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Microcystis aeruginos strain [D−Leu¹] Mcyst−LR producer, from Buenos Aires province, Argentina

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PEER REVIEW

ABSTRACT

Objective: To show the toxicological and phylogenetic characterization of a native *Microcystis aeruginosa* (*M. aeruginosa*) strain (named CAAT 2005−3) isolated from a water body of Buenos Aires province, Argentine.

Methods: A *M. aeruginosa* strain was isolated from the drainage canal of the sewage treatment in the town of Pila, Buenos Aires province, Argentina and acclimated to laboratory conditions. The amplification of *cpcBA*−IGS Phcocyanin (PC, intergenic spacer and flanking regions) was carried out in order to build a phylogenetic tree. An exactive/orbitrap mass spectrometer equipped with an electrospray ionization source (Thermo Fisher Scientific, Bremen, Germany) was used for the LC/ESI−HRMS microcystins analysis. The number of cell/mL and [D−Leu¹] Mcyst−LR production obtained as a function of time was modelled using the Gompertz equation.

Results: The phylogenetic analysis showed that the sequence clustered with others *M. aeruginosa* sequences obtained from NCBI. The first Argentinian strain of *M. aeruginosa* (CAAT 2005−3) growing under culture conditions maintains the typical colonial architecture of *M. aeruginosa* with profuse mucilage. *M. aeruginosa* CAAT 2005−3 expresses a toxin variant, that was identified by LC−HRMS/Orbitrapas as [D−Leu¹] microcystin−LR ([M+H]+=1037.8 m/z).

Conclusions: [D−Leu¹] microcystin−LR has been also detected in *M. aeruginosa* samples from Canada, Brazil and Argentina. This work provides the basis for technological development and production of analytical standards of toxins present in our region.

1. Introduction

The cyanobacteria, cyanophytes, or blue−green algae are photosynthetic prokaryotes distributed worldwide. Species of the genus *Microcystis*, (Order Chroococcales) are known for their potential ability to synthesize toxins, mainly microcystins (Mcyst)[1].

The typical structure of Mcyst is cyclic (D−alanine¹−X²−D−MeAsp³−Y−4−Adda⁵−D−glutamate⁶−Mdha⁷), in which X and Y are variable L−amino acids, D−MeAsp³ is D−erythro−β−methyl aspartic acid, and Mdha is N−methyldehydroalanine. The Adda amino acid [(2S,3S,8S,9S)−3−amino−9−methoxy−

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2,6,8–trimethyl–10–phenyldeca–4,6–dienoic acid] has only been found in cyanobacterial hepatotoxins. There are known over 85 different varieties of this toxin[2], differing by Adda methylation, or configuration and/or identity of X and Y amino acids[3,4].

Mcyst are non–ribosomally synthesized by a multifunctional enzyme complex, consisting of both a peptide synthetase and a polyketide synthase coded by the *mcy* gene cluster[5]. Microcystin synthetase genes *mcyA, mcyB, mcyC* and *mcyD* have been identified in *Anabaena, Planktothrix* and *Microcystis* strains[6].

Mcyst constitute a family of analogous cyclic heptapeptides that specifically inhibit eukaryotic protein phosphatases of types 1 and 2A and increase free radicals, affecting various functions in cell metabolism such as changes in the cytoskeleton, mitochondrial activity and cellular communication[7,8]. In acute exposure, these toxins can lead to hepatocyte necrosis and liver hemorrhage[1]. In addition to acute hepatotoxicity, effects of sub–chronic exposure to Mcyst has been reported in mice[9,10]. Also, exposure to low concentrations of Mcyst in drinking water can cause chronic effects in mammals due to their potent tumor–promoting activity[11].

Microcystis sp. blooms are frequently found at eutrophic waters affecting fishes[12], zooplanctonic communities[13], aquatic plants and vertebrates[14].

Microcystis blooms decrease water quality and impacts nautical sports development and recreacional activities[15,16]. Also, when toxic cyanobacterial blooms occur in drinking water sources, Mcyst could reach the net water[17,18]. It is widely accepted that Mycs exposure occurs mainly by chronic oral intake of contaminated water and could produce liver damage[18,19]. Human exposure to Mcyst has been linked to high incidences of liver cancer in certain regions of China[20]. However, lung functions may result when aerosols, containing cells and toxin, are aspirated during water sports or recreational activities[16].

Even when toxic cyanobacteria have been identified in 13 out of 24 provinces along Argentina[21–23] and the presence of Mcyst synthesized by *Microcystis aeruginosa* (*M. aeruginosa*) (Kützing) Kützing, 1846, in Río de la Plata basin has been reported[18,24–26]; there are only few reports of this issue in Argentina.

[D–Leu¹] Mcyst–LR was evidenced as a component of a toxic bloom appearing in the urban area of Buenos Aires city during the summer of 2001[27].

In cyanobacterial blooms collected over 4 years (1998–2002)

in San Roque Reservoir (Córdoba, Argentina) the presence of Mcyst–LR and Mcyst–RR in 97% of these blooms was reported[21]. Four variants, Mcyst–LR, –RR, –LA and –YR, were identified in water samples of Los Padres Lake, Buenos Aires, Argentina. Mcyst–RR was the main toxin found in both cellular and dissolved fractions respectively[13].

