TOXIGENIC CYANOBACTERIA IN VOLCANIC LAKES AND HOT SPRINGS OF A NORTH ATLANTIC ISLAND (S. MIGUEL, AZORES, PORTUGAL)

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ABSTRACT

Increased occurrence of blooms of cyanobacteria producing toxins due to eutrophication of lakes and rivers has been alerting the world to the problem of water quality, because of the risk they pose to environmental and animal health. In this study, we sampled cyanobacteria from volcanic lakes and hot springs of the North Atlantic S. Miguel Island (Azores, Portugal), and identified them using a molecular approach. Cyanobacteria were also scanned for the presence of toxigenicity using specific primers targeting genes involved with toxin production. Sequences of 16S rDNA were related to six genera: *Microcoleus, Leptolyngbya, Limnothrix, Microcystis, Pseudanabaena,* and *Gloeothece*, being similar to those of other volcanic areas in Mexico, Australia and Hawaii. In some of the samples, genes responsible for the production of the toxins microcystin, cylindrospermopsin and saxitoxin were identified, indicating the potential toxicity of the species.

KEYWORDS:
Cyanobacteria; azores; cyanotoxins, volcanic lakes, hot springs

1. INTRODUCTION

Planktonic and epilitic cyanobacteria from islands in the North Atlantic area are not well-known. Some studies were done in Iceland, in lake Myvatn, where *Anabaena flos-aquae* blooms occur synchronized with zoobenthos cycles but no information regarding toxins was given [1]. In Azores, Madeira or Canarian arquipelagos not much is known concerning cyanobacteria and cyanotoxins, although problems of severe eutrophication were detected in some Azores lakes [2]. Santos et al. [3] detected blooms of *Microcystis aeruginosa* in Lake Azul in S. Miguel Island, but low toxin levels were found. It is also interesting because all these islands, formed by tectonic activity and still having surface volcanic phenomena, such as hot springs, boiling mud ponds and geysers, develop microbiological communities that are quite unique. On the other side, being in the Atlantic, they may help us to understand the species dispersion between North America and Europe as well as Africa. Cyanobacteria are known for the production of secondary metabolites that may be of pharmaceutical interest [4, 5], or have toxic properties – cyanotoxins [6, 7].

The cyanotoxins, secondary metabolites produced by many cyanobacteria, as other toxins, may enter the body by different pathways and damage cells, organs and tissues. One way to classify cyanotoxins reflects their biological effects on the systems and organs they affect most strongly. Nowadays, known cyanotoxins are classified as hepatotoxins, neurotoxins, cytotoxins and dermatotoxins [8, 9]. Each cyanotoxin can be produced by more than one cyanobacterial species, and the same species is able to produce more than one toxin. Moreover, within a single species, different genotypes occur, some of which possess the gene for a given toxin and others do not. There are also members of the same species that present genes for toxin production (toxigenic ones) but do not produce the toxin under some conditions, or do not produce it at all [9]. The toxicity of a given bloom is determined by its strain composition, in other words, the relative share of toxic versus nontoxic genotypes.

Nowadays, there is not much information about freshwater cyanobacteria from Azores Archipelago, and few reports on cyanotoxin production have been published. To get data on these organisms is important to understand the migration patterns and their phylogeny. In this study, we isolated and identified cyanobacteria collected in lakes of Azores Archipelago – S. Miguel Island (Portugal), to investigate a possible production of cyanotoxins by the application of molecular probes.

2. MATERIAL AND METHODS

2.1. Sampling

All the sampling sites were located in S. Miguel Island (Azores) in the 37ºN 25ºO zone, and in altitudes ranging

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from 207 to 758 m above sea level. Sampling was done in March 2008, in all the lakes and hot springs (Table 1) by collecting water from the margin avoid disturbing the sediment [10]. In some lakes (Canário, Fogo, Terra Nostra), superficial sediment samples were also taken because cyanobacterial development was clearly visible. Samples in the hot springs Furnas Caldeira, Furnas Hot Spring and Caldeira Velha were done by scraping epilythic material from the hot spring zone. In Furnas, material from 8 different hot springs was collected and numbered consecutively. Water temperature was measured *in situ* with a thermometer. Environmental samples were refrigerated, transported to the lab, and aliquots were frozen before molecular analysis.

