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Reassessment of the toxin profile of *Cylindrospermopsis raciborskii* T3 and function of putative sulfotransferases in synthesis of sulfated and sulfonated PSP toxins

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ABSTRACT

The toxigenic freshwater cyanobacterium *Cylindrospermopsis raciborskii* T3 has been used as a model to study and elucidate the biosynthetic pathway of tetrahydropurine neurotoxins associated with paralytic shellfish poisoning (PSP). There are nevertheless several inconsistencies and contradictions in the toxin profile of this strain as published by different research groups, and claimed to include carbamoyl (STX, NEO, GTX2/3), decarbamoyl (dcSTX), and N-sulfocarbamoyl (C1/2, B1) derivatives. Our analysis of the complete genome of another PSP toxin-producing cyanobacterium, *Raphidiopsis brookii* D9, which is closely related to *C. raciborskii* T3, resolved many issues regarding the correlation between biosynthetic pathways, corresponding genes and the T3 toxin profile. The putative *sxt* gene cluster in *R. brookii* D9 has a high synteny with the T3 *sxt* cluster, with 100% nucleotide identity among the shared genes. We also compared the PSP toxin profile of the strains by liquid chromatography coupled to mass spectrometry (LC-MS/MS). In contrast to published reports, our reassessment of the PSP toxin profile of T3 confirmed production of only STX, NEO and dcNEO. We gained significant insights via correlation between specific *sxt* genes and their role in PSP toxin synthesis in both D9 and T3 strains. In particular, analysis of sulfotransferase functions for SxtN (N-sulfotransferase) and SxtSUL (O-sulfotransferase) enzymes allowed us to propose an extension of the PSP toxin biosynthetic pathway from STX to the production of the derivatives GTX2/3, C1/2 and B1. This is a significantly revised view of the genetic mechanisms underlying synthesis of sulfated and sulfonated STX analogues in toxigenic cyanobacteria.

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1. Introduction

Cylindrospermopsis raciborskii was described as a planktonic cosmopolitan filamentous cyanobacterium (Order Nostocales) from freshwater, able to fix nitrogen in terminal heterocysts (Woloszynska, 1912). *C. raciborskii* has become one of the most notorious blue-green algal species (Padisák, 1997) because of its toxicity and tendency to form dense blooms that interfere in multiple ways with water use. The species comprises strains that can produce either the hepatotoxin cylindrospermopsin (CYN), a potent protein synthesis inhibitor, or the neurotoxins saxitoxin and its analogues. The latter toxins are associated with paralytic shellfish poisoning (PSP), which can cause illness and even death of humans after consumption of seafood contaminated with these toxins, and are also responsible

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for deleterious effects on organisms in aquatic ecosystems (Ressom et al., 1994; Zingone and Enevoldsen, 2000). Nevertheless, some strains of *C. raciborskii* do not produce any known toxins.

The first report of PSP toxin production in *C. raciborskii* characterized the toxin profile of three strains T1, T2 and T3 (Lagos et al., 1999). Strains T2 and T3 were isolated in 1996 from the same location in Brazil, a branch of Billings water reservoir called Taquacetuba. The toxin profiles of cultured isolates were determined by liquid chromatography with fluorescence detection (LC-FD) after post-column derivatization. Confirmation of toxin identity was provided by liquid chromatography with detection by electro-spray ionization mass spectrometry (LC-ESI-MS). Lagos et al. (1999) mentioned that strains T2 and T3 had identical toxin profiles, comprising saxitoxin (STX), and the epimers gonyautoxin 2 and gonyautoxin 3 (GTX2/3) in a 1:9 ratio, but showed only the LC-chromatograms of strains T1 and T2. Curiously, despite the lack of definitive chromatographic evidence of the toxin profile of T3, this strain was subsequently selected by different researchers to investigate the biosynthetic pathway for PSP toxins. The first published chromatogram for *C. raciborskii* T3 (Pomati et al., 2003) was based upon a pre-column oxidation method followed by liquid chromatographic separation of the oxidation products (Lawrence et al., 1996). Furthermore, although Pomati et al. (2003) used a mixture of STX and GTX2/3 as a calibration standard and claimed to find only STX in T3, they noted that the strain also produces the low potency N-sulfocarbamoyl derivatives C1/2, by referencing the work of Lagos et al. (1999). This was a misquotation of the T3 toxin profile as described by Lagos et al. (1999), who reported only the presence of STX and GTX2/3. This confusion was later compounded by Pomati et al. (2004), who considered the "STX and C1/2" profile of T3 as the basis for experimentation and hypothesis of a possible new pathway for synthesis of C1/2 analogues, thereby modifying the biosynthetic pathways proposed by Shimizu (1996) and Sako et al. (2001).

