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Modern Method for Sampling and Detection of Cyanobacterial Toxins Microcystins

ORIGINAL ARTICLE

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Abstract

Cyanobacteria are the predominant phototrophs in freshwater ecosystems of the polar regions where they commonly form extensive benthic mats. Despite their major biological role in these ecosystems, little attention has been paid to their physiology and biochemistry. An important feature of cyanobacteria from the temperate and tropical regions is the production of a large variety of toxic secondary metabo lites. In Antarctica, and more recently in the Arctic, the cyanobacterial toxins microcystin and nodularin (Antarctic only) have been detected in freshwater microbial mats. To date other cyanobacterial toxins have not been reported from these locations. Five Arctic cyanobacterial communities were screened for saxitoxin, another common cyanobacterial toxin, and microcystins using immunological, spectro scopic and molecular methods. Saxitoxin was detected for the first time in cyanobacteria from the Arctic. In addition, an unusual microcystin variant was identified using liquid chromatography mass spectrom etry. Gene expression analyses confirmed the analytical findings, whereby parts of the sxt and mcy operon involved in saxitoxin and microcystin synthesis, were detected and sequenced in one and five of the Arctic cyanobacterial samples, respectively. The detection of these compounds in the cryosphere improves the understanding of the biogeography and distribution of toxic cyanobacteria globally. The sequences of sxt and mcy genes provided from this habitat for the first time may help to clarify the evolutionary origin of toxin production in cyanobacteria.

Key Words

Cyanobacteria, toxin, organisms.

Introduction

Several phototrophic organisms survive the harsh climate of the high Arctic regions, including some higher plants, mosses, lichens, various algal groups and cyanobacteria. Cyanobacteria are globally distributed, but in high Arctic freshwater ecosystems they represent the dominant primary producers. Special features such as resistance to ultraviolet (UV) radiation, freeze thaw cycle adaptation and nitrogen fixation allow their survival in these extreme environments. During the polar summer, when both light and temperatures above the freezing point prevail, cyanobacterial communities thrive. They develop highly diverse benthic or floating mats in freshwater streams, ponds and on soils continuously fed with melt water that can be several centimeters

thick and extend over large areas. These extensive mats form the basis of a small but diverse and dynamic ecosystem accommodating a variety of organisms such as nematodes, rotifers, tardigrades, mosses and moss infecting oomycetes such as the recently de scribed Pythium polare.

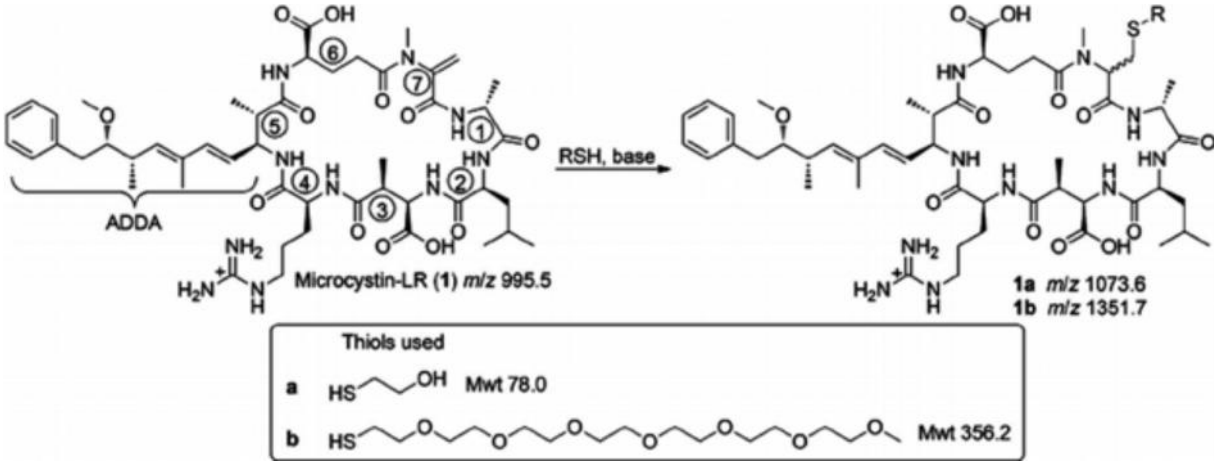
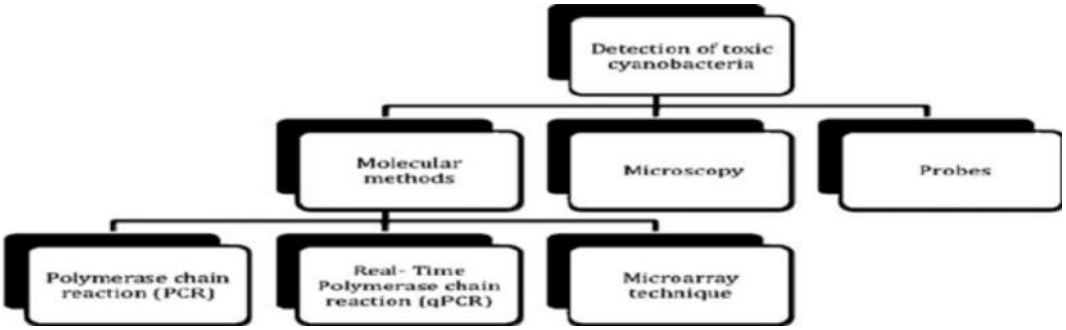


