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Highly Toxic *Microcystis aeruginosa* Strain, Isolated from São Paulo—Brazil, Produce Hepatotoxins and Paralytic Shellfish Poison Neurotoxins

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Abstract While evaluating several laboratory-cultured cyanobacteria strains for the presence of paralytic shellfish poison neurotoxins, the hydrophilic extract of *Microcystis aeruginosa* strain SPC777—isolated from Billings's reservoir, São Paulo, Brazil—was found to exhibit lethal neurotoxic effect in mouse bioassay. The in vivo test showed symptoms that unambiguously were those produced by PSP. In order to identify the presence of neurotoxins, cells were lyophilized, and the extracts were analyzed by HPLC–FLD and HPLC–MS. HPLC–FLD analysis revealed four main Gonyautoxins: GTX4(47.6%), GTX2(29.5%), GTX1(21.9%), and GTX3(1.0%). HPLC–MS analysis, on other hand, confirmed both epimers, with positive Zwitterions M^+ 395.9 *m/z* for GTX3/GTX2 and M^+ 411 *m/z* for GTX4/GTX1 epimers.

The hepatotoxins (Microcystins) were also evaluated by ELISA and HPLC–MS analyses. Positive immunoreaction was observed by ELISA assay. Alongside, the HPLC–MS analyses revealed the presence of [L-ser^7] MCYST-RR. The *N*-methyltransferase (NMT) domain of the microcystin synthetase gene *mcyA* was chosen as the target sequence to detect the presence of the *mcy* gene cluster. PCR amplification of the NMT domain, using the genomic DNA of the SPC777 strain and the MSF/MSR primer set, resulted in the expected 1,369 bp product. The phylogenetic analyses grouped the NMT sequence with the NMT sequences of other known *Microcystis* with high bootstrap support. The taxonomical position of *M. aeruginosa* SPC777 was confirmed by a detailed morphological description and a phylogenetic analysis of 16S rRNA gene sequence. Therefore, co-production of PSP neurotoxins and microcystins by an isolated *M. aeruginosa* strain is hereby reported for the first time.

Keywords Cyanobacteria · Paralytic shellfish poison neurotoxins · Gonyautoxins · Microcystins · *McyA*

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Introduction

Cyanobacteria genus *Microcystis* Kützing comprises 25 planktonic species that are frequently found in freshwater ecosystems all over the world. The ability to form colonies of irregularly arranged coccoid cells within a fine mucilaginous envelope, cell division in three perpendicular planes, and the presence of aerotopes (Komárek 2003) are the main morphological features of this genus. Perhaps the most common species is *Microcystis aeruginosa* Kützing, which is widely known for both, its capacity to form water blooms and the production of hepatotoxins named microcystins. Microcystins are cyclic heptapeptides and are known to be toxic to aquatic biota, livestock, and humans (Carmichael 1996; Jochimsen et al. 1988; Falconer et al. 1999). Microcystins were first described in 1959, in a culture of *M. aeruginosa* (Bishop et al. 1959), and their

structure was elucidated in 1984 (Botes et al. 1984). At present, more than 70 structural variants of these peptides are known (Sivonen and Jones 1999; Codd et al. 2005). Various *Microcystis* strains also produce other linear and cyclic peptides such as micropeptins (Okino et al. 1993), aeruginosins (Murakami et al. 1995), anabaenopeptins (Namikoshi and Rinehart 1996), cyanopeptolins (Martin et al. 1993), and microviridin (Ishitsuka et al. 1990). These peptides may not be acutely toxic, but they have other bioactivities (Namikoshi and Rinehart 1996).

Production of several microcystin variants by individual *Microcystis* strains is well documented in the literature (Harada et al. 1991; Lawton et al. 1999a, b; Lee and Chou 2000; Luukkainen et al. 1994; Metcalf and Codd 2000; Oliveira et al. 2005). The number of variants of a single peptide class that can be found in an individual strain is higher than 10, although a few variants are often dominant (Welker et al. 2004). Co-production of microcystins and other oligopeptides in several *Microcystis* strains has also been reported (Birk et al. 1989; Ishida et al. 1995; Martin et al. 1990; Murakami et al. 1997; for an overview, see Welker and von Döhren 2006). The ability of a strain to produce typical peptides can be maintained for decades under laboratory conditions, as observed in *Microcystis aeruginosa* PCC7806, a culture that was deposited in the Pasteur Culture Collection in 1978, and still produce the same microcystin and cyanopeptolin peptides (Welker and von Döhren 2006). In this strain, the non-ribosomal synthesis of microcystins was demonstrated by site-directed mutagenesis and sequencing (Dittmann et al. 1997; Tillett et al. 2000). The *mcy* gene cluster contains 10 genes (*mcyA* to *J*) that encode non-ribosomal peptide synthetases (NRPSs), type I polyketide synthetases (PKSs), and tailoring enzymes. Since then, *mcy* gene clusters were characterized from *Planktothrix agardhii* CYA126/8 (Christiansen et al. 2003) and *Anabaena* sp. 90 (Rouhiainen et al. 2004). Type I PKSs, NRPSs, and their hybrids are involved in the most common bioactive metabolites isolated from cyanobacteria (Welker and von Döhren 2006).

Despite the co-occurrence of several microcystins and other oligopeptides in individual strains, no PSP neurotoxins had yet been reported in *Microcystis* strains. PSP neurotoxins are carbamate alkaloids that inhibit nerve conduction by blocking neuronal voltage dependent sodium channels (Narahashi 1972; Henderson et al. 1973; Strichartz et al. 1995). More than 20 structural analogs of PSP toxins have been described in dinoflagellates, mollusks, and cyanobacteria sample extracts (Bricelj and Shumway 1998; Oshima 1995; Lagos 1998); the most commonly known are classified in three mayor groups: saxitoxins (STXs), gonyautoxins (GTXs), and *N*-sulfocarbamoyl-11-hydroxysulfate (C-toxins) (Lagos 1998, 2003; Llewellyn 2006). Gonyautoxins are the most frequently found in all the PSP toxin profile samples (Bricelj and Shumway 1998; Lagos 1998, 2003). Until now, PSP toxins had been found only in filamentous cyanobacteria (*Anabaena*, *Aphanizomenon*, *Cylindrospermopsis raciborskii*, *Lyngbya*, and *Planktothrix/Limnothrix*) (Alam et al. 1973; Carmichael et al. 1997; Humpage et al. 1994; Lagos et al. 1999; Pereira et al. 2000; Pomati et al. 2000) and in several marine dinoflagellates (Shimizu 1977; Harada et al. 1982; Oshima et al. 1987; Lagos 1998). In this article, co-production of PSP neurotoxins and microcystins by an isolated *M. aeruginosa* strain is reported for the first time. Evidences for morphological and molecular characterization of the cyanobacterium are shown.

Materials and Methods

Cyanobacteria Strain Isolation and Morphological Evaluation

A water bloom sample was collected on February 05, 2000, from the Riacho Grande arm of Billings Reservoir, located in the metropolitan region of São Paulo (23° 47 S 46° 40 W) in southeastern Brazil. This reservoir is a eutrophic water body and has been used for water supply, recreation, fishing, irrigation, and flood control (Carvalho 2003).

