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Biosynthesis of microcystin hepatotoxins in the cyanobacterial genus *Fischerella*

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ABSTRACT

Microcystins (MCs) are serine/threonine phosphatase inhibitors synthesized by several members of the phylum Cyanobacteria. Mining the draft genome sequence of the nostocalean MC-producing *Fischerella* sp. strain CENA161 led to the identification of three contigs containing *mcy* genes. Subsequent PCR and Sanger sequencing allowed the assembling of its complete biosynthetic *mcy* gene cluster with 55,016 bases in length. The cluster encoding ten genes (*mcyA-J*) with a central bidirectional promoter was organized in a similar manner as found in other genera of nostocalean cyanobacteria. However, the nucleotide sequence of the *mcy* gene cluster of *Fischerella* sp. CENA161 showed significant differences from all the other MC-producing cyanobacterial genera, sharing only 85.2 to 74.1% identities. Potential MC variants produced by *Fischerella* sp. CENA161 were predicted by the analysis of the adenylation domain binding pockets and further investigated by LC-MS/MS analysis. To our knowledge, this study presents the first complete *mcy* cluster characterization from a strain of the genus *Fischerella*, providing new insight into the distribution and evolution of MCs in the phylum Cyanobacteria.

1. Introduction

Microcystins (MCs) are small cyclic heptapeptides synthesized by several members of the phylum Cyanobacteria with global significance due to their toxicity to humans and other animals (Jochimsen et al., 1998; Sivonen and Jones, 1999). Their toxicity is exerted through inhibition of members of the protein phosphatase families PP1 and PP2A (MacKintosh et al., 1990; Gulledge et al., 2002). Despite best known for their acute hepatotoxicity, MCs are of interest as possible anti-cancer drug development targets (Niedermeyer et al., 2014; Kounnis et al., 2015). The general structure of MCs can be summarized as cyclo-D-Ala^1-X^2-D-MeAsp^3-Z^4-Adda^5-D-Glu^6-Mdha^7 (see Fig. 1) (Botes et al., 1985), where X and Z are variable L-amino acids, while D-MeAsp corresponds to D-erythro-β-methyl-aspartic acid, Mdha to N-methyl-α-β-dehydroalanine and Adda to (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-(4*E*,6*E*)-dienoic acid. The latter is exclusive to these toxins and nodularins and contributes to the molecule toxicity (Gulledge et al., 2002; Kounnis et al., 2015).

MCs are synthesized through enzymatic modification of short precursor peptides in the nonribosomal pathway. This process is driven by a multifunctional modular enzyme complex consisted of a combination of nonribosomal peptide synthetases (NRPS), type I polyketide synthases (PKS-I), hybrid NRPS/PKS-I and tailoring enzymes (Nishizawa et al., 2000; Tillett et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004; Fewer et al., 2013). The microcystin gene cluster (*mcy*) is composed of nine to ten genes depending on taxa and the involvement of several *mcy* genes in MC biosynthesis was established by gene inactivation studies (Dittmann et al., 1997; Pearson et al., 2004; Christiansen et al., 2008; Fewer et al., 2008). The closely related nodularin (*nda*) synthetase gene cluster from *Nodularia* was also elucidated, indicating that it derived from MC synthetase genes through a deletion event and a change in substrate specificity (Moffitt and Neilan, 2004; Rantala et al., 2004).

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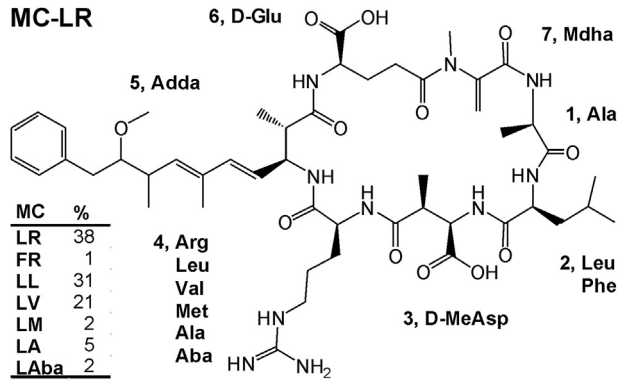

Fig. 1. Structure of MC-LR. The *Fischerella* sp. CENA161 MC variations encountered are shown schematically according to their position. Abbreviations: Adda, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-(4*E*,6*E*)-dienoic acid; D-Glu, glutamic acid; Mdha, N-methyl-α-β-dehydroalanine; Ala, alanine, Leu, leucine, Phe, phenylalanine, D-MeAsp, D-erythro-β-methyl-aspartic acid, Aba, Aminoisobutyric acid, Arg, arginine, Val, valine and Met, methionine.

The biological role of cyanobacterial MC is not currently understood, but several hypotheses have been suggested such as contributing in photosynthesis, environmental adaptation, protection against oxidative stress, nutrient metabolism and storage, quorum sensing, colony formation, defense against zooplanktonic grazers, iron uptake or transfer and allelopathy (Omidi et al., 2017). These authors stated that conflicting results, unstandardized experimental design, strain-specific behavior and differences between conditions in laboratory and nature hinder generalizations on microcystin functions.