In this work we show the characterization of the first Argentinean *Microcystis* strain. It was isolated from a cyanobacteria natural bloom, collected from an eutrophicated freshwater body located near Pila town, province of Buenos Aires, Argentina. The only Mcyst variant produced by the strain was identified as [D–Leu¹] Mcyst –LR by LC–Orbitrap–MS system.

2. Material and methods

2.1. Natural bloom sampling

Bloom sample was obtained on September 17th of 2005 from the drainage canal of the sewage treatment in Pila town (35°59′49″S, 58°08′11″W), Buenos Aires province, Argentina.

Phytoplankton samples were obtained using 30 μm mesh plankton net and immediately transported to the laboratory. Quantitative samples were collected with a Van Dorn bottle and fixed in situ with 1% lugol solution.

The samples were observed using a Wild M20 inverted microscope Zeiss Axiovert.

2.2. Strain isolation

A *M. aeruginosa* strain was isolated from bloom samples and acclimated to laboratory conditions. The isolated *M. aeruginosa* strain was named CAAT 2005–3. Isolation was carried out by capillary isolation. We isolated a single cell and was grown under sterile conditions in batch culture in BG11 medium[28] at 20 °C under continuous illumination with an average photon flux density of 30 $\mu E/m^2 s^1$.

2.3. Identification and characterization of isolated strain

Microcystis strain was taxonomically identified by means of Komarek and Anagnostidis[29]. Quantifications were done with an inverted microscope, following Utermöhl methodology[30].

For negative staining, one drop of culture sample was mixed with four drops of undiluted latex base drawing ink

(3084–F; Koh–i–noor), and was observed at microscope. The optic microscope was furnished with a drawing and photographic camera.

2.4. Extraction and quantification of Mcyst

In HPLC analyses, samples were extracted with 5% acetic acid while being emulsified by 30 min ultrasonication (Omni ruptor 400), then were centrifuged for 15 min at 5 000 r/min to eliminate cell debris.

The supernatant was passed through conditioned (10 ml 100% methanol, 50 mL 100% distilled water) Sep–Pak C18 cartridges (Waters). The toxins were eluted with 80% methanol.

Quantitative chromatographic analysis of MCs was performed by HPLC with a photodiode array detector (Shimadzu 2010) and C18 column (Hyperprep HS, 5–μm pore, 250 mm×10 mm). The column was equilibrated with a mixture composed by 65% of A solution [water with 0.05% (v/v) trifluoroacetic acid] and 35% of B solution [acetonitrile with 0.05% (v/v) trifluoroacetic acid]. The mobile phase consisted of a discontinuous gradient of A and B solutions. The flow rate was 1.0 mL/min. Mcyst were identified on the basis of their UV spectra and retention time. Standard of Mcyst–LR was purchased from Sigma (St Louis, MO, USA).

2.5. Analysis of Mcysts by LC/ESI–MS

An Exactive/Orbitrap mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Fisher Scientific, Bremen, Germany) were used for the LC/ESI–HRMS analysis.

The chromatographic separation was performed on a reversed–phase Phenomenex Luna C18 (2) column (150 mm× 2.1 mm, 5μm).

The mobile phase was composed of Milli Q water as solvent A and acetonitrile as solvent B, both containing 0.08% (v/v) formic acid at a flow rate of 200 μL/min. The linear gradient elution program for the intracellular analysis was: 10%–30% B 10 min, 30%–35% B 20 min, 35%–55% B 15 min, 55% B 5 min, 55%–90% B 2 min, 90% B 3 min and return to initial conditions for re–equilibrate (10% B 5 min). The injection volume was 10 μL.

The analyses were carried out in positive ion ESI with the spray voltage at 4.5 KV and the tube lens voltage at 120 V. The ion transfer tube temperature was set at 250 °C. Nitrogen (purity>99.98%) was used as sheath gas, ion sweep gas and auxiliary gas at flow rates of 30 psi, 0 and 5 a.u. (arbitrary

units), respectively. Full scan and high energy collision dissociation fragmentation experiments (high energy collision dissociation voltage 70 eV) were performed over a mass range from m/z 60 to 1 200 at high resolving power (50 000 FWHM, m/z 200).

2.6. Mathematical modelling of M. aeruginosa growth and [D–Leu^1] Mcyst–LR production

The number of cell/mL and [D–Leu^1] Mcyst–LR production obtained as a function of time was modelled using Gompertz equation (equation 1), a double exponential function based on 4 parameters which describe an asymmetric sigmoid curve.

$$\text{Log}(N) \ o \ [D–Leu^1]–Mcyst–LR \ a \ c \ \exp\{-\exp[-b \ (t–m)]\} \quad (\text{Eq. 1})$$

Where log N is the decimal logarithm of the cyanobacteria counts (log (cell/mL)), [D–Leu^1]–Mcyst–LR is the concentration of [D–Leu^1] Mcyst–LR (μg/L), t is time (d), a is the logarithm of the asymptotic counts when time decreases indefinitely (roughly equivalent to the logarithm of the initial levels of bacteria (log (cell/mL)) or the initial level of concentration of [D–Leu^1] Mcyst–LR (μg/L); c is the logarithm of the asymptotic counts when time is increased indefinitely (it is the number of log cycles of growth) [log (cell/mL)] or the increased the [D–Leu^1]–Mcyst–LR concentration (μg/L); m is the required time to reach the maximum growth rate (day), b is the growth rate relative to time (1/d).