The sequence of the PSP toxin biosynthesis gene cluster, known as the *sxt* gene cluster, in strain T3 was recently published (Kellmann et al., 2008). Based upon their LC–MS analysis, Kellmann et al. (2008) described yet another toxin profile for T3: STX, neosaxitoxin (NEO), decarbamoyl saxitoxin (dcSTX) and the N-sulfocarbamoyl toxin B1 (GTX5). These authors did not refer to synthesis of C1/2 analogues, but confusingly in the discussion they mentioned that both *Anabaena circinalis* AWQC131C and *C. raciborskii* T3 produce N-sulfonated and O-sulfated analogues of STX (B1, C2/C3, dcGTX3/dcGTX4). Kellmann et al. (2008) analyzed the structure of the gene cluster and the possible function of each open reading frame (ORF) based on the toxin profile they reported.

Obviously the interpretation of existing reports on the toxin profile and molecular genetic evidence for the biosynthetic pathway to STX and analogues in strain T3 has been complicated if not confounded by these discrepancies. The inconsistencies could conceivably have arisen via a combination of cross-contamination, miss-identification, inappropriate application or interpretation of analytical methodologies, and/or errors in citation of the literature. Furthermore, the Brazilian group responsible for the distribution of the original strain T3 did not specify the toxin profile of this strain, and only expressed the sum of all peak areas of toxins as concentration equivalents of STX (Ferrão Filho et al., 2008).

We recently sequenced the genome of *Raphidiopsis brookii* D9 (Stucken et al., 2010), a PSP toxin-producing cyanobacterium closely related to and formerly assigned to *C. raciborskii* (Stucken et al., 2009) and later to *Raphidiopsis* (Plominsky et al., 2009). The toxin profile for strain D9, based upon detailed LC–MS/MS analysis, comprises STX, dcSTX, GTX2/3 and dcGTX2/3. We identified a gene cluster for PSP toxin synthesis that is highly similar to that published for T3, but in D9 the cluster is not flanked at one end by the proposed regulatory genes *sxtY*, *sxtZ* and *ompR* and lacks four open reading frames (ORFs) related to PSP toxin production in T3 (*sxtN*, *sxtX*, *sxtW* and *sxtV*) (Stucken et al., 2010). Considering the putative differences in toxin profiles between T3 and D9, these four ORFs are of interest to evaluate and correlate the toxin profile in *C. raciborskii* T3.

The primary aim of this study was to clarify the toxin profile of *C. raciborskii* T3, a reference strain in the study of PSP toxin biosynthesis, and to assess the coherence between the PSP toxin profile and the gene content, with reference to other cyanobacterial strains. We provide further evidence on the role of sulfotransferase-like genes in the synthesis of sulfated and sulfonated analogues in cyanobacteria.

2. Materials and methods

2.1. Cyanobacterial cultures and growth conditions

The non-axenic *C. raciborskii* T3 strain was kindly provided by Sandra Azevedo (Universidade Federal do Rio de Janeiro, Brazil). *C. raciborskii* CS-505, 506 and 511 were isolated from Australia and obtained from the CSIRO collection, Hobart, Tasmania. *R. brookii* D9 was isolated by re-cloning from the multiclonal isolate SPC338 collected in 1996 from the Billings freshwater reservoir near Sao Paulo (Brazil) (Castro et al., 2004). Cyanobacteria were cultured in MLA medium according to Castro et al. (2004), at 25–28 °C under fluorescent light at a photon flux density of 40 μmol m^{-2} s^{-1} on a light/dark photocycle of 12:12 h without aeration.