The monoculture of *Microcystis* clone was obtained and isolated since February 05, 2000, which was collected from the Riacho Grande arm of Billings Reservoir, Brazil. In order to obtain the monoculture of *Microcystis* used in this study, 1 ml of the bloom sample was inoculated in 125 ml Erlenmeyer containing 50 ml of ASM-1 liquid medium (Gorham et al. 1964). Then, it was allowed to grow up and again this sequence was repeated 12 times. The purity of the strain was checked every time during each of these sequences and several times during the continuous culture, always by microscopic observations. The monoclonal strain was kept under 14/10-h dark/light cycle with white fluorescent light (40–50 μmol photon μm^{-2} s^{-1}) at 23 ± 1°C. Since then, this isolated strain had been maintained in the culture collections of the Institute of Botany Algal Culture Collection (SPC) in São Paulo, Brazil (from where we obtained and tested), and in the culture collection of CENA/USP in Piracicaba, SP, Brazil, under the number SPC777. The strain was identified following the classification system devised by Komárek and Anagnostidis (1999). The morphological studies and photo documentation were carried out using a Zeiss Axioplan-2 optical microscope (Carl Zeiss, Jena, Germany).

Analyses of Toxins

Cyanobacteria biomass was obtained from 0.2 l of culture grown during 2 weeks in four Erlenmeyers of 1 l of

ASM-1 medium. The whole volume was concentrated by centrifugation at 5,000 × g for 5 min. The pellet was resuspended in 5 ml of distilled water and lyophilized. Lyophilized cells (0.2 mg) were extracted twice with 4 ml of 0.5 M acetic acid using sonication and centrifugation at 5,000 × g for 5 min. The supernatant was collected, and the residues were re-extracted with 8 ml of chloroform/methanol (1:1, v/v). Both extracts were combined and centrifuged (5,000 × g, 5 min), and then the aqueous layer was removed. The organic layer was washed three times with 4 ml of 0.5 M acetic acid. All aqueous layers were combined and backwashed with 1/3 volume of chloroform/methanol (1:1, v/v) and then concentrated in a SpeedVac (SC210A, Savant, Farmingdale, NY, USA). The residue was dissolved in 1 ml of 50 mM acetic acid and kept frozen until use.

The mouse bioassay was performed as described by Turner et al. 2009.

HPLC-Fluorescent Detection On-Line

PSP toxins were detected and quantified using HPLC–FLD analyses according to a procedure described previously (Lagos 1998; Lagos et al. 1999; Pereira et al. 2000; Lagos 2003). In brief, 20 µl of the extract sample were injected by the Rheodyne injector model 7725i, with a 20-µl loop, into a silica-base reversed phase column (Symmetry 5 µm, C_8, 4.6 × 150 mm, Waters Corp., Milford, MA, USA). A mobile phase of 2 mM 1-heptenesulfonic acid in 30 mM ammonium phosphate buffer, pH 7.1: acetonitrile (100:5) was used at a flow rate of 0.7 ml/min for detection of saxitoxin group. For gonyautoxin group, 2 mM 1-heptenesulfonic acid in 10 mM ammonium phosphate buffer, pH 7.1, was used. Finally, in order to detect the C-toxin group, the mobile phase consisted of 1 mM tetrabutylammonium phosphate (pH 5.8). In all the three cases, the eluate from the column was mixed continuously at 0.4 ml/min with 7 mM periodic acid in 10 mM potassium phosphate buffer, pH 9.0, and heated at 65°C by letting it pass through a Teflon tubing coil (0.5 mm i.d., 10 m long), and then mixed with 500 mM acetic acid at a flow rate of 0.3 ml/min before entering the fluorescence detector. The fluorescence detector was set at an excitation wavelength of 330 nm and an emission wavelength of 390 nm. HPLC chromatographic equipment was a Shimadzu LC-10AD liquid chromatograph apparatus with an on-line JASCO FP-2020 PLUS Fluorescent detector. The oxidizing reagent and the acid were pumped by a dual head pump (model SP-D-2502, Nihon Seimitsu Kagaku). Data acquisition and data processing were performed with ChromPass software. Toxin concentrations were measured by comparing peak area of each toxin with those of the analytic standard. Pure toxin solutions, calibrated by combustion analysis nitrogen measurements and HPLC–MS, were used as an external standard (Lagos 1998, 2003).

In order to avoid false identification of PSP toxins, further analyses were carried out. The sample was re-analyzed after a pre-purification using Sep-Pak C_{18} cartridge column (Waters Corp.) and a 5-kDa cut-off ultrafiltration filter (Ultrafree-MC C3GC, Millipore, Bedford, MA, USA). Next, the pre-purified sample was re-analyzed with the same HPLC–FLD equipment and procedure, but replacing the oxidizing reagent by distilled water. Under these conditions, oxidation of toxins does not occur (Lagos et al. 1999; Pereira et al. 2000).

HPLC–MS Analysis for PSP toxins

Samples obtained from the cyanobacterium extract were analyzed by HPLC–MS. The analysis was performed using a C-8 column (Waters 5 µm, 4.6 × 150 mm) at room temperature and isocratic conditions, with mobile phase of aqueous 90% acetonitrile at a flow rate of 1 ml/min. The system was equipped with an electrospray mass spectrometer with ionic trap Esquire 4000 ESI-IT (Bruker Daltonics, Inc., MA-USA), and high purity 99% nitrogen was used as the nebulizing gas (ca. 0.2 l/min). A potential of 2.5 kV was applied at the corona, and voltage cones of 5 and 50 V were used. Source temperature was set at 350°C, and probe temperature was set at 150°C. Positive ion mode was used with a full scan from 0 to 1,000 *m/z*. Mass calibration of the instruments was carefully checked (Lagos et al. 1999.

Microcystins Analysis

Microcystin contents was first analyzed by ELISA assay using a commercially available diagnostic kit (Beacon Analytical Systems Inc., Portland, ME, USA), following the manufacturer's recommendations. Toxins were extracted as follows: 2 ml of water (Milli-Q, Millipore) were added to 0.02 g of lyophilized cells; this solution was boiled in a microwave oven for 1 min. The extract was then centrifuged (10,000 × g for 15 min.), and the collected supernatant was used for the ELISA assay.

HPLC–MS Analysis for Microcystins

The *M. aeruginosa* strain SPCC777 was also analyzed by liquid chromatography with mass spectrometry (HPLC–MS) for Microcystins. Lyophilized cells (40 mg) were extracted adding 2 ml of water and boiling in a microwave oven for 1 min. The supernatant was collected by centrifugation (10,000 × g for 15 min) and lyophilized. The residue was dissolved in 1 ml of methanol, and then the solution was mixed for 30 s and maintained at 10°C for

24 h. The supernatant was filtered (Chromafil RC 45/25—Regenerated Cellulose 45 mm). The pellet was re-extracted in 1 ml of methanol and stirred for 30 s. The same procedure was repeated. HPLC analysis was performed on a system consisting of a liquid chromatograph (model 2695, Waters Corp.) with a photodiode array (model 2996, Waters Corp.) coupled to a Micromass ZQ4000 quadrupolar mass spectrometer (Waters Corp.). The preparative column (Atlantis, dC_{18}, 3 µm, 2.1 × 150 mm) was gradient eluted with at flow rate of 0.29 ml/min. The solvent gradient system consisted of isocratic mode for 2 min with an initial solution of 88% ultrapure water (Milli-Q):2% acetonitrile:10% trifluoroacetic acid (TFA, 0.2%); gradient mode for 30 min until the solution reached 20% ultrapure water (Milli-Q):70% acetonitrile:10% TFA (0.2%); and gradient mode for 6 s until the solution reached 10% ultrapure water (Milli-Q):80% acetonitrile:10% TFA (0.2%). The column remained in this last solution for 2 min and then returned to the initial solution in 6 s, as a gradient; it then remained in this solution for 3 min for a total of 35 min of analysis. The LC/MS-ZQ quadrupole mass spectrometer (mod. Micromass ZQ4000, Waters Corp.) was equipped with an electrospray ionization (ESI) source operating in positive ion mode. The ESI probe temperature was set to 150°C, the capillary voltage was set to 3.5 kV, and the source block voltage was set to 40 V. The flow rate of nebulizer gas was 4.6 l/min. The MCYST-LR was monitored at its base peak of $m/z = 995.60$ $[M+H]^+$. Chromatographic data acquisition and analyses were performed using Empower software.

