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Characterization of microcystin production in an Antarctic cyanobacterial mat community

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Abstract

Cyanobacteria are well known for their production of non-ribosomal cyclic peptide toxins, including microcystin, in temperate and tropical regions, however, the production of these compounds in extremely cold environments is still largely unexplored. Therefore, we investigated the production of protein phosphatase inhibiting microcystins by Antarctic cyanobacteria. We have identified microcystin-LR and for the first time [D-Asp^3] microcystin-LR by mass spectrometric analysis in Antarctic cyanobacteria. The microcystins were extracted from a benthic microbial community that was sampled from a meltwater pond (Fresh Pond, McMurdo Ice Shelf, Antarctica). The extracted cyanobacterial cyclic peptides were equivalent to 11.4 ng MC-LR per mg dry weight by semi-quantitative analyses using HPLC-DAD and the protein phosphatase inhibition assay. Furthermore, we were able to identify the presence of cyanobacterial non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) genes in total DNA extracts from the mat community.

Keywords: Cyanobacteria; Microcystin; Mass spectrometry; Non-ribosomal peptide synthetase; Antarctica

1. Introduction

Bloom-forming cyanobacteria are well known for the production of microcystins in temperate and tropical regions (Carmichael, 2001; Chorus and Bartram, 1999), but the production of cyclic peptides in microbial mats from extreme environments, such as Antarctica, is largely unexplored (Hitzfeld et al., 2000). Microcystins inhibit protein-phosphatases and acute exposure can lead to liver failure and death (Chorus and Bartram, 1999). Microcystins have also been linked to tumour promotion after long-term subchronic exposure (Falconer et al., 1994). The general structure of microcystin is cyclo- (D-alanine-*X*-D-MeAsp-*Z*-Adda-D-glutamate-Mdha), where D-MeAsp is D-erythro-beta-methyl-aspartic acid and Mdha is *N*-methyldehydroalanine. *X* and *Z* are variable L-amino acids (Sivonen and Jones, 1999). All microcystins are synthesised non-ribosomally by a multi-enzyme complex consisting of non-ribosomal peptide synthetases and polyketide synthases (Tillett et al., 2000). So far, almost 80 different types of microcystins have been identified, however, the most common isoforms are microcystin-LR, -RR and -YR (Sivonen and Jones, 1999). The ecological role of these

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compounds is a topic of much discussion. Possible putative functions of the molecule include a feeding deterrent, quorum-sensing, or iron-scavenging molecule (Kaebernick and Neilan, 2001; Kaebernick et al., 2001; Lukac and Aegerter, 1993; Mikalsen et al., 2003; Rohrlack et al., 1999) to ancestral relict (Moffitt and Neilan, 2004; Rantala et al., 2004).

Cyanobacterial species belonging to the genera *Microcystis*, *Anabaena* and *Planktothrix* are often associated with microcystin production in temperate or tropical habitats (Carmichael, 1997; Codd et al., 1997; Fastner et al., 1999; Sivonen et al., 1992a). Other genera, such as *Phormidium*, *Oscillatoria* and *Nostoc* have also been found to have microcystin-producing species, however, they are less commonly associated with large toxic bloom events (Carmichael et al., 1990; Hitzfeld et al., 2000; Izaguirre et al., submitted for publication; Moffitt and Neilan, 2001; Sivonen et al., 1990).

In Antarctic microbial mat communities motile cyanobacteria, including several species belonging to *Oscillatoria* and *Phormidium*. (order Oscillatoriales), as well as the nitrogen-fixing *Nostoc* sp. and *Nodularia* sp. (order Nostocales), are the predominant species (Howard-Williams et al., 1990; Jungblut et al., 2005). They are abundant in the top layers of the mat communities (Stal et al., 1985). Species of *Microcystis* and *Planktothrix*, that are most often associated with hepatotoxic microcystin blooms are, however, not found in these Antarctic communities (Howard-Williams et al., 1990; Jungblut et al., 2005).