In order to demonstrate the morphological identity of *C. raciborskii* T3, as directly received from S. Azevedo, who is responsible for maintenance and distribution of the original stain, the isolate was examined and compared with our previous results. The morphological identification was assessed by light microscopy with a Nikon ECLIPSE TS100 inverted microscopes at 1000× magnification with oil immersion.

2.2. Genomic DNA isolation, amplification and sequencing

The DNA was extracted with the CTAB method described by Wilson (1990). For PCR amplification of 16S rDNA, the general primers CYA106a (forward) and 1492 (reverse) were used as described by Nübel et al. (1997) and Lane et al. (1985), respectively. Amplification of the putative *sxt* gene cluster was carried out with *sxtA*, *sxtN*, *sxtX*, *sxtW*, *sxtV*, *sxtD*-context, and *sxtO*-context primers (forward

Table 1
PCR primers used for amplification and sequencing. The length of PCR products and the 5′-binding sites are given in parentheses.

Primer	Sequence	Identity (%)[a]
16S rDNA (1400 bp)		99.8
CYA106Fa (5′–45)	5′-CGGACGGGTGAGTAACGCGTGA-3′	
1492R (5′–1411)	5′-GGTTACCTTGTTACGACTT-3′	
sxtA (3600 bp)		100
SXTAF (5′–5146)	5′-CTCCTCTTCGGTATTGGCGG-3′	
SXTAR (5′–8745)	5′-GCGGTTCCCGTTATTCTTGC-3′	
sxtN (1045 bp)		100
SXTNF (5′–18931)	5′-CTGCTAGTTTGCGGCTGGTG-3′	
SXTNR (5′–19975)	5′-CCCCCTCTGAACGGTTACGA-3′	
sxtX (921 bp)		100
SXTXF (5′–20212)	5′-AAAAAGTGCAAGTTAAGAGG-3′	
SXTXR (5′–21132)	5′-TAAAACACGGGGACTACATC-3′	
sxtW (430 bp)		100
SXTWF (5′–21141)	5′-ACTTAGGTAAAGCGGCTTTG-3′	
SXTWR (5′–21512)	5′-GCGCTACACCACCAGTATTA-3′	
sxtV (1829 bp)		100
SXTVF (5′–21511)	5′-CCTGCTACAACTTTAATACT-3′	
SXTVR (5′–23338)	5′-AGCTTAGTAAAGAACAACT-3′	
sxtD′ (853 bp)		100
SXTDcontF (5′–868)	5′-ATACCTCCAGAGCGAGGACA-3′	
SXTDR (5′–1720)	5′-ATGGAAGGGAGAGCGAATTT-3′	
sxtO′ (1240 bp)		100
SXTOF (5′–29976)	5′-TCCTGACTCCGCAAAGAGTT-3′	
SXTOcontR (5′–31215)	5′-CGGGGTTGGATATGTTTTTG-3′	

[a] Percentage identity of sequences obtained in this work compared with those described for *C. raciborskii* T3.

and reverse). Primer sequences and PCR amplicon sizes are shown in Table 1. The PCR reaction contained 50–100 ng of genomic DNA. Reagents for each amplification were: 0.25 U Taq DNA polymerase (Invitrogen®, California, USA); 3 μl 10× PCR buffer (Invitrogen®); 2.5 mM MgCl (Invitrogen®, California, USA); 0.4 mM primers; and 0.93 mM of each deoxynucleoside triphosphate (Promega®, Madison, Wi-USA). Thermal cycling for 16S rDNA was performed in an Eppendorf Mastercycler (Westbury, NY-USA), under the following conditions: initial DNA denaturation at 92 °C for 2 min; 30 cycles at 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min and a final elongation at 72 °C for 5 min.