Fig. 1: Chemical structures of saxitoxin (STX) and microcystin (MC). General structure of the STXs (A); adapted from Humpage et al. and the general structure of the MCs (B); adapted from Puddick.

The molecular basis for the production of both toxins are large and variable gene clusters, encoding enzymes involved in second ary metabolite production such as polyketide synthetases and/or non ribosomal peptide synthetases. The distribution of these gene clusters among different strains of cyanobacteria does not necessarily correlate with the actual production of the toxins. Therefore the presence of these genes in a given ecosystem is only an indication for the presence of the toxins and is therefore considered in this study as providing evidence for the 'potential to produce toxins'. These biosynthetic steps are energetically expensive for cyanobacteria, and this has prompted considerable speculation on their ecological function. To date the physiological function and ecological regulation of both STXs and MCs are poorly understood. The current hypotheses aiming to explain this relatively enormous investment of energy in the synthesis of these toxins, include protection against grazing pressure, UV radiation, and reactive oxygen species, as well as their function as signaling molecules in a quorum sensing like manner. The development of toxins as protection against grazers appears to be the least plausible hypothesis as the corresponding gene clusters ap pear to have been present in ancestral cyanobacteria species that have existed prior to the mesoproterozoic period, i.e. millions of years prior to the emergence of potential eukaryotic grazers of cyanobacteria. However it cannot be excluded that new functions have developed in the course of evolution.



In view of the ancient origin and the high conservation of the toxin gene clusters it is not surprising that cyanobacteria inhabiting remote and pristine areas, e.g. the rudimentary environments of the polar regions, could produce toxins. The presence of MCs in cyanobacterial mats has been reported for several locations in Antarctica, whereas this has only recently been demon strated for Arctic cyanobacteria from northern Baffin Island. Kleinteich et al. demonstrated that culturing of cyanobacterial mats in the laboratory at increased temperatures caused a marked rise in the concentration of MCs in concert with shifts in the diversity of the

cyanobacterial mat community composition. Whether the latter is a response to temperature stress, changing diversity of community structures or indeed a marker of a growth advantage of toxin producing cyanobacteria still needs to be ascertained. Saxitoxins, on the other hand, have never been reported in polar environments and cyanobacterial toxins in general remain under studied in this habitat. In this study five cyanobacterial communities from the Arctic were screened for the presence of STX using enzyme linked immunosorbent assay (ELISA) and further confirmation was undertaken using high performance liquid chromatography with fluorometric detection (HPLC FLD). Furthermore an unusual MC variant was identified using liquid chromatography mass spectrometry (LC MS). Samples were also screened for selected genes involved in the synthesis of MC and STX, thereby providing evidence of the toxin producing potential of Arctic cyanobacterial communities.