The specific growth rate for *M. aeruginosa* growth μ=b. c/e with e=2.718 2, log (cell/mL/d) and [D–Leu^1] Mcyst–LR production rate (μg/mL/d), were calculated.

In addition, the duration of the lag phase LPD=m/b, (day) and maximum population density of *M. aeruginosa* MPD=a+c [log (cell/mL)] or maximum production concentration of [D–Leu^1 Mcyst–LR] MPC=a+c (μg/L) were derived from these parameters[31].

The equation was applied to cyanobacteria growth data and [D–Leu^1] Mcyst–LR production by nonlinear regression using the program Systat[32]. The selected algorithm calculates the set of parameters with the lowest residual sum of squares and a 95% confidence interval for cyanobacteria growth and [D–Leu^1] Mcyst–LR production.

2.7. DNA extraction, PCR amplification, cloning and phylogenetic determination

PCR amplification was carried out from 5 mL *M. aeruginosa* CAAT 2005–3 cultures. DNA from culture cells was extracted

and purified using DNeasy Plant Mini kit (Qiagen). PCR amplifications were carried out using approximately 1 000 cells per reaction. DNA quality was determined by agarose (0.8%) gel electrophoresis[33].

The amplification of *cpcBA*–IGS– Phcocyanin (PC, intergenic spacer and flanking regions) was carried out according to[34].

Products were cloned in the pGEM–T Easy vector. For RFLP analyses, the insert DNA fragments were PCR–amplified using the primer pair PCβF/PCαR (Table 1). Aliquots of those amplification mixtures were digested with HaeIII at 37 °C overnight.

Table 1
Gompertz equation parameters a, c, b and m and, kinetics parameters (μ, LPD and MPD) for *M. aeruginosa* CAAT 2005–3 in culture conditions.

	A	C	b	M	μor [D–LeuMC–LR] [day^{-1}]	Lag [day]
*Microcystis*aeruginosa	6.39±0.04	0.76±0.05	0.69±0.12	2.34±0.35	0.19±0.05	0.89±0.10
[D–Leu^{1}] Mcyst–LR	0.23±0.02	1.79±0.06	0.40±0.03	4.46±0.10	0.26±0.02	1.96±0.01

Inserts of different selected clones were sequenced (Macrogen, Korea) for phylogenetic analysis.

M. aeruginosa UTEX 2666, obtained from the culture collection of University of Texas, was used as toxic strain reference.

Comparisons of *cpcBA*–IGS sequences were performed using nucleotide sequences available in the databases at the National Center for Biotechnology Information. Sequence alignments were generated using the CLUSTAL W software and graphic representations of phylogenetic trees were performed using the MEGA4 software. The trees were statistically evaluated with non–parametric boot–strap–analysis (number of replicates=1 000).

Multiplex PCR was performed using primer sets for amplification of *mcyC*, *mcyD* and *mcyG* regions, corresponding to the genes coding for a thioesterase, a β–ketoacyl synthase and a C–methyltransferase, respectively (Table 1)[5]. Reactions were carried out in a volume of 25 μL containing: 2.5 μL of 10X PCR buffer (Biotools, Spain),

0.75 μL of 50 mmol/L MgCl$_2$, 0.5 μL of 10 mmol/L of each deoxynucleoside triphosphate (dNTPs), 2 μL of 10 mg/mL bovine serum albumin, 3 pmol of each primer (*mcyC*, *mcyD* and *mcyG*), 1 IU of DNA polymerase (Biotools, Spain), and 5 μL of template. Thermal cycling was carried out with an initial denaturation step at 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 seconds, annealing at 52 °C for 30 seconds and elongation at 72 °C for 1 min, and ending with an extension step at 72 °C for 5 min. PCR products were separated by electrophoresis on 1% agarose gel (Invitrogen Life Technologies, Brazil) in TAE 0.5X buffer and visualized after ethidium bromide staining (Promega Corp., USA).

3. Results

3.1. Strain isolation, culture growth and toxin production

Microcystis strain (named CAAT 2005–03) was isolated from the bloom using capillary isolation techniques and identity was confirmed by taxonomic descriptions of *M. aeruginosa* (Kützing) Kützing.

Colonies were irregularly, globose, usually with clathros and with colorless mucilage, unstratified and diffluent. Cells generally spherical, with aerotopes are present in Figure 1.

The Gompertz model was used as mathematical model of cyanobacteria growth curve.

At least eight points were obtained for each curve. A good agreement between experimental data and predicted values was obtained with a correlation coefficient of R^2=0.999 for *M. aeruginosa* growth and R^2=0.987 for [D–Leu^{1}] Mcyst–LR production (Figure 2A). *M. aeruginosa* CAAT2005–3 expresses one toxin variant, that was identified by LC–HRMS/Orbitrapas as [D–Leu^{1}] Microcystin–LR ([M+H]+=1 037.8 m/z).