Thermal cycling amplification for the *sxt* gene cluster was performed in an Eppendorf Mastercycler (Westbury, NY-USA), under the following conditions: initial DNA denaturation at 99 °C for 1 min, 30 cycles at 94 °C for 15 s, 53 °C for 1 min, 72 °C for 1.5 min and a final extension at 72 °C for 7 min.

All PCR products were checked by electrophoresis in 1.0% agarose gel electrophoresis and visualized under UV transillumination after staining with ethidium bromide.

PCR-generated fragments of the 16S rDNA and *sxt* gene cluster were purified, with a Wizard®*Plus* SV Miniprep, DNA Purification System (Promega®, Madison, Wi-USA). PCR primers were used for sequencing of both DNA strands (Macrogen, Korea). All sequences were checked manually by Vector NTI software (Invitrogen®, California, USA).

2.3. GenBank accession numbers

Published sequences were obtained from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) under the accession numbers: *C. raciborskii* T3 16S rDNA gene, partial sequence (EU439566); *C. raciborskii* T3 *sxt* gene cluster, complete sequence (DQ787200); *R. brookii* D9 *sxt* gene cluster, complete genome sequence (ACYB00000000); *Lyngbya wollei sxt* gene cluster, complete sequence (EU603711); *C. raciborskii* CSIRO (CS) collection 16S + ITS-1(S): CS-505 (EU552062), CS-506 (EU552063), CS-511 (EU552068); *R. brookii* D9 16S + ITS-1(S) (EU552070). *A. circinalis* ACMB13 *sxtSUL* gene, partial sequence was submitted in this work under the accession number HM163164.

2.4. Toxin extraction of C. raciborskii T3

Cyanobacteria were harvested in exponential growth phase by centrifugation at 16,000×*g* for 15 min to yield a cell pellet. The cell pellets were dried in a DNA Speed Vac, SAVANT mod. DNA 110-230 (NY, USA) and extracted in 300 μl of 0.05 M acetic acid. Samples were disrupted three times (20 s each) with an ultrasonic cell disruptor (Microson XL, Misonix, Farmingdale, USA). The extracts were then centrifuged at 5,000×*g* for 10 min, filtered through a 0.45 μm membrane filter and stored at −20 °C until analysis.

2.5. Liquid chromatography with fluorescence detection (LC-FD)

The LC-FD analysis was carried out on an LC1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a PCX 2500 post-column derivatization system (Pickering Laboratories, Mountain View, CA, USA). The LC-system consisted of a degasser (G1379A), a quaternary pump (G1311A), an autosampler (G1229A), an

autosampler thermostat (G1330B), a column thermostat (G1316A) and a fluorescence detector (G1321A).

Chromatographic conditions were as follows: mobile phase A: 6 mM 1-octanesulphonic acid and 6 mM 1-heptanesulfonic acid in 40 mM ammonium phosphate, adjusted to pH 7.0 with diluted phosphoric acid and 0.75% of tetrahydrofurane (THF) for the gonyautoxin group; mobile phase B: 13 mM 1-octanesulphonic acid in 50 mM phosphoric acid, adjusted to pH 6.9 with ammonium hydroxide, and 15% (v/v) acetonitrile and 1.5% THF for the saxitoxin group. The flow rate was 1 mL min^{-1} with the following gradient: 0 min, 100% A isocratic to 15 min, switch to 100% B until 16 min, isocratic B until 35 min, switch to 100% A until 36 min, isocratic 100% A until 45 min (= total run time). The autosampler was cooled to 4 °C and the injection volume was 20 μL. The separation of analytes was performed on a 250 × 4.6 mm i.d., 5 μm, Luna C18 reversed-phase column (Phenomenex, Aschaffenburg, Germany) equipped with a Phenomenex SecuriGuard pre-column. The eluate from the column was continuously oxidized with 10 mM of periodic acid in 550 mM ammonium hydroxide at a flow rate of 0.4 ml min^{-1} in a reaction coil set at 50 °C. Subsequently, the eluate was continuously acidified with 0.75 N nitric acid at a flow rate of 0.4 ml min^{-1} and the toxins were detected by a dual monochromator fluorescence detector (λ_{ex} 333 nm; λ_{em} 395 nm). Data acquisition and processing was performed with the HP ChemStation software. PSP toxin concentrations were determined by external calibration.