The production of cyclic peptides in cyanobacterial mat communities from Antarctica should be correlated with protein phosphatase inhibition in order to confirm the presence of microcystin (Hitzfeld et al., 2000). Here, we present a detailed investigation of a benthic cyanobacterial mat community obtained from an Antarctic meltwater pond. This pond has a low conductivity (158 μS/cm) and is situated in a network of meltwater ponds on the McMurdo Ice Shelf, Antarctica. Protein phosphatase 2A inhibition assays, high performance liquid chromatography (HPLC), and time-of-flight tandem mass spectrometry analyses were used for the identification, quantification, and characterisation, of microcystins from an Antarctic cyanobacterial mat community. PCR screening was performed to identify non-ribosomal peptide synthetase and polyketide synthase genes potentially involved in the production of hepatotoxic cyclic peptides by Antarctic cyanobacteria.

2. Material and methods

2.1. Sampling and sample site

Benthic microbial mat material was collected from the meltwater pond Fresh Pond (unofficial name) on the McMurdo Ice Shelf, located south of Bratina Island (78.00′S, 165.30′E) in January 2002 as previously described (Hitzfeld et al., 2000).

2.2. Chemical analysis of water and mat sample

Water chemistry parameters, including conductivity were as previously reported (Jungblut et al., 2005).

An aliquot of the microbial mat sample was lyophilised and the organic content was determined by furnace incineration at 600 °C for 14 h (Hitzfeld et al., 2000).

2.3. Sample extraction, high performance liquid chromatography (HPLC)

The sample was resuspended in 70% methanol, sonicated for 60 min, centrifuged at 48,000 × *g* for 60 min, and the supernatant retained for analysis. The methanol/sonication/centrifugation steps were repeated twice. The supernatant was collected and dried under a continuous stream of nitrogen. The extract was then purified by solid phase extraction phase chromatography (Chromabond C18 ec, Dueren, Germany). The cartridge was conditioned with 6 ml of methanol (100%), washed with 6 ml ultra-pure water, the sample loaded, washed with 6 ml deionised water and peptides eluted with 6 ml 100% methanol. Methanol was evaporated and the extract dissolved in a defined volume of 20% methanol. Fractionation of the 20% methanol extract of the cyanobacterial mat was carried out by HPLC-DAD (solvent module 125 and autosampler 507e, Beckman, Krefeld, Germany; photodiode array detector SPD-M10A, Shimadzu, Duisburg, Germany) using a C18-reversed phase-column (Grom-Sil-ODS4 He) in 1 min steps (equivalent to 3 ml). As mobile phase an isocratic 27% acetonitrile/0.0135 M ammonium acetate buffer (v/v) was used. Each fraction was dried and redissolved in ultra-pure water.

2.4. Protein phosphates 2A inhibition assay (PPIA)

PPIA was performed as described by Heresztyn and Nicholson (2001) with MC-LR (Alexis, Germany) as a standard for relative inhibition capacity. The degree of protein phosphatase inhibition (PP-inhibiting capacity) was calculated as a percentage of the protein phosphatase 2A (PP2A) (Promega, Mannheim, Germany) activity of the control. The PP-inhibiting capacity was compared to the MC-LR standard curve and expressed as MC-LR-equivalents per mg organic matter. The standard is not a certified analytical standard, but had a purity of more than 95% with a deviation of ±20% in the lot weight supplied.

2.5. Mass spectrometric analysis

Mass spectrometric analysis was performed on a Q-Star quadrupole-TOF hybrid mass spectrometer (ABI,

Foster City, California). Data acquisition was performed using ABI Analyst QS software. An electrospray source was used with sample introduction by direct infusion using medium length nano-electrospray capillaries from Proxeon Biosystems (Odense, Denmark). Prior to analysis, the samples were diluted (1:1 or 1:4, v/v) with an aqueous mixture of 50% acetonitrile and 0.1% formic acid. All analyses were carried out in the positive ion mode. Two-point mass calibration was performed prior to analysis using MSMS fragments (*m/z* 175.1190 and 1285.5444) of glufibrinopeptide B (Sigma, Australia). MSMS analysis of microcystins with parent masses $[M+H]^+$ of 981 and 995 were performed using an ion spray voltage of 1100. The CAD gas pressure, collision energy and electron multiplier voltage settings were 5, 60 and 2300, respectively.