The [D–Leu^{1}] Mcyst–LR production was proportional to number of cells. We found a direct linear correlation
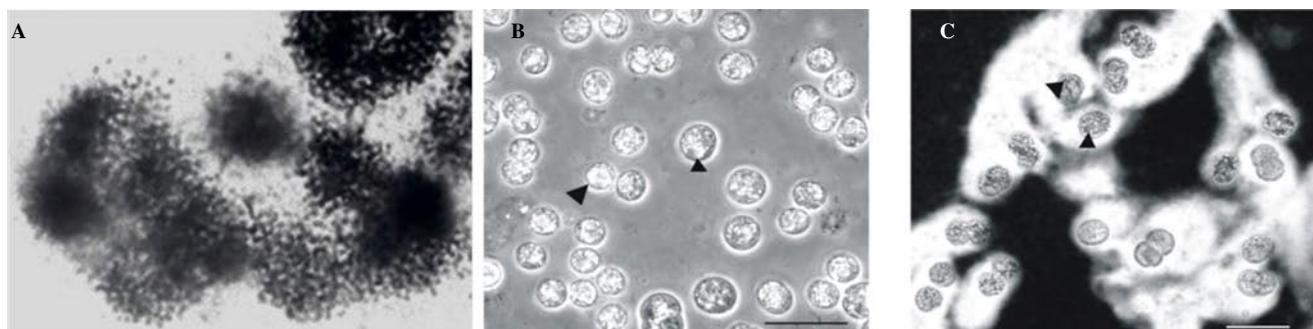

Figure 1. Appearance of *M. aeruginosa* cells and mucilage (A) Photomicrograph of CAAT 2003–5 colonies. (B) cells in culture showing aerotopes (arrows) and (C) the mucilage surrounding cells. Black background, drawing ink negative stain; bar, 10 μm.

(R=0.94) between cell concentration and [D–Leu^1] Mcyst–LR production by CAAT 2005–3 calculated according to a simple first–order equation (y=a+b) The intercept was 1.49±0.90 and the slope 8.63±0.80 (Figure 2B).
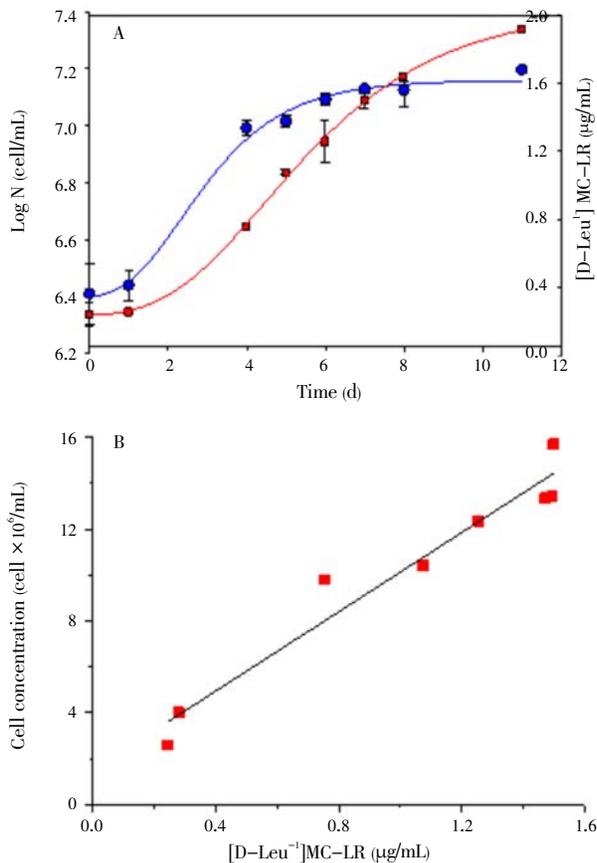

Figure 2. A: Modeling of (●) *M. aeruginosa* growth on culture media and (■) [D–Leu^1] Mcyst–LR production, solid lines correspond to Gompertz model fitted to the experimental data; B: Direct linear correlation (R=0.94) between cell concentration and [D–Leu^1] Mcyst–LR production by CAAT 2005–3 were observed.

Table 1 shows Gompertz parameters a, c, b and m. From these, derivatives parameters (μ, LPD and MPD) were obtained for *M. aeruginosa* growth and [D–Leu^1] Mcyst–LR production in culture conditions.

Specific growth rate for *M. aeruginosa* μ=0.19±0.05 log (cell/mL)/d lag phase duration (LPD=0.81±0.10 d) and the maximum population density (MPD=7.15±0.09 cell/mL) were the kinetic parameters obtained for *M. aeruginosa* growth.

For [D–Leu^1] Mcyst–LR, the production rate was (0.26± 0.02) μg/mL/d, the lag phase duration for the production was (1.96±0.01) d and the maximum production concentration was (2.02±0.08) μg/mL.

The relationship between cell number and the amount of toxin produced by CAAT 2005–3 shows a linear correlation (R=0.94) (Figure 2B) and holds a stable toxin production of (95.5±14.9) fg/cel (mean±SD).