2.2. DNA extraction

All samples were homogenized. Small quantities (2 to 6 ml) were collected by centrifugation in 1.5-ml microcentrifuge tubes (2000 rpm, 15 min) and the genomic DNA extracted using Purelink Genomic DNA Mini Kit (Invitrogen) following the instructions provided for gram-negative bacteria. The purification of extracted DNA was confirmed using 1.5% agarose gel in 1× TAE buffer (20 ml of 50X TAE buffer to 980 ml of deionized water) [11]. The gels were stained with ethidium bromide and photographed under UV transillumination.

2.3. PCR conditions and primers

PCR was carried out on the DNA extracts using specific primer pairs to amplify the 16S rDNA region shared by all cyanobacteria, as a positive control for subsequent analyses [12, 13] (Table 2). In order to detect potentially toxic cyanobacteria, different primer pairs previously described, were used to detect different targets known to be involved either in the biosynthesis of microcystin (MC), nodularin, cylindrospermopsin (CYN) or saxitoxin (STX). DNA extracted from *Microcystis aeruginosa* M6 strain was used as positive control for the detection *16s rRNA*, *mcyA*, *mcyB* and *mcyE* genes. *Cylindrospermopsis raciborskii* AQS strain was used as positive control for the detection of *PS* and *PKS* genes, and *Aphanizomenon gracile LMECYA040* strain DNA was used as positive control for the detection of *sxt1* gene (Table 2).

TABLE 1 - Coordinates (latitude/longitude), height above sea level (H) and water temperature (T) of the sampled lakes in S. Miguel Island, Azores, Portugal (L = Lake, Hs = hotspring, E = Eutrophic, M = Mesotrophic).

Lake	Type of system		Latitude/ longitude	H (m)	pH [31, 33]	Cond µS/cm [31-35]	Trophic state [33, 34]	T (ºC)
A	Azul	L	37º51'36''N/25º46'46''O	266	7.5	114	E	17
V	Verde	L	37º51'12''N/25º47'22''O	266	7.5	114	M	17
F	Furnas	L	37º45'10''N/25º20'17''O	285	7.5	110	E	18
FG	Fogo	L	37º45'21''N/25º28'67''O	572	6.7	48	M	13
SB	S. Brás	L	37º47'30''N/25º24'30''O	615	6.3	45	E	19
C	Canário	L	37º50'09''N/25º45'32''O	758	6.4	62	E	13
TN	Terra Nostra	L	37º46'20''N/25º18'53''O	207	5.6	785	-	35
CF	Caldeira das Furnas	Hs	37º46´42''N/25º18'40''O	247	8.7	1312	-	54
FH	Furnas Hot Springs	Hs	37º45'10''N/25º20'17''O	285	6.4	1352	-	40
CV	Caldeira Velha	Hs	37º46'52''N/25º29'58''O	416	4.6	293	-	30

TABLE 2 - Primers used in this study to detect cyanobacteria and amplify genes involved in cyanotoxin production.

Primer	*Sequence* (5' → 3')	AT (ºC)	Size (Bp)	Target gene	Ref.
27F	AGAGTTTGATCCTGGCTCAG	50	780	*16s rDNA*	[13]
809R	GCTTCGGCACGGCTCGGGTCGATA				
740F	GGCYRWAWCTGACACTSAGGGA	50	754	*16s rDNA*	[12]
1494R	TACGGTTACCTTGTTACGAC				
mcyA - CD1F	AAAATTAAAAGCCGTATCAAA	59	297	*mcyA*	[14]
mcyA - CD1R	AAAAGTGTTTTATTAGCGGCTCAT				
mcyB2959F	TGGGAAGATGTTCTTCAGGTATCCAA	59	350	*mcyB*	[15]
mcyB3278R	AGAGTGGAAACAATATGATAAGCTAC				
PKEF1	*CGCAAACCCGATTTACAG*	52	755	*mcyE*	[!7]
PKEF2	*CCCCTACCATCTTCATCTTC*				
Sxt1-F	*GCTTACTACCACGATAGTGCTGCCG*	60	1669	*sxt*	[18]
Sxt1-R	*GGTTCGCCGCGGACATTAAA*				
M13	GGCAAATTGTGATAGCCACGAGC	55	597	*ps*	[21]
M14	GATGGAACATCGCTCACTGGTG				
K18	CCTCGCACATAGCCATTTGC	45	422	*pks*	[21]
M4	GAAGCTCTGGAATCCGGTAA				