2.6. DNA isolation

Freeze dried material was used for DNA isolation. An aliquot of freeze-dried material containing 20 mg of the organic partition of freeze-dried material was suspended and ground with a sterile micro pestle in 900 µl SLP-buffer (50 mM Tris–HCl (pH 8.3), 40 mM EDTA, 0.75 mM sucrose). After resuspending, 50 µl of proteinase K (10 mg/ml SLP) and 50 µl 20% w/v SDS was added. After each addition the extract was vortexed. The mixture was incubated and shaken for 2 h at 55 °C. The extract was then centrifuged at $3000\times g$ for 3 min to remove the cell debris. DNA was extracted with an equal volume of phenol chloroform-isoamyl alcohol (25:24:1). DNA was precipitated with a standard NaCl-ethanol precipitation and resuspended in 100 µl sterile water.

2.7. Polymerase chain reaction (PCR), gel-purification and sequencing

All PCR reactions were performed using 0.2 unit *Taq* polymerase (Fischer Biotech, Perth, Australia) in a 20 µl reaction mix containing 2.5 mM $MgCl_2$, 1× *Taq*-Polymerase buffer (Fischer Biotech, Perth, Australia), 0.5 mM dNTPs (Fischer Biotech) and 2.5 pM each of forward and reverse primers. Thermal cycling was performed in a GeneAmp PCR System 2400 thermocycler (Perkin Elmer, Norwalk, CT). PCR amplification was carried out with the MTF2 and MTR2 primer-set (Neilan et al., 1999), and DKF/DKR primers (Moffitt and Neilan, 2001) using an annealing temperature of 50 °C. PCR products were visualised using 2% agarose gels with 1× TAE-buffer and stained with ethidium bromide (1 µg/ml). For photo documentation, a Gel-DOC Bio-RAD System with Quantity One 4.1R software (BIO-RAD, Hercules, CA) was used. The PCR fragments were excised from the gel and purified (Promega, Sydney, Australia).

Automated sequencing was performed using the PRISM Big Dye cycle sequencing system and an ABI 3730 Capillary (Applied Biosystem, Foster City, CA.) using Polymer 7. Sequence data was analysed using the Applied Biosystem Auto-Assembler computer program. The identities of the sequenced amplicons were determined using the BLAST search option on GenBank. All identified sequences were manually checked for possible contamination of the PCR products.

3. Results

3.1. Protein phosphatase inhibition assay (PPIA)

To determine whether the investigated cyanobacterial mat community from the Antarctic Fresh Pond contains compounds with protein phosphate inhibitory capacity, the PPIA was carried out with HPLC fractionated extracts. Protein phosphatase inhibition was observed in HPLC-fractions eluting between minutes 16 and 19. The highest inhibitory activity of undiluted extract, as well as the 4- and 16-fold dilutions, was observed in the 18-min fraction (Fig. 1).

No significant inhibition was observed in any other fraction (data not shown). Calculation of the relative toxin concentration of the 18-min fraction yielded 11.4 ng of MC-LR equivalents per mg dry weight of microbial mat extract. The used standard had a purity of more than 95% with a deviation of ±20% in the lot weight supplied. The total concentration of PP inhibitors would be assumed to be slightly higher than the calculated value for the 18-min fraction, since a weak PPI capacity was also detected in the 15, 16, 17 and 19 min fractions. A subsequent HPLC run with an MC-LR standard (Calbiochem, Germany) resulted in maximal absorption (238 nm) after approximately 18 min retention (Fig. 1). The absorption peak coincided with the observed PPI capacity of the microbial mat extract.

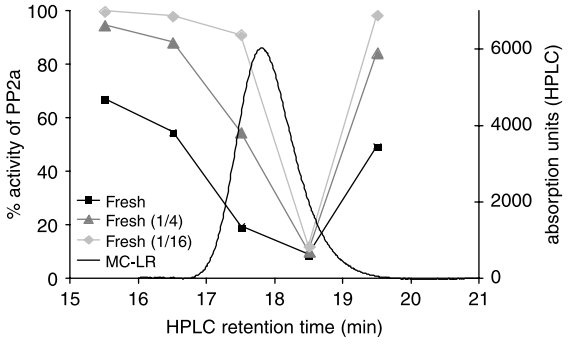

Fig. 1. PP2A/HPLC fraction-diagram: % inhibition of HPLC-fractionated extract samples pure and in 4- and 16-fold dilution (left *y*-axis); retention time of a separately injected MC-LR standard (right *y*-axis).