DNA Extraction

Total genomic DNA from *M. aeruginosa* SPC777 was isolated from cultured cyanobacteria cells using a previously described procedure (Fiore et al. 2000).

PCR Amplification and Sequencing of NMT Domain of *mcyA*

The *N*-methyltransferase domain (NMT) of the *mcyA* gene was amplified using the MSF and MSR primers (Tillett et al. 2001). PCR mixture contained 10 ng of genomic DNA, 0.2 µM of each primer, 200 µM dNTPs, 3.0 mM $MgCl_2$, 1× PCR buffer, 1.5 U of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), and water to a final volume of 25 µl. Thermal cycling was performed at 94°C for 4 min followed by 30 cycles of 94°C for 20 s, 55°C for 30 s, 72°C for 1 min, followed by one cycle of 72°C for 7 min. Gel was viewed on a Fluor-S MultiImager (BioRad, Hercules, CA, USA) and recorded. Fresh PCR product was cloned using the pGEM®-T Easy Vector Systems kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. *Escherichia coli* DH5α competent cells were used for the transformation process. Clones were grown for duration of 14–16 h, and recombinant plasmids were isolated from white colonies using an alkali extraction procedure (Birnboim and Doly 1979). DNA sequencing was performed using the cloned PCR product and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Piscataway, NJ, USA). Primers used for the cycle sequencing were the T7 promoter and M13 of the vector, and internal primer MSI (Tillett et al. 2001). Cycle sequencing reaction was performed using the GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA, USA) and reaction conditions were 25 cycles of the following sequence: 20 s at 95°C, l5 s at 50°C, and 1 min at 60°C. After sequencing reaction was completed, residual dye terminators were removed by washing with 75% isopropanol followed by a 70% ethanol rinse. The purified reaction was then resuspended in HiDi formamide (Applied Biosystems), and samples were read in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Cloned PCR product was bidirectionally sequenced and each set of sequencing data was obtained from at least two independent experiments. Sequenced fragments were assembled into one contig using the Phred/Phrap/Consed software program (Philip Green, Univ. of Washington, Seattle, USA) and only bases with a value >20 were considered.

PCR Amplification and Sequencing of 16S rRNA

The 16S rRNA gene from the genomic DNA of the SPC777 strain was amplified by PCR using the 27F1 and 1494Rc oligonucleotide primers (Neilan et al. 1997). Amplification was performed in a GeneAmp PCR System 2400 (Applied Biosystems) using 25-µL reactions containing 10 ng of genomic DNA, 0.2 µM of each oligonucleotide primer, 200 µM dNTPs, 3.0 mM $MgCl_2$, 1× PCR buffer, and 1.5 U Platinum *Taq* DNA polymerase (Invitrogen). Reactions were cycled with an initial denaturation step at 94°C for 4 min, followed by 30 cyc1es of DNA denaturation at 94°C for 20 s, primer annealing at 50°C for 30 s, strand extension at 72°C for 2 min, and a final extension step at 72°C for 7 min. PCR product was analyzed on 1% agarose gels stained with ethidium bromide.

Cloning and transformation procedure were performed as described above. Primers used for the cycle sequencing of 16S rRNA were the T7 promoter and M13 of the vector and the following internal primers 341–357F (5′-CCTACGGG AGGCAGCAG-3′), 357–341R (5′-CTGCTGCCTCCCGT AGG-3′), 685–704F (5′-GTAGSGGTGAAATSCGTAG A-3′), 704–685R (5′-TCTACGSATTTCACCSCTAC-3′), 1099–1114F (5′-GCAACGAGCGCAACCC-3′), and 1114–1099R (5′-GGGTTGCGCTCGTTGC-3′) (Lane 1991).

Phylogenetic Analyses

The nucleotide sequences of *mcyA* and 16S rRNA obtained in this study and related sequences retrieved from GeneBank were aligned, refined, and used to generate a phylogenetic tree. Trees were constructed by neighbor-joining (NJ) and maximum-parsimony (MP) algorithms using the MEGA version 3.1 program package (Kumar et al. 2004). The NJ and MP stability of relationships was assessed by bootstrapping (1,000 replicates). GenBank accession numbers for the *mcyA* and 16S rRNA nucleotide sequences obtained from the cyanobacterium *M. aeruginosa* SPC777 are EU239931 and EF121241, respectively.

Results

Morphological Characterization

The unicellular *Microcystis* strain SPC777 isolated in February 2000 in the Billings Reservoir, São Paulo, the largest city in Brazil, presented typical characteristics of this genus: e.g., coccoid-shaped cells held together by fine hyaline mucilage and the presence of aerotopes (Fig. 1). The strain was identified as *Microcystis aeruginosa* (Kützing) Kützing through morphological observation under light microscope following Komárek's and Anagnostidis' (1999) classification system. According to the classification system used, the cell diameter of this species varies between 4.0 and 6.0 µm. The cell diameter of SPC777 strain is 3.6 µm in average. The colonial form disaggregated after 1 year of subculture showing floating detached cells.

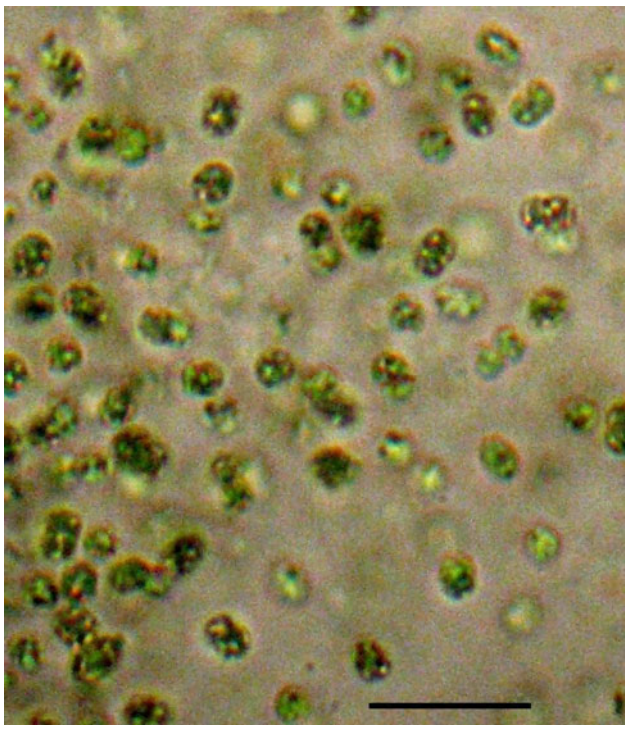

Fig. 1 Photomicrograph of a *Microcystis aeruginosa* SPC777 culture under the microscope. *Scale bar* 10 µm

PSP Neurotoxins Analysis

HPLC-Fluorescent Detection on Line

HPLC–FLD chromatograms of gonyautoxin standards and the *M. aeruginosa* SCP777 extract are shown in Fig. 2. Six major peaks were found in the sample extract (Fig. 2b). Four of them showed the same retention time as the four GTX standards (Fig. 2a). They were the GTX4/GTX1 and GTX3/GTX2 epimers. As for the other two peaks, the first one close to the void volume corresponds to pigment of the sample, and the second, marked with an asterisk (*), did not match any known analytical standard used as internal marker.