For potential MC-producing cyanobacteria detection, a battery of primer pairs was used to better characterize the samples assayed. The mcyA CD1F/mcyA CD1R primer pair [14] was designed to amplify a section of *mcyA* gene coding for the condensation domain from MC-synthesizing *Microcystis* and *Planktothrix* strains, previously proved to be suitable to detect MC-producing cells from the genera *Anabaena, Microcystis* and *Planktothrix* [14]. To determine the presence of potentially MC-producing *Microcystis* strains, the mcyB 2959F/3278R primer pair was used [15]. The mcyE PKEF1/PKER1 primer pair was used to amplify a 755-bp fragment of the glutamate-1-semialdehyde aminotransferase domain – the *mcyE* gene [16, 17]. The SXTI-F/ SXTI-R primer pair was used to amplify a region responsible for transfer of a carbamoyl group to the hydroxymethyl side chain of STX precursor - *sxt1* gene [18]. CYN is produced by species such as *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* [19] and, besides MC, is the most widely occurring hepatotoxin produced by cyanobacteria [20]. Production of CYN by other species, such as *Anabaena bergii* [21], *Aphanizomenon flos-aquae* [22], *Aphanizomenon ovalisporum* [23], *Raphidiopsis curvata* [24] and *Umezakia natans* [25], has been reported. Two sequence determinants likely to be involved in CYN production, *ps* and *pks*, were identified by degenerating PCR amplification of conserved regions in polyketide synthase genes, and correlated well with toxin production [21, 26].

All PCR reactions were prepared in a volume of 20 µl containing 1× PCR buffer, 2.5 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate, 10 pmol of each of the primers, 0.5 U of Taq DNA polymerase, and 5–10 ng of DNA. Thermal cycling was carried out using a Bio-Rad MyCycler™, or a Biometra TProfessional standard thermocycler. Amplifications were performed as decribed before (Table 2). PCR products were separated by 1.5% agarose gel in 1× TAE buffer. The gels were stained with ethidium bromide and photographed under UV transillumination.

2.4. DNA Sequencing

PCR was carried out on the DNA extracts using the 16S 27F/16S 809R and 16S 704F/16S 1494R [13] primer pairs to amplify, respectively, a 780 and 754-bp fragment of 16S rRNA shared by all cyanobacteria. All PCR reactions were prepared in a volume of 100 µl containing 1× PCR buffer, 2.5 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate, 10 pmol of each of the primers, 0.5 U of Taq DNA polymerase, and 5–10 ng of DNA. Thermal cycling was carried out using a MyCycler (Bio-Rad). For the 16S 27F/ 809R and 16S 704F/ 1494R primer pairs, amplification was performed with an initial denaturation step of 92 °C for 2 min, followed by 35 cycles of 92 °C for 20 s, annealing temperature of 56 ºC for 30 s, 72 °C for 60 s, and a final extension step at 72 °C for 5 min. A portion of PCR products (8 µl) was separated by 1.5% agarose gel in 1× TAE buffer. The gels were stained with ethidium bromide and photographed under UV transillumination

PCR products were cleaned using Nucleospin II kit (Macherey-Nagel, Germany) following the manufacturer's instructions, and sent for sequencing performed in an ABI Prism following the Big Dye Terminator Protocol.

3. RESULTS

The method used for genomic DNA extraction was efficient for all the samples analyzed as shown by the detection of cyanobacterial DNA in all the samples from lakes and hot springs of S. Miguel Island, confirmed by the 16S rDNA PCR amplification (Table 3). The results presented in Table 4 indicate the potential cyanobacterial species dominant in each sample, obtained by statistical similitude with some genes sequences stored in databases. All samples presented an identity of at least 91 %, and 6 genera were assigned: *Microcoleus, Leptolyngbya, Limnothrix, Microcystis, Pseudanabaena* and *Gloeothece.* Most of the cyanobacteria whose sequence was compared with ours, were collected in similar environments, such as waters rich in sulfur arising from sedimentary rock hypolithic cyanobacteria in Northern Australia, hydrothermal exit in Cenote Verde, México, or cyanobacterial mats in lava caves - Cavernas de Lava, in Hawai (Table 4).

In the lakes, filamentous forms such as *Leptolyngbya* were found, as well as *Limnothrix redekei* in Lake Azul, but also *Microcystis aeruginosa* was identified in the benthos sample of Lake Canário. It is interesting because the species was not identified in the planktonic sample and, in fact, the water was transparent till the bottom. In the hot springs, we found mostly *Pseudanabaena*, *Gloeothece* and *Microcoleus.* Apart from these, we could match our species with clones from similar environments in Mexico (hydrothermal exits in Cenote Verde) or in Havaii (cyanobacterial mats in Cavernas de Lava).