Materials and Methods

Study sites and sampling

Five samples of cyanobacterial communities were collected during an expedition to northern Baffin Island in the vicinity of Cape Hatt (72300 N and 79470 W) in August September 2009 from microbial communities on wet soil, small streams and ponds (see Supplemental Fig. 1 for GPS coordinates). Samples for DNA extraction and toxin analysis were sealed in sterile tubes and those for RNA analysis immersed in RNAlater (Qiagen, Hilden, DEU). DNA and RNA samples were frozen (20 C) within 24 h after collection and stored for approximately 6 months until further analysis. 2.2. Screening for saxitoxin and microcystin

Microcystin Analysis

The extract of cyanobacterial sample A, which had previously tested positive for MC by ADDA ELISA, was analyzed by LC MS in order to identify the MC congener/s present. LC MS was undertaken on a HPLC system (UltiMate 3000; Dionex) coupled to an AmaZon X (Bruker Daltonics) electrospray ionization ion trap mass spectrometer (ESI IT MSn). Samples (20 lL) were separated on a C18 column (Ascentis Express C18, 100 2.1 mm, 2.7 l; Supl eco Analytical) using a gradient system of 98% H_2O + 0.1% formic acid (v/v; solvent A) and 98% acetonitrile + 0.1% formic acid (v/v; solvent B) with the following gradient program; the sample was loaded in 10% B; 10% B was held for 1 min and increased to 100% B over 12 min; 100% B was held for 2 min; the solvent concentra tion was returned to 10% B in 1 min and the column re equilibrated for 4 min. The eluting compounds were transferred into the IT MS using a capillary voltage of 3.5 kV and a nebulizer pressure of 3.0 bar. Desolvation was accomplished with a nitrogen flow of 8 L/min at 220 C. Tandem MS (MS/MS) spectra were gathered using the doubly or singly protonated ions of the target com pounds and collision induced dissociation (CID) to induce frag mentation of the parention (collision amplitude of 1.0).

A recently developed thiol derivatization technique was used to determine whether the position seven amino acid in the MC ob served was N methyldehydroalanine (Mdha) or dehydrobutyrine (Dhb). Sample A (1420 lL) was mixed with 200 mM $NaHCO_3$ (pH 9.7; 360 lL) in a septa capped vial and left to equilibrate to 30 C. Following LC MS injection of the original extract, b mercaptoethanol (20 lL) was added to the extract of sample A and the vial inverted to mix. The reaction mixture was maintained at 30 ^{0}C in the sample tray of the LC MS apparatus and injections were made periodically over a 90 h period.

Screening for Genes Involved in Toxin Synthesis

Nucleic acid extraction

DNA was extracted from 5-10 mg of frozen material using the MO BIO Power Soil DNA Isolation Kit following the manufacturer's recommendations. Due to the heterogeneity of the sample material, three individual extractions were performed and the pooled extracts used for downstream applications. RNA was extracted from 5 to 10 mg of material stored in RNAlater (Qiagen, Hilden, DEU). RNA later was removed by patting the material on a dry stack of paper, and RNA extracted using the MOBIO Power Biofilm RNA extraction kit following the manufacture's protocol. RNA was eluted with RNase free water and stored at 80 C.