Standard solutions of PSP toxins (STX, NEO, dcSTX, dcNEO, gonyautoxins 1/4 (GTX1/4), GTX2/3, decarbamoyl gonyautoxins 2/3 (dcGTX2/3), B1 and C1/2) were purchased from the Certified Reference Materials Programme of the Institute for Marine Biosciences (National Research Council, Halifax, NS, Canada).

2.6. Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS)

Mass spectrometric analyses for PSP toxins were performed according to a hydrophilic interaction liquid ion-chromatography (HILIC) method (Diener et al., 2007) with slight modifications. Mass spectral experiments were performed on an ABI-SCIEX-4000 Q Trap (Applied Biosystems, Darmstadt, Germany), triple quadrupole mass spectrometer equipped with a TurboSpray® interface coupled to an Agilent model 1100 LC. The LC equipment included a solvent reservoir, in-line degasser (G1379A), binary pump (G1311A), refrigerated autosampler (G1329A/G1330B), and temperature-controlled column oven (G1316A).

The analytical column (150 × 4.6 mm) was packed with 5 μm ZIC-HILIC (SeQuant, Lund Sweden) and maintained at 35 °C. The flow rate was 0.7 ml min^{-1} and gradient elution was performed with two eluants, where eluant A was 2 mM formic acid and 5 mM ammonium formate in acetonitrile/water (80:20 v/v) and B was 10 mM formic acid and 10 mM ammonium formate in water. The gradient was as follows: 20 min column equilibration with 80% A, linear gradient until 5 min to 65% A, then until 10 min to 60% A, then until 20 min 55% A, subsequent isocratic elution with 55% A until 24 min, and finally return to initial 80% A until 25 min. Total run time: 45 min. 5 μl of sample was injected in triplicates.

Selected reaction monitoring (SRM) experiments were carried out in positive ion mode by selecting the following transitions (precursor ion > fragment ion), period 1 (B-, C- and gonyautoxins): m/z 412 > 332 and m/z 412 > 314 (for GTX1/4 and C3/4), m/z 396 > 316 and m/z 396 > 298 (for GTX2/3, C1/2 and B2), m/z 380 > 300 and m/z 380 > 282 (for B1 = GTX5), m/z 353 > 273 (for dcGTX2/3), m/z 369 > 289 (for dcGTX1/4); period 2 (STX, NEO and their decarbamoyl derivatives): m/z 300 > 282 and m/z 300 > 204 (for STX), m/z 316 > 298 and m/z 316 > 196 (for NEO), m/z 257 > 196 and m/z 257 > 156 (for dcSTX) and m/z 273 > 255 (for dcNEO). Dwell times of 100–200 ms were used for each transition. For these studies the source parameters were as follows: curtain gas: 30 psi, temperature: 650 °C, ion-spray voltage: 5,000 V, gas 1 and 2: 70 psi, interface heater: on, collision gas: high, declustering potential: 66 V, entrance potential 10 V, collision energy: 30 V and collision cell exit potential: 12 V.

3. Results and discussion

3.1. Morphological and genetic analysis of C. raciborskii T3

Morphological analysis of *C. raciborskii* T3 by light microscopy was consistent with previous observations. As distinctive for *C. raciborskii*, filaments showed typical vegetative cells (Fig. 1A) and specialized cells, specifically cone-shaped heterocysts located at the end of the filament (Fig. 1B). Characteristic ovoid-shaped akinetes (Fig. 1C) were also present, sometimes in a tandem of four akinetes cells (inset in Fig. 1C).