Concerning gene analysis, using the primers for the detection of MC, NOD, CYN and STX genes, only in one sample C (b) (Table 3), the result was positive for mcy genes. In the sediment sample of Lake Canário, we identified a *Microcystis aeruginosa* clone, and all the specific genes responsible for the production of MC in this genus (*mcyA*, *mcyB* and *mcyE*) gave positive amplification. In some other lakes, some genes could be detected as *mcyA* in Lakes Azul and Terra Nostra, *mcyB* in Lake Fogo but, as shown for other lakes and other regions in the world, in some non toxigenic strains of cyanobacteria, parts of the gene cluster responsible for the production of MC may be detected. Concerning STX production, no amplification products were found for any of the lake samples. Concerning CYN occurrences, the molecular results confirmed that in one sample – Lake Terra Nostra – both *ps* and *pks* genes were amplified indicating the possibility to produce CYN. In some other samples, only one of the genes was present, *pks* in lakes Azul and Fogo (b) as well

TABLE 3 - Results of PCR analysis for cyanobacterial identification and screening of toxin-producing genes from S. Miguel Lake samples ((b) benthic cyanobacteria, (+) positive, (-) negative).

Lake/ Hotspring	16S	*mycA*	*mcyB*	*mcyE*	*sxt*	*pks*	*ps*
A	+	+	-	-	-	+	-
V	+	-	-	-	-	-	-
F	+	-	+	-	-	-	-
FG	+	-	-	-	-	-	-
FG (b)	+	-	-	-	-	+	-
SB	+	-	-	-	-	-	+
C	+	-	-	-	-	-	-
C (b)	+	+	+	+	-	-	
TN	+	+	-	-	-	+	+
TN (b)	+	-	-	-	-	-	-
CF	+	+	-	-	-	-	-
FH1	+	+	-	-	-	-	+
FH2	+	-	-	-	-	-	-
FH3	+	+	-	-	-	-	-
FH4	+	-	-	-	-	-	-
FH5	+	-	-	-	-	-	-
FH6	+	+	-	-	-	+	+
FH7	+	-	-	-	-	-	-
FH 8	+	+	-	+	-	-	-
CV	+	-		-	-	-	-

TABLE 4 - Sequencing results, percent identity (%) and size of the fragment (bp) of lake and hotspring samples from Azores, and putative identification of cyanobacteria ((b) benthic cyanobacteria).

Lake/ Hotspring	Sequencing	%	Size (bp)	Observations
A	*Limnothrix redekei* CCAP 1443/1	99	741	-
V	Cf. *Leptolyngbya* sp. Greenland_9	95	1124	-
FG	Uncultured cyanobacterium clone Z4MB16	97	1391	freshwater; collected in 4 m depth; El Zacaton, México
FG (b)	Not determined	-	-	-
SB	*Leptolyngbya* SP. PCC 7410	99	741	-
C	Not determined	-	-	-
C (b)	*Microcystis aeruginosa* PCCC 7806	95	737	-
TN	Uncultured cyanobacterium clone BAC3_B12	99	752	collected in waters rich in sulfur arising from sedimentary rock
TN (b)	Uncultured bacterium clone QuartzC18	93	1445	Hypolithic cyanobacteria; Northern Australia
CF	Not determined	-	-	-
FH1	*Pseudanabaena* PCC6802	96	657	-
FH2	Uncultured cyanobacterium clone VERDEA65	99	738	Freshwater; collected in 1 m depth; hydrothermal exit; Cenote Verde, México
FH3	Uncultured cyanobacterium clone VERDEA65	98	736	Freshwater; collected in 1 m depth; hydrothermal exit; Cenote Verde, México
FH4	Uncultured cyanobacterium clone HAVO-mat113	98	727	collected in a cyanobacterial mat; Cavernas de Lava; Havai;
FH5	*Gloeothece* sp. PCC 6909/1	93	740	-
FH6	Uncultured cyanobacterium clone VERDEA69	93	681	Freshwater; collected in 1 m depth; hydrothermal exit; Cenote Verde, México
FH7	Uncultured cyanobacterium clone VERDEA69	97	737	Freshwater; collected in 1 m depth; hydrothermal exit; Cenote Verde, México
FH8	*Microcoleus* sp. SAG 2212	91	719	-
CV	Uncultured cyanobacterium clone VERDEA68	95	1110	Freshwater; collected 1 m depth; hydrothermal exit; Cenote Verde, México

as *ps* in Lake S. Brás. As in the case of MC, the presence of only one gene does not usually allow the strain to synthesize the toxin.