Detection of Genes Involved in Toxin Synthesis

PCRs targeting the mcy and sxt operon for MC and STX synthesis respectively were performed with primer pairs and at annealing temperatures as listed in Supplemental Table 2; primers were from MWG eurofins (Ebersberg, DEU). For the reactions either the Master Mix™ (Fermentas, St. Leon Rot, DEU) or the Phusion™ poly merase mix (NEB, Ipswich, USA) was used supplemented with BSA, DMSO and MgCl2. Bands were excised from a 1.5% agarose gel (TAE) using a sterile scalpel, purified with a gel extraction kit (Fermentas, St. Leon Rot, DEU) and sequenced bi directionally using the primers listed in Supplemental Table 1 at MWG eurofins (Ebersberg, DEU). Messenger RNA of the sxt operon was reverse transcribed into cDNA using gene specific reverse primers and a standard protocol for reverse transcription (20 U RNAse Inhibitor, 0.8 mM dNTPs, 7 lL of extracted RNA, 70 U M MuLV). Enzyme and chemicals for RT PCR were from NEB (Ipswich MA, USA). The cDNA produced was used as template for PCR as described above. Microcystis aeruginosa CCAP 1450/16 served as a positive control for mcy genes, but no positive control of cyanobacterial origin was available for the sxt genes. The obtained sequences were analyzed using Geneious™ software (Gene ious Pro 5.3.6) and the closest matches identified using NCBI's BLAST tools (mega BLAST and BLASTn). Phylogenetic trees using sxtA sequences were built using the Geneious™ tree builder (Jukes Cantor, Neighbour joining method). The obtained 657 bp product of the sxtA gene was deposited in the GenBank database under the accession JX887897. The obtained 128 bp product of the sxtA gene is displayed in Supplemental Table 5 since GenBank does not allow deposition of sequences shorter than 200 bp.

Identification of Toxin Producer/s

Cloning of 16S rRNA and intergenic spacer region (ITS)

Two samples that returned positive results from toxin analyses were selected for construction of 16S rRNA gene and intergenic spacer region (ITS) region clone libraries. Amplification was achieved with the cyanobacteria specific primer pair 27F and 23S30R in a 50 lL PCR reaction (4 min, 95 C; 35 [92 C, 60 s; 55 C, 60 s; 72 C, 120 s]; 72 C, 10 min) containing the Fermentas (St. Leon Rot, DEU) Master Mix, 2.5 mM MgCl2, 0.2 lg/lL BSA, 3% DMSO and 0.5 lM of each primer. The PCR products were separated on a TAE 1.5% agarose gel and the bands excised using a sterile scalpel. After purification with the GeneJET™ Gel Extraction Kit (Fermentas, St. Leon Rot, DEU) PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, USA) following the standard protocol with a DNA to vec tor ratio of four to one. For each sample approximately 40 clones were retrieved and conserved in sterile TE buffer. Each clone insert was amplified in a PCR reaction (95 C, 4 min; 35 [92 C, 30 s; 52 C, 30 s; 72 C, 70 s]; 72 C, 5 min) using the Fermentas PCR Master Mix (St. Leon Rot, DEU), 0.25 lM of each TOPO primer M13, 2 mM MgCl2, 3% DMSO and 0.3 lL of DNA template. Products were subjected to a Restriction Fragment Length Polymorphism (RFLP) analysis using restriction enzymes AluI and ScrF1 (NEB, Ips wich, USA) (37 C, 2 h) and subsequently visualized on an agarose gel (TAE, 2.5% agarose) in order to identify groups of multiple clones of the same phylotype. Two to three representative clones of each individual RFLP pattern (phylotype) were selected and sequenced at GATC Biotech, Konstanz, Germany using the TOPO primers T3 and T7 as well as the 16S rRNA gene specific primers 27F, 359F and 23S30R for verification. The resulting sequences were analyzed using Geneious™ software (Geneious Pro 5.3.6) and their closest matches identified via a Mega BLAST search of the GenBank database. Sequences were deposited in GenBank un der the accession numbers as indicated in Supplemental.

Morphological Identification

Microscopic analysis was performed using a Nikon Eclipse TS 100 Microscope and images documented with a Nikon Digital Sight DS 5M camera. Image quality was improved using XnView for Windows Software (version 1.97.6; Libformat version 5.70) and scale bars included by Corel Photo Paint 11 for Windows (version 11.633). Species identification down to genera level was made using the taxonomic guides of Komarek and Anagnostidis.

Data Evaluation

Data were evaluated using Graphpad Prism™ Software (Prism 5 for Windows, Version 5.04).