Amplification and sequencing of the 16S rDNA and five genes belonging to the *sxt* gene cluster (*sxtA*, *sxtN*, *sxtX*, *sxtW*, *sxtV*) confirmed the genetic identity of *C. raciborskii* T3. In the other four cyanobacterial species from which the cluster is known, the genetic context of the *sxt* gene cluster is different from that of T3 strain allowing for a clear

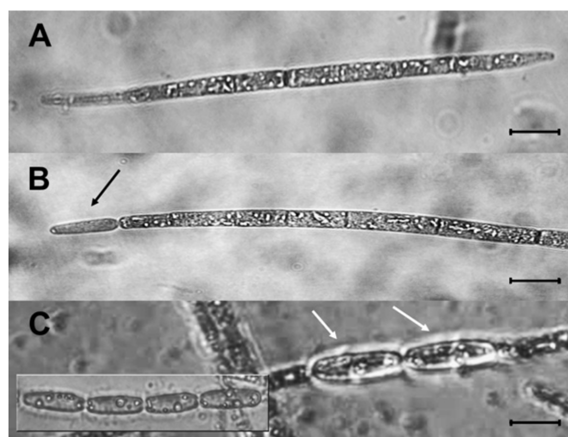

Fig. 1. Filaments of *C. raciborskii* T3. (A) Vegetative cell. Scale bar: 10 μm. (B) Solitary trichome with heterocyst (black arrow) in terminal position of the filament. (C) Trichome with two adjacent oval-shaped akinetes (white arrows).

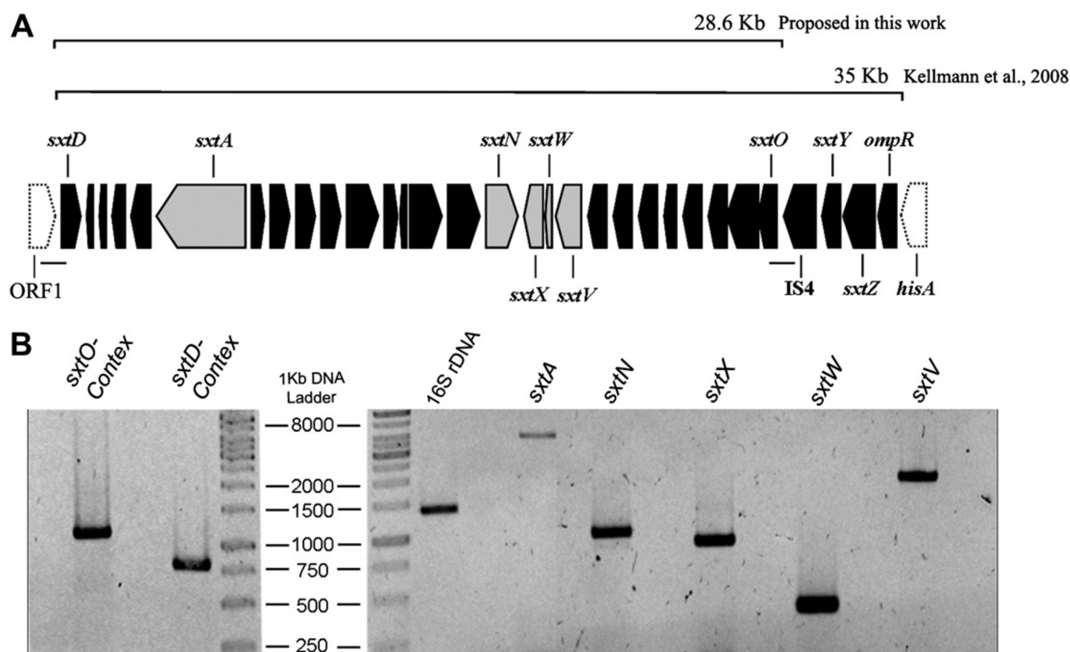

Fig. 2. Schematic organization of the *sxt* gene cluster in *C. raciborskii* T3 (A) and PCR amplicons of *sxt* genes (B). Genes amplified to confirm the cluster structures in T3 are shown in grey. *sxtD* and *sxtO* genes (both at the flanking regions of the cluster) and their context are indicated by a black bar. The scale of the gene cluster lengths is indicated as the number of base pairs. Electrophoresis in 1.0% agarose gel stained with ethidium bromide, showing amplification fragment of 16S rDNA, *sxt* genes from *C. raciborskii* T3 and genetic context of the *sxt* gene cluster ends.