GTX4 was the major toxin component, representing 47.6% of the total PSP neurotoxins, followed by GTX2 (29.5%), GTX1 (21.9%), and GTX3 (1.0%). GTX 4/1 epimers account for 69.5% of total sample (Fig. 3 and Table 1). No further peaks were observed when chromatographic HPLC runs were performed to detect PSP neurotoxins within saxitoxin and C-toxin groups, showing that *M. aeruginosa* SPC777 strain contains only GTX 3/2 and GTX 4/1 epimers, both PSP neurotoxins belonging to the gonyautoxins group.

Presence of gonyautoxin epimers was confirmed by spiking the sample with the analytic standards. The co-injection experiments with sample extracts and analytic standards are regular control experiment in the HPLC–FLD procedures in our Laboratory. The sample extracts are co-injected in similar amounts to the analytic standards (keeping the same 10 microliters volume of each). So, a double amount in the Fluorescent signal is obtained.

Moreover, they were also analyzed replacing the oxidizing reagent by distilled water (Lagos 1998; Pereira et al. 2000). Under this mild oxidative condition, fluorescence intensities of sample peaks changed as the respective toxin standards did, i.e., GTX3/2 epimers disappeared and GTX4/1 epimers increased their fluorescence signal (data not shown). Fluorescence intensity of the peak marked with an asterisk (*) also changed, and essentially disappeared, as that of GTX3/2 epimers did, suggesting that this compound is also a tetrahydropurine belonging to the type of PSP neurotoxins not yet described. Albeit, GTX4/1 epimers accounted for 69.5% of total PSP neurotoxins of the *M. aeruginosa* SCP777 strain, these epimers accounted for 83.4% of total toxicity of sample extract, which was expressed as Mouse Units (MU) per cells total (Table 1).

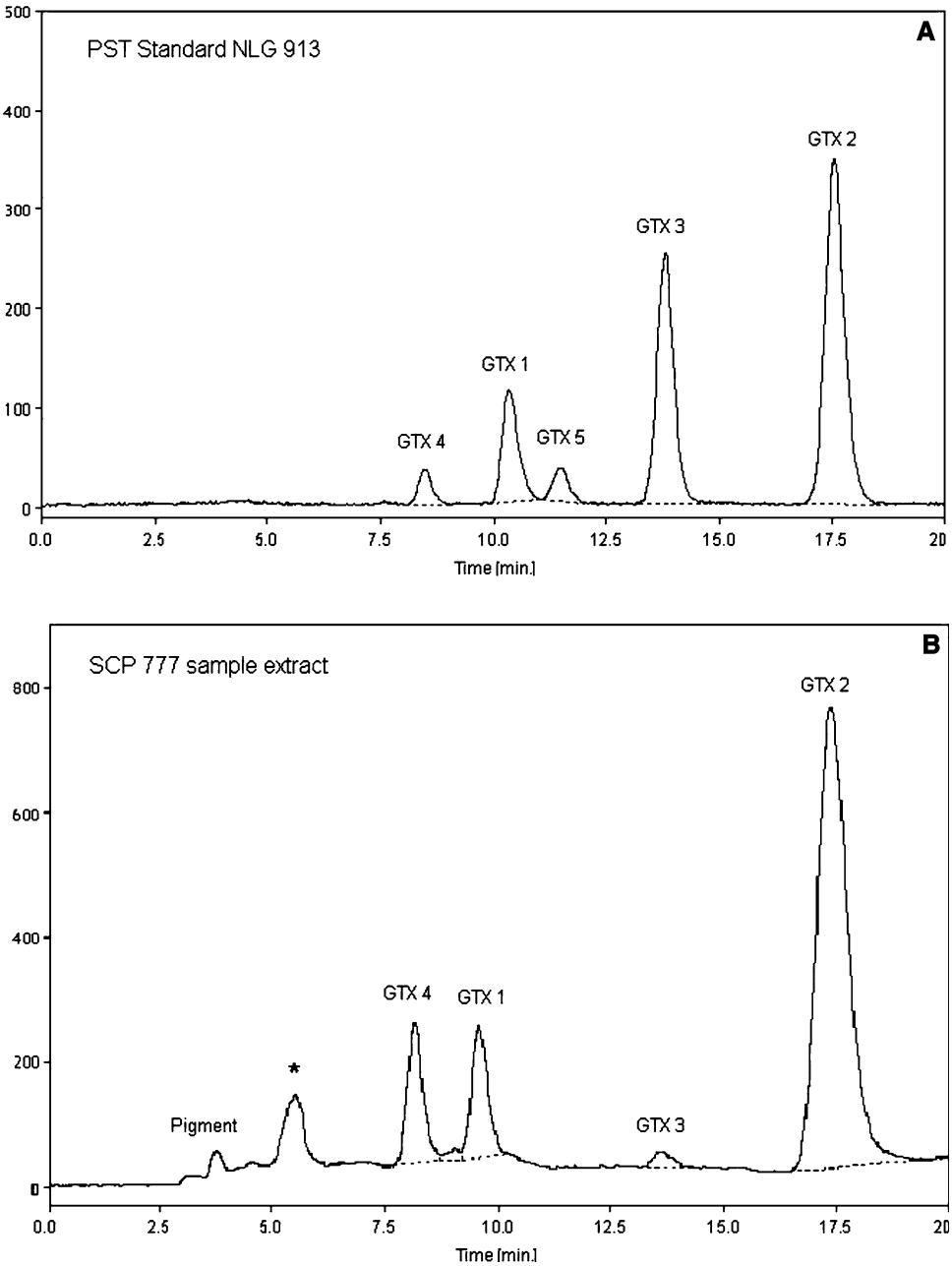

Fig. 2 PSP neurotoxins HPLC–FLD chromatograms. **a** Analytic standard of GTX mixture (NLG913) and **b** *M. aeruginosa* SCP777 sample extract. Both were analyzed under the conditions for GTXs. The units for the vertical axes in both chromatograms are millivolts

The high amounts of GTX4 and GTX1 were responsible for the unusually high toxicity showed by *M. aeruginosa* strain extract in the mouse bioassay (Fig. 3 and Table 1). This strain extract exhibited a high toxicity, with a 1,088 STX pg equivalent per cell, which amounts to 1.7×10^{-15} mol of PSP toxin per cell (Table 1).

HPLC–MS Analysis

All the molecular ions were detected in the positive ion mode. The mass spectra of both gonyautoxin epimers are shown in Fig. 4.

Figure 4a shows the classical mass spectra of the GTX3/GTX2 epimers. This analysis confirmed the presence of GTX3 and GTX2 since the mass spectrum showed only one major positive Zwitterion peak of M^+ 395.9 *m/z*. Figure 4b shows the mass spectra of GTX4 and GTX1; once again, only one major peak is observed, and it corresponds to M^+ 411 *m/z*, the characteristic positive Zwitterion of Gonyautoxins 4 and 1.