3.2. Molecular identification

The *cpcBA*–IGS amplicons were obtained from total DNA of CAAT 2005–3 cells and cloned. RFLP analysis confirmed the presence of only one restriction pattern in *M. aeruginosa* CAAT 2005–3 cultures (Figure 3). Sequence analysis showed a 97% of identity with previously described sequences from *M. aeruginosa* strains. The phylogenetic analysis showed that the sequence clustered with others *M. aeruginosa* sequences obtained from NCBI (Figure 4).
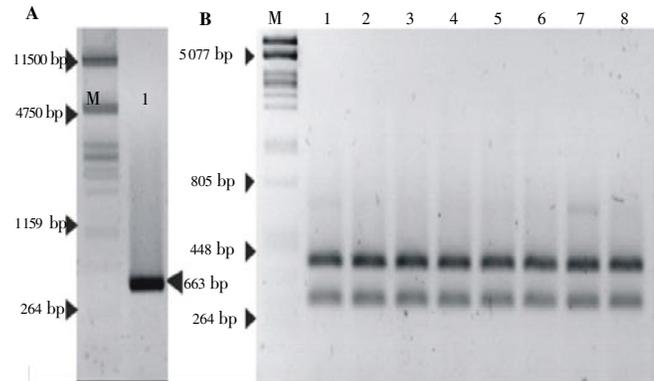

Figure 3. Molecular characterization of *M. aeruginosa* strain CAAT 2005–3. A: Products of *cpcBA*–IGS amplification were separated after electrophoresis on 1% agarose gels, and visualized after ethidium bromide staining; B: HaeIII restriction analysis of *cpcBA*–IGS amplification products, from different positive clones.
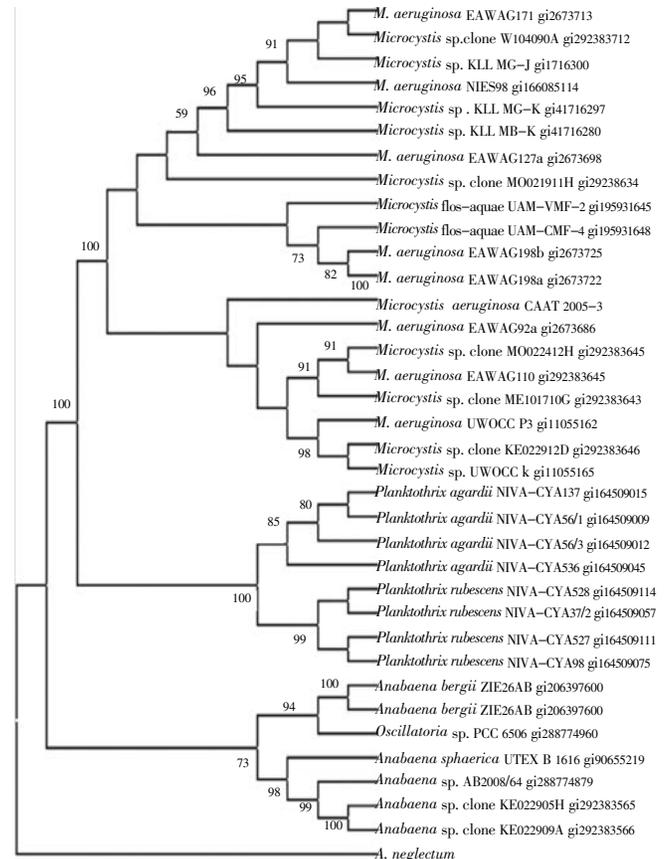

Figure 4. The neighbour–joining phylogenetic tree based on *cpcBA*–IGS sequences of *M. aeruginosa*.
The novel CAAT 2005–3 isolate is indicated in bold letters.

Table 2
Primer pairs used in this study.

Gene	Primer pair	Sequence (5′ to 3′)	Amplified region[a]	Product size (bp)	References
mcyC	PSCF3	GGT GTT TAA TAA GGA GCA AG	TE	648	[27]
	PSCR3	ATT GAT AAT CAG AGC GTT TT			
mcyD	PKDF2	AGT TAT TCT CCT CAA GCC	KS	859	[25]
	PKDR2	CAT TCG TTC CAC TAA ATC C			
mcyG	PKGF1	ACT CTC AAG TTA TCC TCC CTC	CM	425	[25]
	PKGR1	AAT CGC TAA AAC GCC ACC			
cpcBA–IGS	PCβF	GGC TGC TTG TTT ACG CGA CA	IR	685	[34]
	PCαR	CCA GTA CCA CCA GCA ACT AA			

[a], Domain abbreviations: TE, thioesterase; KS, β–ketoacyl synthase; CM, C–methyltransferase[5] and IR, intergenic region[34].

Since the molecular identification of *M. aeruginosa* CAAT 2005–3 by PCR amplification of *cpcBA*–IGS does not give information about toxigenic potential of the strain, we tested the presence of Mcyst biosynthesis genes (*mcyC*, *mcyD* and *mcyG*) using a PCR multiplex approach, primer pairs are described in (Table 2). As shown in Figure 5, the three genes were successfully amplified from *M. aeruginosa* CAAT 2005–3 sample and, the three amplification products had the expected sizes.
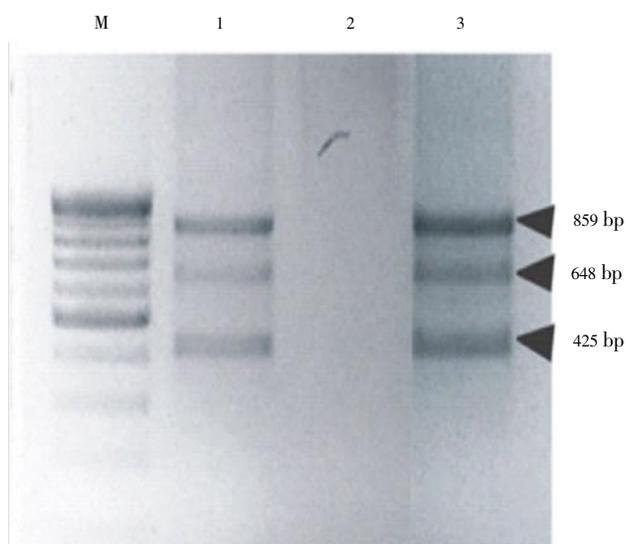