Results

Detection of Toxins in Arctic Cyanobacterial Communities

Saxitoxin Detection

One of the five samples analyzed with the STX ELISA tested positive (Sample E; 21 (SD = 16) lg STX/kg dry weight; n = 6) well above the detection limit of the STX ELISA and in the center of the standard curve. However, the concurrent HPLC FLD analyses were not able to confirm this finding, possibly due to the detection limit of the individual STX variants (LOD between 0.5 and 13 lg/kg). Additionally different sample matrices may result in matrix suppression which may increase limits of detection (Pers. comm. Michael Boundy, Cawthron, October 2012). None of the other samples tested positive in the STX ELISA, either as original samples or when cultured in the laboratory at various temperatures (see Kleinteich et al. for laboratory culture conditions). Subsequent PCR amplification of two segments (128 bp and 657 bp) of the sxtA gene provided for a positive signal in sample E but not for the other four samples (Table 1). Additionally, the sxtA gene mRNA was detected in sample E.

The sxtA gene encodes for a polyketide synthetase, which is part of the recently identified cyanobacterial sxt gene cluster. The amplified 128 bp and 657 bp products of sample E were sequenced and compared to the GenBank data base. The 657 bp gene segment shared a high similarity with known sxtA genes from the fresh water cyanobacteria Scytonema cf. crispum (97%, HM629429) and Lyn gbya wollei (95%, EU603711.1). In contrast, the 128 bp sequence was most similar to the sxtA gene of Aphanizomenon (99%, HQ338481.1) and Anabaena circinalis (99%, HQ338478.1) (Supple mental Table 3). A phylogenetic tree was constructed using the 657 bp product of the sxtA gene in sample E, and the sequence grouped closest to the Lyngbya wollei (EU629174) and Scytonema (HM629429) sequence. One Cylindrospermopsis (EU629178) and several Anabaena and Aphanizomenon sequences clustered differently.

Microcystin Detection

Liquid chromatography MS analysis of sample A that contained 106 lg MC/kg dry weight in the ELISA assay, showed that multiple MC variants were present in the extract. One of these compounds had a similar retention time to that of MC RR, but yielded a doubly protonated ion with m/z 526.7. Tandem MS of the m/z 526.7 ion revealed fragment ions resembling a di arginated MC (minus CN2H2;). Also observed in the fragment ions was a loss of 60 Da which suggested that the MC contained ADMADDA instead of the generally observed ADDA (minus HOAc;). Assignment of the fragmentions indicated that this MC also con tained alanine (Ala), arginine (Arg), aspartic acid (Asp), glutamic acid (Glu) and an 83 Da moiety (Mdha or Dhb) in the sequence Ala Arg Asp Arg ADMADDA Glu Mdha/Dhb.

The amino acid Mdha is generally observed at position seven of MCs, however, the isometric Dhb has also been observed. Therefore, a simple thiol derivatization was used to discriminate between the two amino acids. A MC containing a terminal alkene, such as found in Mdha, will readily react with b mercaptoethanol under alkaline conditions. When Dhb is present, the reaction rate is hundreds of times slower. The b mercaptoethanol derivatization of an Mdha containing MC (MC RR) progressed with a half life of 2.6 min. Derivatization of the MC present in Sample A progressed much more slowly ($t_{½}$ = 1579 min), which suggested that the MC in Sample A contained Dhb in position seven and thus appeared to be the same MC [Asp3 , ADMADDA5 , Dhb7] MC RR described previously in Nostoc.

There were several minor compounds present in Sample A that showed some structural similarity to MCs, but could not be identified using the current sample due to insufficient individual compound quantity. The four other samples which previously tested negative for MC by ELISA tested negative for MC by LC MS. All five samples were tested for the presence of genes of the mcy gene cluster, responsible for MC synthesis. Three different genes involved in MC synthesis (mcyA, mcyE and polyketide synthase regions)

were amplified for sample A, which had tested positive for MC in the ELISA and LC MS analysis, providing a product of the correct size (Table 1). Subsequent sequencing of the products and GenBank comparison however resulted in only one product annotated to a gene involved in secondary metabolite production, i.e. an amino acid adenylation domain of Clostridium. The other two products did not result in a specific identification of an annotated gene.