These spectra show GTX3/GTX2 and GTX4/GTX1 epimers fingerprints. Therefore, the identities of GTX3, GTX2, GTX4, and GTX1 are confirmed by HPLC–MS analysis, proving that these four Gonyautoxins are the only

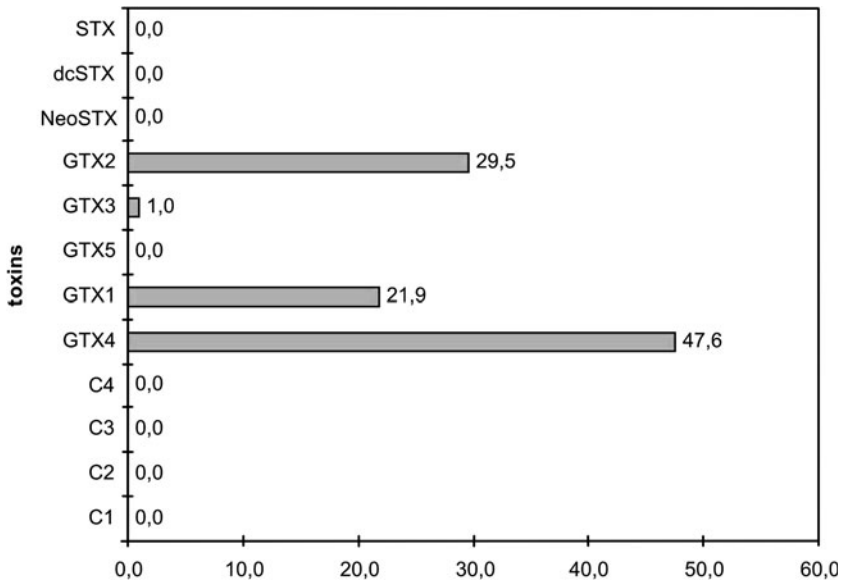

Fig. 3 PSP neurotoxins profile (mol.% of total PSP toxins content) of *M. aeruginosa* SCP777

Table 1 HPLC analysis of paralytic shellfish toxins in *M. aeruginosa* SCP777

Toxins	Specific toxicity[a] (MU μmol^{-1})	Standard concentrations (μmol)	Standard areas (1×10^3)	Sample areas (1×10^3)	Sample concentration (μmol)	PS Toxin profile (%)	Toxicity (MU per total cells)
C1	15	1.96	846.00	0.00	0.00	0.0	0.00
C2	239	0.44	403.00	0.00	0.00	0.0	0.00
C3	33	3.63	537.00	0.00	0.00	0.0	0.00
C4	143	0.82	200.00	0.00	0.00	0.0	0.00
GTX4	1803	0.38	184.62	6524.63	13.43	47.6	24.21
GTX1	2468	1.49	1450.20	6001.73	6.17	21.9	15.22
GTX5	160	0.00	0.00	0.00	0.00	0.0	0.00
GTX3	1584	0.57	2018.60	984.53	0.28	1.0	0.44
GTX2	892	1.33	6497.80	40705.59	8.33	29.5	7.43
neoSTX	2295	0.977	92.00	0.00	0.00	0.0	0.00
dcSTX	1274	0.265	42.00	0.00	0.00	0.0	0.00
STX	2483	0.450	98.00	0.00	0.00	0.0	0.00
Total					28.21	100.0	47.30

[a] The specific toxicity used in Table 1 was taken from Oshima (1995)

PSP neurotoxins produced by this cyanobacteria strain, and that they are responsible for the high toxicity exhibited by this cyanobacterium extract.

Microcystins Analysis

Microcystis aeruginosa SPC777 cell extract elicited a positive immunoreaction for microcystins in the ELISA test (data not shown); a total amount of 1.3 μg/l of microcystin was determined in the sample extract. Furthermore, the LC-MS analyses allowed the identification of the [L-ser^7] microcystin-RR (Fig. 3). The fragment ion peaks at *m/z* 135, and M, 906 showed the presence of the Adda unit. The sequence Adda-Glu-Ser-Ala was suggested from the fragment ion peaks *m/z* 313 and 217 (weak intensity), and Arg-MeAsp was revealed by the peak at *m/z* 286 (weak intensity) (Fig. 5).

Genetic Analyses

Sequencing of the cloned *N*-methyltransferase (NMT) domain of the *mcy*A gene from *M. aeruginosa* SPC777 produced a 1,369-bp fragment. Results from BLAST searches confirmed that the cloned sequence was originated from *mcyA* gene of the microcystin synthetase complex. The former sequence is closely related to *Microcystis aeruginosa* K-139 (98% match to a partial *mcyA* gene, accession no. AB019578, 98% query coverage), *Microcystis aeruginosa*