Figure 5. Amplification of mcy sequences by multiplex PCR.
Lane 1, *M. aeruginosa* UTEX 2666 used as positive control; lane 2, negative control; lane 3, *M. aeruginosa* CAAT 2005–3. M: DNA marker (100 bp ladder). Arrowheads indicate the position of the amplicons from *mcyG* (425 bp), *mcyC* (648 bp) and *mcyD* (859 bp).

3.3. RP–HPLC and LC/ESI/MS

HPLC–DAD analysis of cell extract from *M. aeruginosa* CAAT 2005–3 revealed the presence of only one variant of Mcyst, with a retention time consistent with that of [D–Leu¹] Mcyst–LR purified from natural blooms collected from Río de la
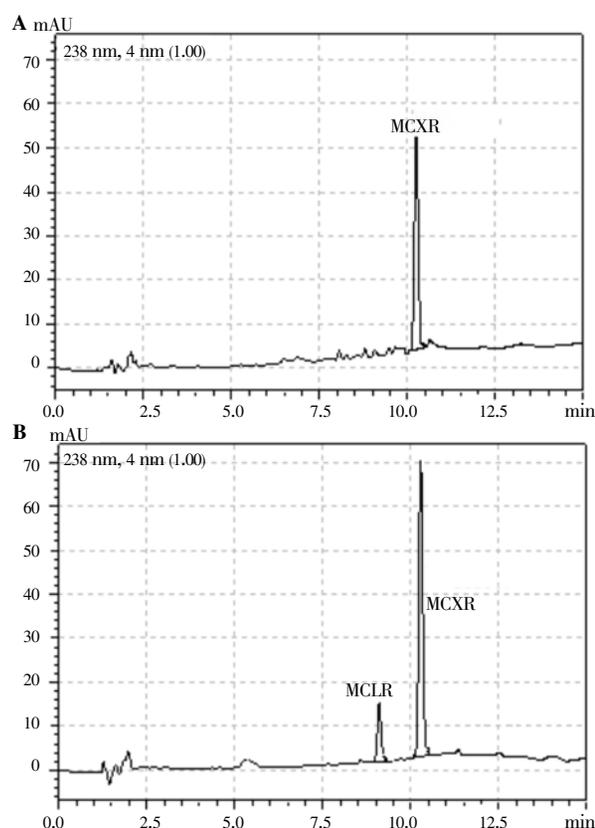
Plata river (Figure 6).

Figure 6. Analytical HPLC chromatograms with DAD detection of A: *M. aeruginosa* CAAT 2005–3 culture with a peak that have de same retention time than [D–Leu¹] Mcyst–LR; B: test mixture for Mcyst–LR (R_t=9.15 min) and [D–Leu¹] Mcyst–LR (R_t=10.24).

HPLC–DAD analysis was confirmed on the basis of ions formed in mass spectrometry. LC/ESI–HRMS data shown a molecular weight of 1036 and detect the Adda unit and others characteristic fragments. The ion peak at m/z 135, resulting from _–cleavage of the methoxy group of the Adda chain side, denotes an unmodified Adda side chain [Ph–CH2CH(OMe)]

The m/z 213 and 169 ions could be assigned to [Glu–Mdha+H] and [Mdha–Leu–CO+H] respectively.