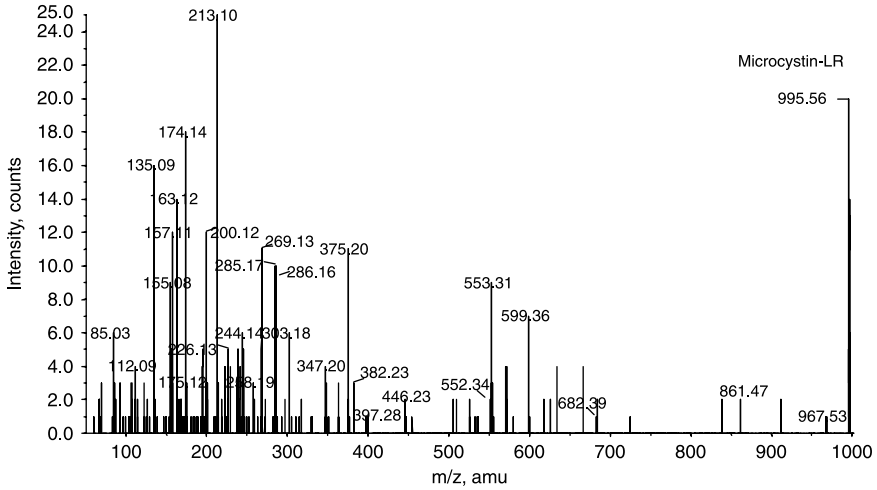

Fig. 2. Ion spectra from the electrospray MS–MS analysis of the methanolic extract of the cyanobacterial mat community from Fresh Pond (Antarctica). Product ion spectra from *m/z* 995 $[M+H^+]$ is microcystin-LR.

3.2. Mass spectrometric analysis

A total extract of the Antarctic cyanobacterial mat community was analysed by time-of-flight tandem mass spectrometry for the presence of microcystins. Two different microcystin isoforms were identified. Both isoforms revealed the characteristic fragment ion peak *m/z* 135 $[M+H^+]$ (Table 1). This ion peak originated from the Adda side-chain ($PhCH_2CH\ (OMe)^+$), which is a key indicator for the presence of microcystins. Further investigation of the fragment ion peaks enabled the identification of the isoform microcystin-LR. This microcystin isoform has a molecular weight of 994. The mass spectrum is shown (Fig. 2) and L-leucine and L-arginine are present at the variable positions of the cyclic heptapeptide structure. A complete list of the detected fragment ion peaks for the Antarctic microcystin-LR is shown in Table 1.

The second microcystin isoform identified in the Antarctic cyanobacterial extract was [D-Asp^3] microcystin-LR at *m/z* 981 $[M+H^+]$. The detected fragment ion peak at *m/z* 539 $[Arg\text{-}Asp\text{-}Leu\text{-}Ala\text{-}Mdha+H]^+$ is characteristic for this demethylated microcystin-LR isoform (Table 1).

3.3. Polymerase chain reaction (PCR) and sequence analysis

Genomic DNA from the Antarctic mat material was screened for the presence of genes encoding non-ribosomal peptide synthetases and polyketide synthases. PCR products were amplified using both the NRPS and PKS primer sets

Table 1
Assignment of fragment ions observed in the product spectra of *m/z* 995 $[M+H^+]$ for microcystin-LR and *m/z* 981 $[M+H^+]$ from [D-Asp^3] microcystin-LR in a methanolic extract of the Antarctic microbial mat from fresh pond

Fragment ions	MC-LR *m/z*	[D-Asp^3] MC-LR *m/z*
MC-LR $[M+H^+]$	995	–
[D-Asp^3] MC-LR $[M+H^+]$	–	981
$PhCH_2CH(OMe)^+$	135	135
$[Mdha\text{-}Ala+H]^+$	155	–
$[C_{11}H_{14}O+H]^+$	–	163
$[Arg+NH_3+H]^+$	174	–
$[Glu\text{-}Mdha+H]^+$	213	213
$[C_{11}H_{14}O\text{-}Glu\text{-}Mdha\ +H]^+$	375	375
$[C_{11}H_{14}O\text{-}Glu\text{-}Mdha\text{-}Ala\ +H]^+$	466	–
$[Arg\text{-}Masp\text{-}Leu\text{-}Ala\ +H]^+$	470	–
$[Arg\text{-}Masp\text{-}Leu\text{-}Ala\text{-}Mdha+H]^+$	553	–
$[Arg\text{-}Asp\text{-}Leu\text{-}Ala\text{-}Mdha+H]^+$	–	539
$[Adda\text{-}Arg\text{-}Masp+H]^+$	599	–
$[Glu\text{-}Mdha\text{-}Ala\text{-}Leu\text{-}Masp\text{-}Arg+H]^+$	682	–
Loss of PhCH=CH (OMe)	861	–