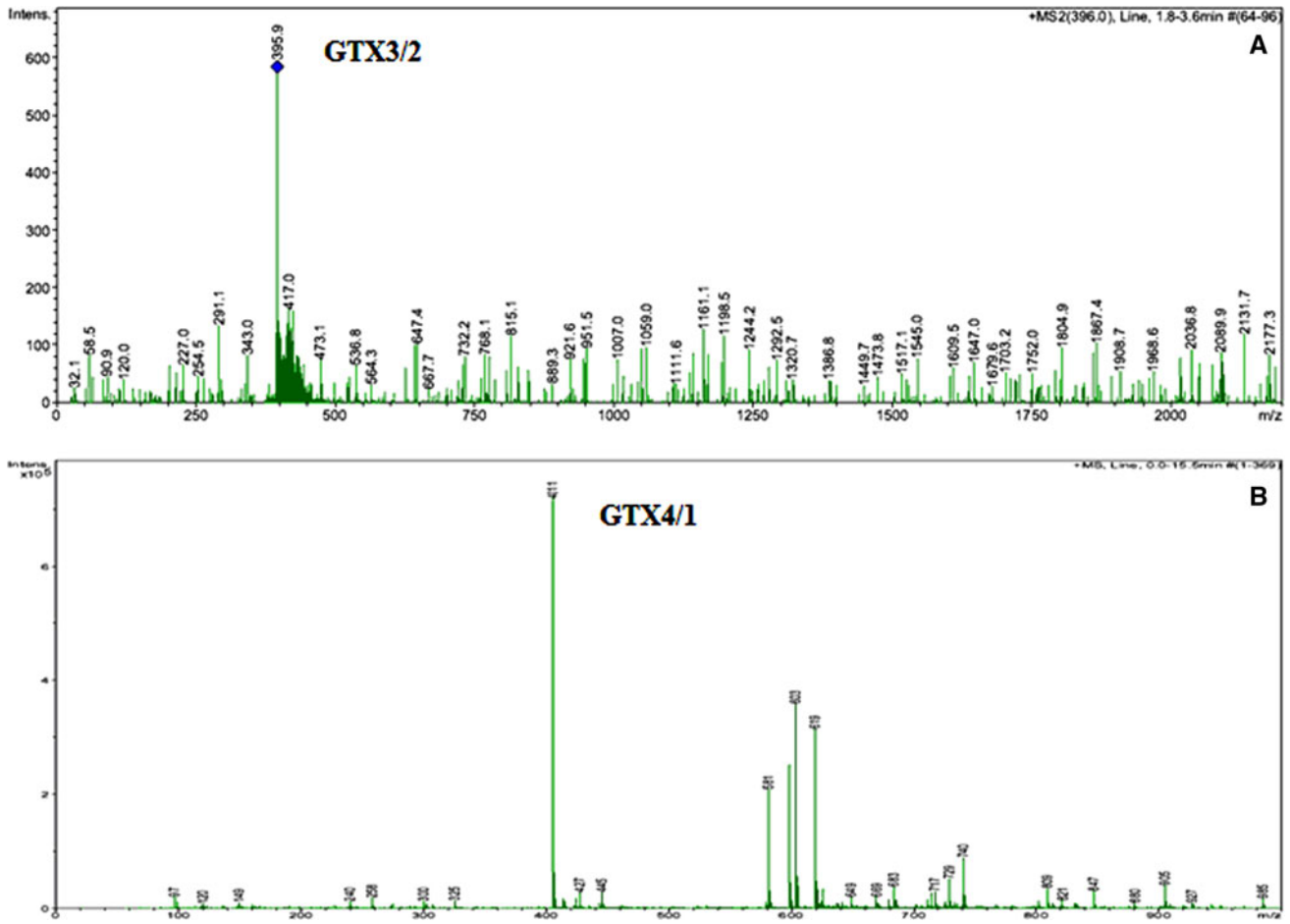

Fig. 4 Positive ion electrospray mass spectra of PSP toxin sample extract. **a** Mass spectrum of GTX3/GTX2 epimers with M^+ 395.9 *m/z*. **b** Mass spectrum of GTX4/GTX1 epimers with M^+ 411 *m/z*

PCC7806 (96% match to a partial *mcyA* gene, accession no. AF183408, 98% query coverage) and other *mcyA* gene sequences from GenBank. A phylogenetic tree was then constructed, using *mcyA* gene sequences from a variety of cyanobacteria with emphasis on the *Microcystis* genus (Fig. 6). *M. aeruginosa* SPC777 strain was positioned in a cluster containing only *Microcystis mcyA* gene sequences with a high bootstrap value (95 and 99% in NJ and MP analyses, respectively).

Scores based on the nearly complete 16S rRNA gene region (1,487 bp) were high among our newly sequenced strain and sequences of *Microcystis* from the GenBank. A comparison of the *M. aeruginosa* SPC777 with both *M. aeruginosa* PCC7941 and *M. aeruginosa* NIES834 revealed 99.6 and 99.4% sequence identities, respectively. The 16S rRNA sequence of *M. aeruginosa* SPC777 not only shared high similarity with several other sequences of *M. aeruginosa* strains (>99%), but also with the *M. ichthyoblable* OBB39S02 (99.7%) isolated from Bubano Basin, Imola, Italy (Castiglioni et al. 2004). The phylogenetic trees constructed using NJ and MP methods were largely congruent and, therefore, only NJ tree was presented with bootstrap values for both methods (Fig. 7). Representative sequences of Chrooccocalean taxa were retrieved from GenBank database, and the 16S rRNA of *Escherichia coli* K-12 was chosen as out group. The data show that *M. aeruginosa* SPC777 was grouped within the *Microcystis* clade with high bootstrap values (99% MJ and MP). The *Microcystis* strains formed a tight group that did not allow delineating inter- and intraspecific variations because sequences were highly similar.

Discussion

According to literature, PSP neurotoxins have been found only in filamentous cyanobacteria *Aphanizomenon* (Alam et al. 1973, Pereira et al. 2000), *Anabaena* (Humpage et al. 1994), *Lyngbya* (Carmichael et al. 1997), *Cylindrospermopsis raciborskii* (Lagos et al. 1999), and *Planktothrix*/*Limnothrix* (Pomati et al. 2000). The findings reported in this study revealed that the unicellular genus *Microcystis*

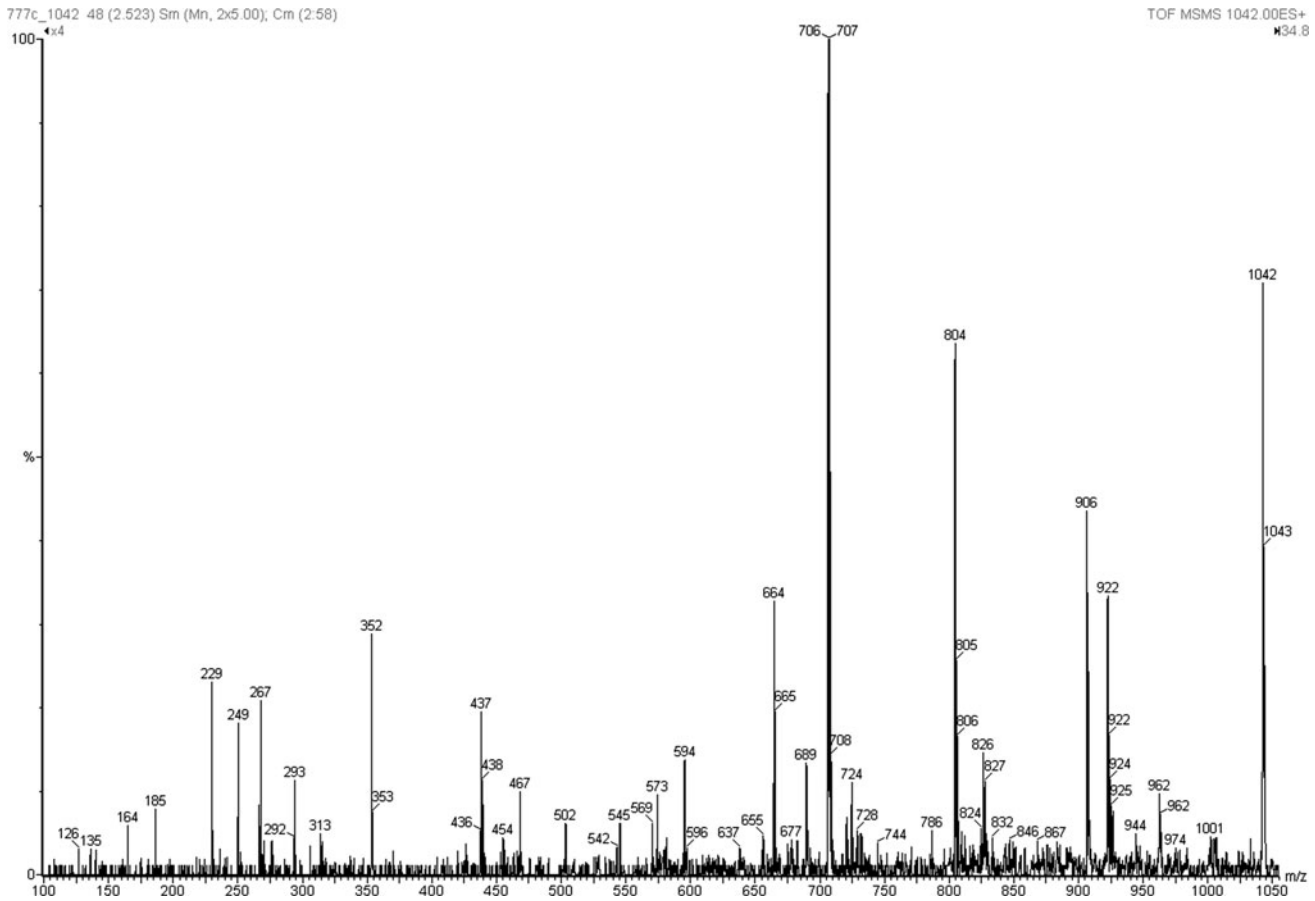

Fig. 5 Fragment ion mass spectra for [L-ser^7] microcystin-RR produced by *M. aeruginosa* SPC777 measured on a quadrupole ion trap mass spectrometer at *m/z* 100–1,100 after fragmentation of *m/z* 1,042