Table 1: Genes for toxin production in five Arctic cyanobacterial samples. Detection of the mcy and sxt operon in five Arctic cyanobacterial communities suggesting the potential for microcystin and saxitoxin production.

Sample	16S	mcyA	mcyA	mcyB	mcyE	PKS	sxtA	sxtA
A	+	+	+	-	+	+	-	-
B	+	+	-	+	-	-	-	-
C	+	+	-	-	+	-	-	-
D	+	+	+	+	-	+	-	-
E	+	+	+	+	+	+	+	+

For the other four samples (B E), negative for MC in the ELISA and LC MS analysis, at least one gene involved in MC synthesis was amplified, sequenced and annotated in GenBank to a known gene involved in MC synthesis with similarities ranging between 60% and 99%. In total, seven sequences, annotated to genes involved in MC synthesis, were amplified. The general corresponding to these genes were Microcystis, Nostoc and Microcoleus.

Potential Toxin Producers in Arctic Cyanobacterial Communities

A clone library was constructed for the samples that contained either MC (sample A) or STX (sample E) to identify the potential toxin producers. Genera were also identified using light micros copy. Molecular characterization of the 16S ITS region demon strated that the species present in the STX containing sample E were most similar to: Nostoc puntiforme (CP001037, 95%), Lep tolyngbya (frigida) (AY493573, 97%), Calothrix sp. (JN385289, 92%), Snowella littoralis (AJ781040, 98%), and Tolypothrix distorta (GQ287651, 98%), see Supplemental Table 4. Fewer cyanobacterial signals were obtained for sample A, and the sequences were most similar to; Aphanizomenon gracile (FJ424575, 94%), Leptolyngbya sp. (DQ431004, 94%), and Chroococcus (FR798926, 97%). As a general guideline strains with >97% 16S rRNA gene sequence similarity are considered to belong to the same species, so that annotations based on lower values as described for some of the strains here need to be handled with care. Light microscopy showed that both samples had a similar appearance, with dominating Nostocales embedded in a firm mucilaginous matrix. Other orders, albeit in lower abundance, i.e. Oscillatoriales (e.g. Leptolyngbya) and Chroo coccales were also present. The Nostocales observed had cells of 3 6 lm in diameter and contained heterocytes (Supplemental Fig. 2). Leptolyngbya with a trichome width of approximately 1.7 lm was present in both samples. In Sample A Tolypothrix was characterized by dark brown colored sheaths and a trichome width of approximately 15 lm.

Discussion

Cyanobacterial toxin production is a worldwide phenomenon with concomitant widespread adverse health effects in humans and wildlife of the temperate and tropical regions. The adverse effects are not only of acute nature but can also entail funda mental changes to whole ecosystems upon chronic or intermittent acute exposure events. Despite the high abundance of cya nobacteria in the Arctic, at present there is only a single recent report of MCs in Arctic cyanobacteria and in cyanobacteria associated lichen. Although MC was below the limit of detection in most of the environmental samples analyzed by Kleinteich et al., MC concentrations increased dramatically when cul tured under laboratory conditions and at higher ambient temperatures. This was also associated with a profound change in the species diversity of the cyanobacterial mats investigated. Whether the higher toxin quantities produced and/or the higher temperatures induced the change in species

diversity could not be determined. Nor was it ascertained whether the higher toxin production was associated with a reallocation of energy investment either as an advantageous trait of toxin producing cyanobac teria or as the result of changing temperature stress. Irrespective of the latter, these data suggest that continued climatic change may led to increases in cyanotoxins in polar regions. In an extension in this study, the same cyanobacterial communities from the Canadian Arctic were analyzed for the presence of STX and further characterization of the MC was undertaken.

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