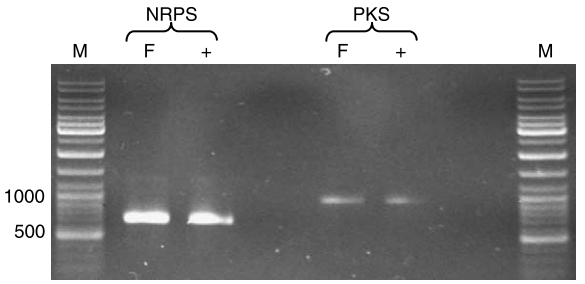

Fig. 3. The gel-purified PCR products obtained from total genomic DNA of the investigated Antarctic cyanobacterial mat community from Fresh Pond using primers targeting NRPS and PKS genes, separated by gel electrophoresis on a 2% agarose gel: M: 1 kb+ DNA-ladder (Fermentas, Australia). Lane 1: NRPS gene fragment from cyanobacterial mat community (Fresh Pond), Lane 2: positive control (*M. aeruginosa* PCC7806) for NRPS screen, lane 3: PKS from cyanobacterial mat community (Fresh Pond), lane 4: positive control (*M. aeruginosa* PCC7806) for PKS gene screen.

(Fig. 3). The PCR amplicon generated using the MTF2/MTR2 primers showed the highest similarity (68%) to cyanobacterial peptide synthetases (Npun02002757) in *Nostoc punctiforme* PCC 73102. The highest similarity of the primed amplicon DKF/DKR primers was 75% to a polyketide synthase (Npun02002761) in *Nostoc punctiforme* PCC 73102.

4. Discussion

In the present study, we confirmed the production of microcystins by an Antarctic benthic cyanobacterial mat community from a meltwater pond situated on the McMurdo Ice Shelf. It was possible to detect the presence of protein phosphatase inhibiting molecules via a combined HPLC/PPIA-system, and also identify MC-LR, and for the first time, [D-Asp^3] MC-LR in an Antarctic cyanobacterial mat community by mass spectrometric analysis. These microbial mats from Fresh Pond have never before been investigated for microcystin production. Even though only two microcystin isoforms were detected in the present study, most species are known to produce various microcystin congeners simultaneously (Fastner et al., 2001; Mikalsen et al., 2003). Further isoforms were possibly not identified because of the detection limit of mass spectrometric analyses. The utility of mass spectrometry (Erhard et al., 1997, 1998) and LC-ESI-MS (Zweigenbaum et al., 2000) for identification of the microcystins from complex communities has been demonstrated, without the need for toxin standards or specific retention times that are required for HPLC analyses. This work confirmed the suitability of this technique also for cyanobacterial communities in Antarctic meltwater ponds.

It was also possible to semi-quantify 11.4 ng MC-LR equivalents per mg dry weight by PPIA. The term semi-quantification indicates that the MC-LR standard used for the PPIA and HPLC-DAD is not a certified analytical standard, but merely a purified toxin (purity > 95%) with a deviation of ±20% in the lot weight supplied. This toxin concentration is approximately two orders of magnitude lower than the toxin levels that are usually associated with planktonic cyanobacterial blooms with levels of more than 1 μg microcystin-LR produced per mg of dry bloom weight (Chorus and Bartram, 1999). However, a previous study of cyanobacteria from 18 Antarctic meltwater ponds of the McMurdo Ice Shelf has shown similarly low values, with microcystin-LR equivalents of 5–100 ng/mg per mg dry weight (Hitzfeld et al., 2000; Späth, 2000). Furthermore, this indicated that the biosynthesis of microcystins has been a continuous process in these Antarctic mats since the previous studies investigated samples from the years 1997 to 1999 while this study was performed on a sample from 2000.