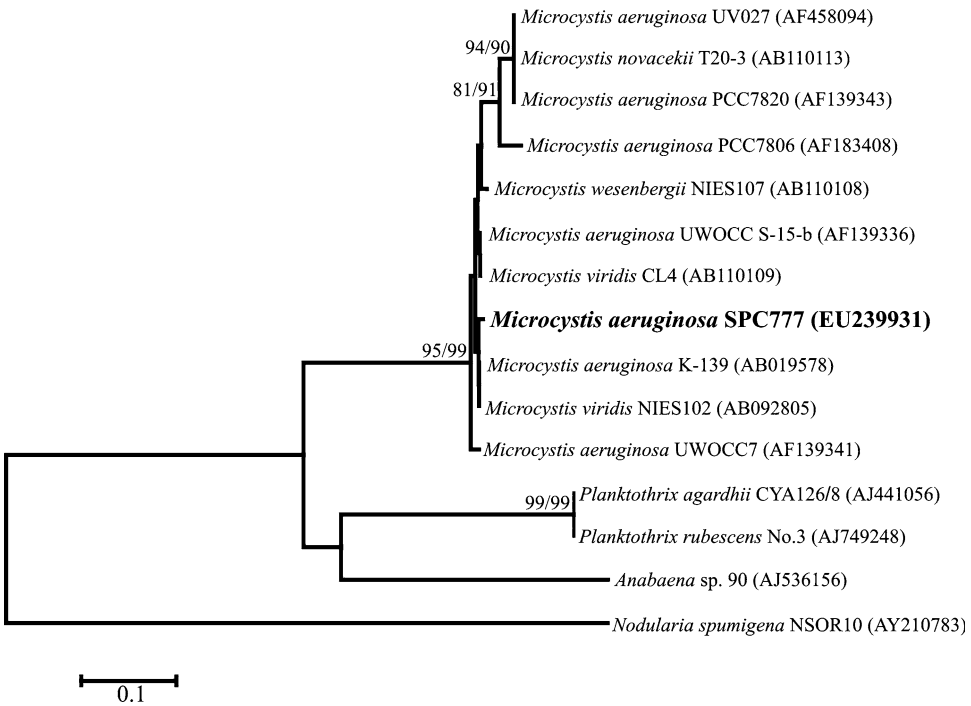

Fig. 6 Phylogenetic analysis displaying the relationship between sequences coding for NMT domain of *mcyA* gene (1,369 bp). Bootstrap values of >50% (for 1,000 iterations) are displayed at the branch nodes. The scale bar represents substitutions per site

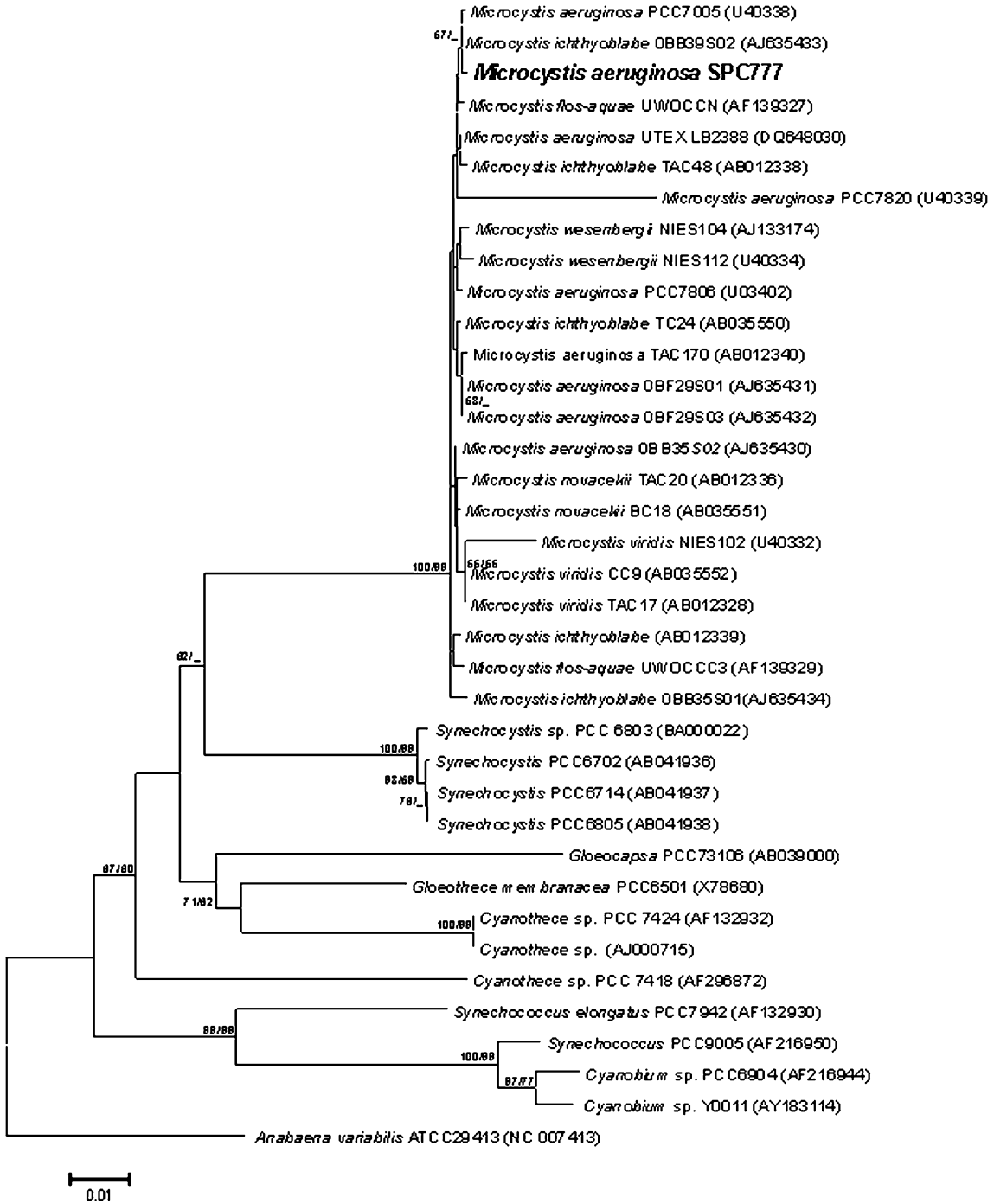

Fig. 7 Phylogenetic analysis based on 16S rRNA sequences (1,417 bp). The clustering of studied *M. aeruginosa* SPC777 is shown in bold. Numbers near nodes indicate bootstrap values over 50% for NJ and MP analyses. The *scale bar* represents substitutions per site

produces four PSP neurotoxins: GTX 1, GTX 4, GTX 2, and GTX 3, all of them encompassed within the gonyautoxin group, the most frequent and major PSP neurotoxins reported in all the samples (Lagos 1998, 2003). They match exactly with the GTX4/1 and GTX3/2 epimer standards. This is the first report of a PSP neurotoxins producer cyanobacterium in Brazil and South America (Lagos et al. 1999). The PSP neurotoxins profile of this *M. aeruginosa* strain looks novel when compared with the *Cylindrospermopsis raciborskii* strain. Besides, HPLC–MS analysis evidenced the presence of both PSP toxin epimers, since the only peaks resulting from the mass spectrum studies were those of M^+ 395.9 *m/z* and M^+ 411 *m/z*, the characteristics Zwitterions of GTX3/GTX2 and GTX4/GTX1 epimers, respectively.