Despite several MC-producing strains have been found in the genera *Microcystis*, *Anabaena*, *Nostoc*, *Fischerella*, *Hapalosiphon*, *Oscillatoria*/*Planktothrix*, and *Phormidium* (Bishop et al., 1959; Botes et al., 1984; Krishnamurthy et al., 1986; Eriksson et al., 1988; Meriluoto et al., 1989; Sivonen et al., 1990; Harada et al., 1991; Prinsep et al., 1992; Izaguirre et al., 2007; Fiore et al., 2009), the MC biosynthetic pathway was only characterized in few strains of the genera *Microcystis*, *Anabaena, Planktothrix* and *Nostoc* (Tillett et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004; Rounge et al., 2009; Fewer et al., 2013). The *mcy* gene clusters of these distantly related cyanobacterial genera have revealed a highly conserved set of multidomain proteins depicting the same basic reaction steps. Differences among these clusters have been observed in gene arrangements, localization and orientation of promoter regions, and in genes coding for tailoring enzymes. Interestingly, a cyanobacterium containing one *mcy* gene cluster can produce more than one MC variant mainly due to the relaxed specificity of adenylation (A) domains of McyB-A_1 and McyC-A (amino acid positions **2** and **4**, Fig. 1). Therefore, the description of novel MC gene clusters from different cyanobacterial taxa offers high potential for isolating variants with unique properties.

Although MC production and fragments of biosynthetic genes have already been identified in strains of the genus *Fischerella* (Fiore et al., 2009; Cirés et al., 2014) and even prediction of an incomplete gene cluster has been reported (Shih et al., 2013), the entire gene cluster remains unsolved. Here we used a genomics-based approach to characterize the complete biosynthetic gene cluster in the MC-producing strain *Fischerella* sp. CENA161. Prediction analysis based on the amino acid residues lining the substrate-binding pockets in NRPS A domains were performed and potential structural variants investigated by high performance liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS).

2. Method

2.1. Cyanobacterial strain

The cyanobacterium *Fischerella* sp. CENA161 was isolated from a water sample collected from a small concrete dam of spring water in the municipality of Piracicaba, São Paulo state, Brazil, as previously described (Fiore et al., 2009). This strain is maintained under culture in CENA/USP, located in Piracicaba, SP, Brazil, in BG–11 (Allen, 1968) liquid medium without inorganic nitrogen (BG–11_0), at 25 ± 1 °C, with a 14:10 h light/dark photoperiod, and photon flux density of 40 μmol photons/m^2/s.

2.2. DNA extraction, PCR amplification and Sanger sequencing

Cells from the cyanobacterial culture were collected and processed as previously described (Heck et al., 2016). Total genomic DNA was extracted using the AxyPrep™ Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences) according to manufacturer instructions. The integrity of the total genomic DNA extracted was verified using 1% agarose gel electrophoresis. The extracted DNA was purified with Axyprep™ PCR Clean-up Kit (Axygen) according to manufacturer instructions. Microcystin codifying genes (*mcy*) were amplified by polymerase chain reaction using a combination of primer sets previously described in literature and designed for this work (Table 1). PCR products were ligated to pGEM®-T Easy Vector Systems (Promega) and inserted into chemically competent *Escherichia coli* DH5α cells. Plasmids that received the PCR products were extracted from cells by alkaline hydrolysis (Birnboim and Doly, 1979). Sequencing reactions were performed using the Big-Dye Terminator Cycle Sequencing Kit (GE Healthcare), with vector primers M13F/M13R in a Techne TC-412 thermocycler (Bibby Scientific Limited) for 25 cycles at 95 °C for 20 s, 52 °C for 15 s, and 60 °C for 1 min. Purified reactions were analyzed in an ABI PRISM 3500 genetic analyzer (Life Technologies). The sequenced reads had their base quality analyzed and consensus sequences were generated with the Phred/Phrap/Consed software package (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998). Sequences were aligned and compared to other sequences available in NCBI GenBank (http://www.ncbi.nlm.nih.gov/) using BLASTn (Altschul et al., 1997).

2.3. Whole genome sequencing and assembly

Genomic DNA extracted from the cells was quantified using Qubit dsDNA Broad BR Assay kit and Qubit®2.0 Fluorometer (Thermo Fisher Scientific). Paired-ends libraries were prepared with the Nextera XT Sample Prep Kit (Illumina), which were sequenced in the MiSeq (Illumina) platform using the MiSeq 600 cycle Reagent Kit v3 (Illumina) according to manufacturer instructions. The quality of the raw Illumina sequence reads were initially assessed using FastQC v0.10.1 (Andrews, 2010). Bases with quality indices lower than Phred 20 and sequences shorter than 50 bp were removed using the program SeqyClean 1.8.10 (Zhbannikov et al., 2015). Overlapping read pairs were merged with PEAR 0.9.6 (Zhang et al., 2014) and genome assembly was performed using SPAdes 3.1.1 (Bankevich et al., 2012).

The complete nucleotide sequence of the *mcy* gene cluster of *Fischerella* sp. CENA161 was deposited in GenBank under accession number KX891213.

2.4. Microcystin gene cluster annotation and phylogenetic analysis

The MC synthetase gene cluster was identified by using BLASTn alignments between the 10 *mcy* gene sequences obtained in Sanger