In the hot springs, the amplification of the 3 MC genes was not seen, but *mcyA* occurred in five and *mcyE* in only one out of 9 Furnas hot springs samples (Table 3), whereas no *mcyB*-positive one was found. No *sxt* was found but two *ps* and one *pks* positive samples were detected. As far as we could search, not much data on toxin-producing cyanobacteria have been published for hot springs.

4. DISCUSSION

The identification of *M. aeruginosa* in the sediment sample of Lake Canário suggests that colonies of this species overwinter on the sediment of the lake, and then can bloom whenever the environmental conditions are adequate. In this lake, the temperature was quite low (13 °C) but colonies of this toxigenic species were alive on the sediment ready to inoculate the water column. In 2005, Santos et al. [27] found *M. aeruginosa* blooming in Lake Azul, from April/May to October. *M. aeruginosa* is also a common cyanobacterium in similar eutrophic crater lakes in Mexico [27]. In Lake Terra Nostra, a ferruginous artificial pond built in the Park with the same name, the cyanobacteria-dominant species could not be identified but it was similar to a clone isolated from waters rich in sulfur and to hypolithic cyanobacteria from Northern Australia. In fact, this lake has waters with high temperature (35 °C), very turbid and with very high concentrations of iron salts giving it a ferruginous color. The species found in the lakes were different from those found in the hot springs as expected. Although the temperatures of the hot springs were quite high, between 30-54 °C, cyanobacteria are some of the few organisms that can survive and develop under such a physical and chemical stress. Mats were quite extended, and most of them were unicyanobacterial as visualized under microscope.

Pseudanabaena strains belong also to the Oscillatoriales, with trichomes solitary or agglomerated in very fine, mucilaginous mats, not very long without any branching. *Microcoleus* has trichomes without heterocysts and with equal diameter throughout the whole length with no branches. As a genus characteristic, it has more than two trichomes within a mucous sheath. *Gloeothece* belong to the Chroococcales, with colonies usually small, composed of sheathed cells or groups of cells enclosed within their own gelatinous envelopes, and sometimes surrounded by common mucilage.

Concerning the toxin-producing clusters, the positive results for the 3 *mcy* genes in Lake Canário sediment allow us to conclude that there is potential for MC production during adequate environmental conditions. This is why it is important to have a molecular approach to the risk analysis in lakes because we can predict that in a certain lake toxin production may occur. Simple phytoplankton analysis of the water column at this stage would not reveal this risk, as we could conclude from the PCR data of the water column in this same lake. Nevertheless, in most of these cases, no toxin production is possible. This may indicate a loss of genes along evolution and by that a loss of toxin production capacity. Santos et al. [3] found *M. aeruginosa* blooming in Lake Azul but MC values were quite low, under 0.1 µg MC-LR eq L^{-1}, in spite of the density of *M. aeruginosa* which could reach 4 x 10^5 cells/ml. This indicates that few strains have the genes involved in MC production, or the environmental conditions are not adequate for the expression of those genes.

The detection of some of the genes involved in MC and CYN production in the hot spring samples is very interesting because of the high environmental temperatures. Nevertheless, in none of the samples all the genes were present, so production of the toxins in not assured, except for the FH6 sample where both *ps* and *pks* were identified on the same sample. Therefore, we may assume that there is potential for CYN production. Mohamed [28] reported the presence of MC in hot spring cyanobacterial mats in Saudi Arabia, with values ranging from 468 to 512 µg MC-LR eq g^{-1}. Krienitz et al. [29] also reported on the diversity and potential toxicity of cyanobacteria in hot springs in Kenya and Tanzania. Later, they also reported that MC values in cyanobacterial mats attained 221 to 845 µg microcystin-LR equivalents g^{-1} DW of mat, and anatoxin-a ranged from 10 to 18 µg g^{-1} DW of mat [30]. They suggested that these toxins could be implicated in the death of Lesser Flamingos in the alkaline Lake Bogoria, Kenya. So, it is evident that cyanotoxins may occur in these extreme environments, and not only in freshwater lakes and reservoirs. So, more information and research is needed.

5. CONCLUSIONS

In this study on volcanic lakes and hot springs of the North Atlantic S. Miguel Island (Azores, Portugal), six cyanobacteria genera were identified based on morphological analysis and confirmed by 16S sequence analysis: *Microcoleus, Leptolyngbya, Limnothrix, Microcystis, Pseudanabaena,* and *Gloeothece.* The detection of some of the genes involved in MC and CYN production in the hot spring samples is very interesting because of the high environmental temperatures. In one of the samples, *ps* and *pks* were identified on the same sample, so we may assume that there is a potential for CYN production.

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