These data lend support to the finding of a new PSP toxin-producing cyanobacterium strain in Brazil. This *M. aeruginosa* strain is also capable of microcystin production.

Up to the present, there is one report of two *Microcystis* strains that produce both type of toxins, microcystins and anatoxin-*a*, at the same time (Park and Watanabe 1996). Anatoxin-a is an alkaloid compound and a potent postsynaptic nicotinic cholinergic agonist, which acts as a depolarizing neuromuscular blocking agent, causing death by respiratory arrest (Carmichael et al. 1975; Carmichael et al. 1979). Both *Microcystis* strains were isolated in Japan, and the samples also showed anatoxin-a-producing *Anabaena* strains. However, those authors reported that after the third cultivation change, the strains lost their ability to produce neurotoxins. This is not the case with *M. aeruginosa* SPCC777 strain described here, since its ability to synthesize PSP neurotoxins, detected in March 2006 during a survey of Brazilian fresh waters, has been kept intact through many sub-cultivation cycles during 2 years.

Until now, Brazil is the only country in South America where PSP neurotoxins production by cyanobacteria has been reported. The first cyanobacterium strain reported was *C. raciborskii* (Lagos et al. 1999; Molica et al. 2002; Castro et al. 2004). This strain produced saxitoxin (STX), neoSaxitoxin (neoSTX), decarbamoylsaxitoxin (dcSTX), gonyautoxin 6 (GTX6), and the gonyautoxin 2/3 epimers (GTX2/3) (Lagos et al. 1999; Molica et al. 2002). Analyses of environmental samples also showed that STX, neoSTX, and dcSTX were present in several Brazilian water bodies (Dos Anjos et al. 2006; Costa et al. 2006; Molica et al. 2005; Yunes et al. 2003). Interestingly, the SPC777 strain was isolated from the same polluted Billings Reservoir where the first PSP toxin-producing *C. raciborskii* strain was isolated (Lagos et al. 1999). The *M. aeruginosa* SPC777 strain described here, produces the GTX 4/1 epimers as the most abundant PSP neurotoxins. However, GTX 3/2 epimers were also detected by HPLC–FLD and HPLC–MS. GTX4/1 epimers account for 83.4% of the total toxicity of the *M. aeruginosa* SCP777 strain, which is expressed as Mouse Units (MU) per total cells (Table 1). The high amount of GTX4/GTX 1 epimers are responsible of the unusual high toxicity showed by *M. aeruginosa* strain extract in the mouse bioassay. Testing the extract of *M. aeruginosa* SPC77 by mouse bioassay showed that mice suffered exactly the same characteristic intoxication symptoms observed when PSP neurotoxins from contaminated shellfish extracts were tested. The mouse bioassay test was the event that drove us to look for PSP neurotoxins in this strain extract. Mice used in the bioassay went through the same sequence of symptoms as those injected with PSP (Lagos 1998, 2003).

The *M. aeruginosa* strain SPC77 also produces microcystin ([L-ser^7] MCYST-RR), determined by ELISA assay and HPLC–MS analysis. The ELISA assay showed the functional identity criteria, absolutely necessary in this case, since the presence of the highly toxic of PSP neurotoxins in the same strain extract, masks the toxicity of hepatotoxins in the mouse bioassay test (Lagos 1998, 2003). The total content of PSP neurotoxins produced by *M. aeruginosa* strain SPC77 was 11.0 μg/l. This amount was higher than the amount of microcystins (1.3 μg/l).

The presence of microcystin synthetase operon in *M. aeruginosa* SPC777 was confirmed by the detection and phylogenetic analysis of the *mcyA* gene involved in microcystin biosynthesis. This gene has been used in a number of studies, aiming to detect the microcystin synthetase operon in diverse isolates of cyanobacteria, and environmental samples (Hisbergues et al. 2003; Oksanen et al. 2004; Ouahid et al. 2005; Ouellette et al. 2006; Richardson et al. 2007; Tillett et al. 2001).

Microcystis aeruginosa is cosmopolitan and frequently appears forming heavy blooms in eutrophic water plankton (Komárek and Anagnostidis 1999). SPC777 strain was isolated from a dense water surface bloom of *Microcystis* sp., occurred in a well-known Brazilian eutrophic reservoir. The morphological characteristics of the strain reported here are in total agreement with the phenotypic description for *M. aeruginosa* (Kützing) Kützing (Komárek and Anagnostidis 1999). Classical *Microcystis* taxonomy follows the traditional botanical approach, which is mainly based on morphological characteristics from field samples, and *Microcystis* taxonomy is normally associated to its transitional morphologies in Nature and to morphological changes in laboratory cultures (Otsuka et al. 2000, 2001). The morphological change of the colonial form of *M. aeruginosa* SPCC777 during subculture was not surprising, since this phenomenon has been well documented elsewhere (Krüger et al. 1981; Komárek 1991; Otsuka et al. 2000; Rippka et al. 1979).

Despite the correspondence of morphological and genetic analyses of *M. aeruginosa* SPCC777 within-genus, no consistent differences were found at the species level. This was expected nevertheless, since it is known that current morphological classification of *Microcystis* species is not supported by phylogenetic analysis based on 16S rRNA sequence (Neilan et al. 1997; Lyra et al. 2001; Willame et al. 2006). The 16S rRNA gene is too highly conserved to use it to estimate *Microcystis* relationships below the genus level. The 16S rRNA gene analysis showed that *M. aeruginosa* SPC777 was very similar to *M. aeruginosa* PCC7941 (accession no. AB035549) recommended by Rippka and Herdman (1992) and also with *M. aeruginosa* NIES834 (accession no. AJ33171) suggested being the type strain by Otsuka et al., (2001). On other hand, according to our data, a high similarity with *M. ichthyoblable* was also found, which agrees with

literature data, since in some cases *M. ichthyoblabe* cannot be distinguished from *M. aeruginosa* (Kato et al. 1991). Besides, the former has also been suggested to be assigned as *M. aeruginosa* (Otsuka et al. 2001). Conversely, *M. ichthyoblabe* occurs in temperate regions and in mesotrophic to slightly eutrophic freshwaters, and never so far in polluted water like the Billings Reservoir (Komárek and Anagnostidis 1999). Although the 16S rRNA sequence obtained from the *M. aeruginosa* SPCC777 morphospecies could not be assigned to any species, it does contribute to the genetic characterization of the first *Microcystis* strain found to be capable of synthesizing both, PSP neurotoxins and hepatotoxins.

Before this study, only marine dinoflagellates, molluscs, and freshwater filamentous cyanobacteria had been found to synthesize PSP neurotoxins (Bricelj and Shumway 1998; Lagos 1998, 2003; Llewellyn et al. 2006). A PSP neurotoxin producing *Microcystis* strain is a major concern, because this genus is distributed worldwide and the most common fresh water bloom promoter. Although PSP neurotoxins were detected in a cultured *Microcystis* strain, this strain was isolated from Billings Reservoir, where the analogs found in this study were also present in environmental samples (Lagos et al. 1999), and indicating high potential hazard to the aquatic ecosystem and to the health of domestic and wild animals, as well as to human beings. Further knowledge of the biosynthetic pathway of PSP neurotoxins is most necessary to understand the spreading of this metabolic capability between related and unrelated species from different domains. Data shown in this article signify a strong wake-up call for authorities and the scientific community of Brazil; a permanent monitoring program of PSP neurotoxin producing cyanobacteria becomes an urgent need, since this is the second such cyanobacteria detected and isolated from São Paulo, the largest city of Brazil and South America.

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