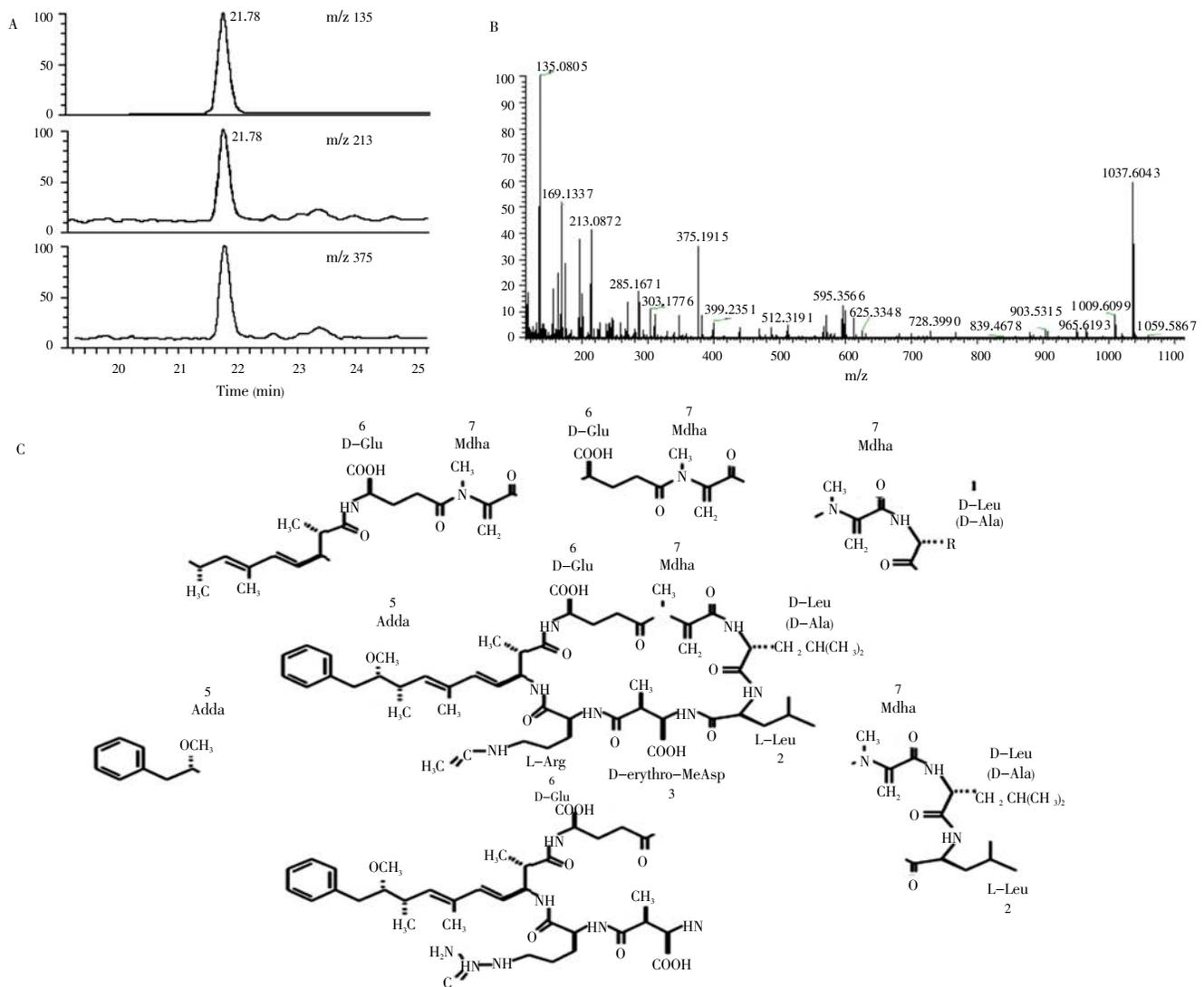The 375, 595 and 728 product ions would correspond to

Figura 7. LC/ESI−HRMS analysis performed to confirm the identity of microcystin produced by CAAT 2005−3.

A: Product ion scan data used to identify several putative metabolites for Mcyst−LRs, 135 m/z, 216 m/z and 375 m/z; B: Mass chromatogram of the [D−Leu¹] Mcyst−LR present in cultured samples of CAAT 2005−3 which showed the molecular ion (1037 m/z) and several metabolites; C; Fragments ions of which the [D−Leu¹] Mcyst−LR molecule is composed. Masp: d−erythro−b−methylaspartic acid. Adda: (2S,3S,8S,9S)−3−amino−9−methoxy−2,6,8−trimethyl−10−phenyldeca−4,6−dienoic acid. Mdha: N−methyldehydroalanine.

[CH (Me)−CH=C(Me)−CH=−CH−CH2−CH(Me)−CO−Glu−Mdha], [Mdha−Leu−Leu−MeAsp−Arg+H] and [MeAsp−Arg−Adda−Glu] as it can been see in Figure 7.

4. Discussion

There are insufficient and fragmented information of cyanobacteria and cyanotoxins presence in South America[35,36].

In this study, we identified and characterized, at phylogenetic, taxonomic and toxicological level a native *M. aeruginosa* strain (named CAAT 2005−3) isolated from a eutrophicated freshwater body located at Pila city, province of Buenos Aires, Argentina, which was cultured in BG11 medium. Morphological analysis had shown that cells have

the typical structure of *M. aeruginosa*colony. Additional, phylogenetic analysis of *cpcBA*−IGS confirms its identity by grouping with sequences from *M. aeruginosa* strains and was named as CAAT 2005−3. Restriction analysis with HaeIII of *cpcBA*−IGS amplification products from DNA of different clones also points to a unialgal culture.

CAAT 2005−3 has shown a low growing specific μ (0.19) in comparison with μ reported by[37], in a study of the relationship between Mcyst production and cell division rates in nitrogen−limited *M. aeruginosa* cultures, which case growing specific μ ranged between 0.68 and 1.3 l/day. In agreement with these authors we found a direct linear correlation between μ and [D−Leu¹] Mcyst−LR production.

In culture condition described, CAAT 2005−3 strain synthesized one variant of Mcyst that has been identified as [D−Leu¹] Mcyst−LR based on molecular ion (C52H80N10O12,

M+H]+:1 037.603 5) and fragmentation spectra by LC–Orbitrap–MS.

The toxin has characteristic fragmentation patterns described by[38]. The Mcyst characteristic fragment ion peak at m/z 135 derived from the Adda residue was observed and the seventh amino acid unit was deduced to be Mdha from the fragment ion peaks at m/z 213 and 375[39,40].

