GAC TAC TGG GGT ATC TAA TCC CAT T	Taton et al., 2003
	16S1494R	TAC GGC TAC CTT GTT ACG AC	Taton et al., 2003