The low level of microcystin could potentially be due to either the low abundance of a particular microcystin producing species, or due to low levels of biosynthesis by one or more members of the community. It was recently reported that the cyanobacterial mat community of Fresh Pond is very diverse, consisting of at least 17 Oscillatoriales and 6 Nostocales phylotypes (Jungblut et al., 2005). These identified phylo- and morphotypes belonged to the filamentous genera *Phormidium*, *Oscillatoria* and *Lyngbya*, or the heterocystous genera *Nostoc*, *Nodularia*, and *Anabaena*. Of these phylo- and morphotypes the cyanobacterial genera *Lyngbya* and *Nodularia* have never been reported to be microcystin producers. The *Anabaena* phylotype was identified by molecular characterisation of the mat community and has often been associated with microcystin production (Carmichael and Gorham, 1977; Rouhiainen et al., 2004; Sivonen et al., 1992a), however, the *Anabaena* phylotypes identified showed the highest similarity to *Anabaena cylindrica* NIES 19, which has not been reported to produce microcystin so far (Jungblut et al., 2005).

Oscillatoria sp., *Phormidium* sp. and *Nostoc* sp. have also been reported to be microcystin-producers, however these three genera are not common bloom-forming species. For example, the species *O. tenuis*, *O. chlorina* and *O. limosa* were identified to be microcystin-producing, however, none of these species have been identified in the mat community of Fresh Pond (Brittain et al., 2000; Mez et al., 1997; Oudra et al., 2002). In the case of *Phormidium* sp. only rare isolates are able to produce microcystin (Izaguirre et al., in preparation) and these morphotypes/phylotypes did not show high similarity to those found in Fresh Pond. Most of the microcystin-producing *Nostoc* isolates have been reported from European countries, such as England and Finland (Sivonen et al., 1992b), being temperate to cold temperate environments.

Microcystin production by *Nostoc* from cool (Oksanen et al., 2004; Sivonen et al., 1990) to warm temperate environments has been reported (Sivonen and Jones, 1999; Davidson, 1959; Dos S. Vieira et al., 2005), however, phylotypes identified in Fresh Pond did not display a high similarity to the characterised microcystin-producing *Nostoc* species. Microcystin production in *Nostoc* has involved the identification and characterisation by of uncommon microcystin variants (Sivonen et al., 1990, 1992b). The identification of an unusual variant would be potentially helpful for the identification of the microcystin-producing species in the Antarctic mat community. It is also interesting that the microcystins identified in this study were extracted from a benthic community since most microcystin-producing species are planktonic (Carmichael, 1997; Sivonen and Jones, 1999). *Oscillatoria limosa* from alpine regions of Switzerland was associated with microcystin-production (Mez et al., 1997, 1998), however *Oscillatoria limosa* was not found in the mat community present in Fresh Pond. This particular species, however, has been identified in benthic mat communities of other meltwater ponds on the McMurdo Ice Shelf (Howard-Williams et al., 1990; Jungblut et al., 2005). Hitzfeld et al. (2000) drew a correlation between the presence of Antarctic *Nostoc* sp. and *Oscillatoria* sp. with the inhibitory capacities of the mats investigated. It could be summarised that either the toxin-producing species in the mat are of low abundance or represent novel microcystin-producing species since no morpho- or phylotypes, that have previously been reported to be a microcystin producer, were identified. An important future step will be the isolation and characterisation of potentially toxic cyanobacteria from Antarctic cyanobacterial mat communities.

The molecular approach of the present study was able to amplify genes encoding cyanobacterial non-ribosomal peptide synthetases (NRPS) and polyketide synthase (PKS). At this stage it was not possible to conclude that these genes were responsible for the microcystin biosynthesis. Further studies on microcystin production in Antarctica, including clone-libraries of the multiple PCR products obtained, could detect a wide range of microcystin synthetases from unique Antarctic taxa. Nevertheless, the present study confirmed that Antarctic cyanobacteria are capable of producing non-ribosomal peptides and could display a similar molecular diversity of non-ribosomal peptides found in cyanobacterial isolates in culture collections (Christiansen et al., 2001). These compounds are a source of chemotherapeutic agents, such as antimicrobial and antifungal compounds (Mankelow and Neilan, 2000) and, these results indicate the presence of peptides of biotechnological interest.

In summary, we were able to identify and semi-quantify the production of the microcystins MC-LR and MC-LR [D-Asp^3] in the benthic Antarctic cyanobacterial mat community from the meltwater pond Fresh Pond. Isolation of members of the community and investigations targeting the genes involved in Antarctic microcystin production will further knowledge regarding the evolution and distribution of cyanobacteria and their toxins.

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