The fragment ion peaks of this pattern at m/z 595 showed the sequence of Mdha–Leu–Leu–MeAsp–Arg[38]. The amino acid components of [D–Leu[1]] Mcyst–LR resembled those of Mcyst–LR except for the presence of two Leu instead of Ala and Leu at the position 1 and 2 of the molecule, respectively. The difference in the amino acid unit between [D–Leu[1]] Mcyst–LR and Mcyst–LR revealed the difference in the molecular weight (42 mass units) and formula (C36) between [D–Leu[1]] Mcyst–LR and Mcyst–LR (MW. 994, C49H74N10O12). These data suggested that [D–Leu[1]] Mcyst–LR is the Leu variant of Mcyst–LR linked to [D–Ala1] unit.

[D–Leu[1]] Mcyst–LR was first reported by Park *et al.*[38] from a water bloom collected in Pakowki Lake, Alberta, Canada and associate with bird mortalities that have occurred there since 1995.

Then, chromatograms obtained from samples collected in the Río de la Plata estuary containing Mcyst–LR, and other unidentified Mcyst with the [M+H]+ m/z 1037.8 ion that was proposed to be [D–Leu[1]] Mcyst–LR[18]. Later[27], [D–Leu[1]] Mcyst–LR was also identified as a unique naturally produced toxin in another *M. aeruginosa* bloom in the Rio de la Plata estuary (2003).

Also, [D–Leu[1]] Mcyst–LR was identified as the most abundant Mcyst produced by a laboratory strain of *Microcystis sp.* isolated from brackish waters in the Patos Lagoon estuary, southern of Brazil and the toxicity of [D–Leu[1]] Mcyst–LR and Mcyst–LR, were described similar according to bioassay and protein phosphatase inhibition assay[36].

Other Brazilian strain *M. aeruginosa* NPLJ–4 isolated from a pond during the rainy season in Paulista (Paraiba, Brazil) was also cited as a producer of [D–Leu[1]] Mcyst–LR[41].

Besides *Microcystis sp.*, other genera of cyanobacteria, Phormidium strain CENA 270, isolated from a pond (Paraiba, Brazil) and Nostoc UK89II, isolated from the lichen Peltigera neopolydactyla sampled in Laukaa (Finland), are [D–Leu[1]] MCYST–LR producers. Nevertheless, Shishido *et al.* showed that these strains have identical binding pocket sequences and conclude that different evolutionary events are involved in the convergence of the [D–Leu[1]] Mcyst–LR[15].

Microcystis cells producer of [D–Leu[1]] Mcyst–LR are widely distributed along the American continent from Canada to Argentina in ponds, lakes, river and brackish water. This is the most austral report for M aeruginosa [D–Leu[1]] Mcyst–LR producer. Considering the above, it could be interesting study the relationship between microcystin's chemical diversity and the ability of these cyanobacteria to colonize the most diverse aquatic environments. Furthermore, the capability to maintain under cultured condition a *M. aeruginosa* strain [D–Leu[1]] Mcyst–LR producer provides the basis for technological development and production of analytical standards of this toxin present in our region.

Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

These data are essential for a complete environmental work up of potential similar outbreaks of this harmful cyanobacterium. This study also lays a very strong multi–disciplinary basis not only for detection, but mitigation, and potential remedial applications in medicine of the outbreaks and their consequences based on the elucidation of the chemical composition of the toxin.

Research frontiers

Harmful algal blooms including cyanobacteria are one of the most significant economic and health–related aquatic phenomena, especially in coastal environments. The complete characterization of the taxonomy, molecular phylogenetics, and molecular characterization of their toxins is essential to better understand the physiological toxic effects and to better predict which taxa are likely to be related and similarly toxic. Accurate identification, elimination, and remediation of their blooms is essential for control and medical treatment in cases of poisoning.

Related reports

The authors provide a very good background on previous published research. In addition to taxonomic prior work on this species, *M. aeruginosa*, the authors cite prior evidence of occurrence in Rio de la Plata (De Leon *et al.*

2001; Andrinolo *et al.* 2007), published work on natural product chemical analytic work such as that of Watanbe *et al.* 1992 on toxic cyclic heptapeptides, of the same class of compounds as in this taxon, and relevant molecular genetic prior work (*e.g.* Neilan, 1995).

Innovations and breakthroughs

The methodologies used here are all well–documented and apparently carefully applied. The innovative quality of the work is not only in the careful documentation of the occurrence of this cyanobacterial species, but more particularly in the combination of modern analytic techniques that have been used, spanning modern light microscopic, chemical analytic (*e.g.*, mass spec.), and molecular phylogenetic approaches. This comprehensive treatment, based on clonal cultures derived from single cell isolates, ensures the taxonomic and physiological validity of the suite of studies they have completed, including a nicely described model of growth kinetics and toxin production.

Applications

These data are essential for a complete environmental work up of potential similar outbreaks of this harmful cyanobacterium. This study also lays a very strong multi–disciplinary basis not only for detection, but mitigation, and potential remedial applications in medicine of the outbreaks and their consequences based on the elucidation of the chemical composition of the toxin.

Peer review

Overall, this is a nicely organized manuscript and in most aspects is clearly presented. It is a very nicely composed and thorough analysis of the outbreak of *M. aeruginosa* in Argentina waters, including a complete taxonomic and physiological analysis.

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