identification and discrimination of the T3 *sxt* cluster. In this strain, an unknown ORF1 is located upstream of the *sxtD* gene (*sxtD*-context) and an insertion sequence from the IS4 family is located upstream of the *sxtO* gene (*sxtO*-context) (Fig. 2A). PCR amplicons of the expected size were obtained for all genes studied (16S rDNA: 1367 bp; *sxtA*: 3600; *sxtN*: 1045 bp; *sxtX*: 921 bp; *sxtW*: 430 pb; *sxtV*: 1653 pb; *sxtD*-context: 853 bp; and *sxtO*-context: 1240 bp) (Fig. 2B).

Comparison of the sequences of the 16S rDNA of *C. raciborskii* T3 and the corresponding genes present in the putative *sxt* gene cluster with those available in the NCBI database as provided by Kellmann et al. (2008) yielded very high similarities. Alignment of the 16S rDNA sequences gave identity matches of 99.8%; the difference of 0.2% corresponded to one gap and a mismatch of five nucleotides (Fig. S1). Further comparison of the 16S rDNA sequence of strain T3 with *C. raciborskii* strains from Australia (CS-505, 506 and 511), showed that the differences among the strains are restricted mostly to specific polymorphic sites (Fig. S1). We found a nucleotide identity of 100% in all genes of the *sxt* gene cluster tested (Table 1).

3.2. Re-assessment of the C. raciborskii T3 toxin profile

Clarification of the *C. raciborskii* T3 toxin profile is an important consideration for defining correct gene functions because the gene cluster responsible for the biosynthesis of PSP toxins was first described in T3, with putative functions assigned based upon the reported toxin profile (STX, NEO, dcSTX and B1) (Kellmann et al., 2008). Nevertheless, the previously described toxin profile of *C. raciborskii* (Lagos et al., 1999; Kellmann et al., 2008) is inconsistent among studies, with the following PSP toxin analogues: STX, GTX2/3, C1/2, B1, NEO, dcNEO and dcSTX, reported in different combinations in various publications. Our analysis of the toxin profile of T3 by LC-FD revealed three fluorescent peaks that coeluted with the toxin standards for NEO-dcNEO, dcSTX and STX, respectively (Fig. 3A,B). However, the analyte peak at the approximate retention time of dcSTX of the T3 chromatogram eluted slightly earlier than the standard dcSTX, which makes it difficult to confirm the presence of dcSTX in this strain. We also observed several peaks with retention times that corresponded with those of standards for various GTXs. True PSP toxins do not possess native fluorescence and must be converted to fluorescent iminopurine derivatives for detection by this method. These “GTX peaks” are therefore artifacts (“false positives”) and do not represent PSP toxins because they continued to appear as fluorescent components, even when post-column oxidation (derivatization) was suspended (Fig. 3C). In contrast, the three peaks corresponding in retention time to NEO-dcNEO, dcSTX and STX were missing in chromatograms without post-column oxidation, supporting their legitimacy as putative PSP toxin peaks.

In any case, without further confirmatory analysis (e.g. by LC–MS), reports of PSP toxin profiles with definitive peak identities for cyanobacteria must be viewed rather sceptically. For example, the pre-column oxidation LC method (Lawrence et al., 1996) generates multiple fluorescent derivatives for certain toxin analogues and was originally designed to estimate overall PSP toxicity in shellfish rather than to directly infer the exact toxin profile.

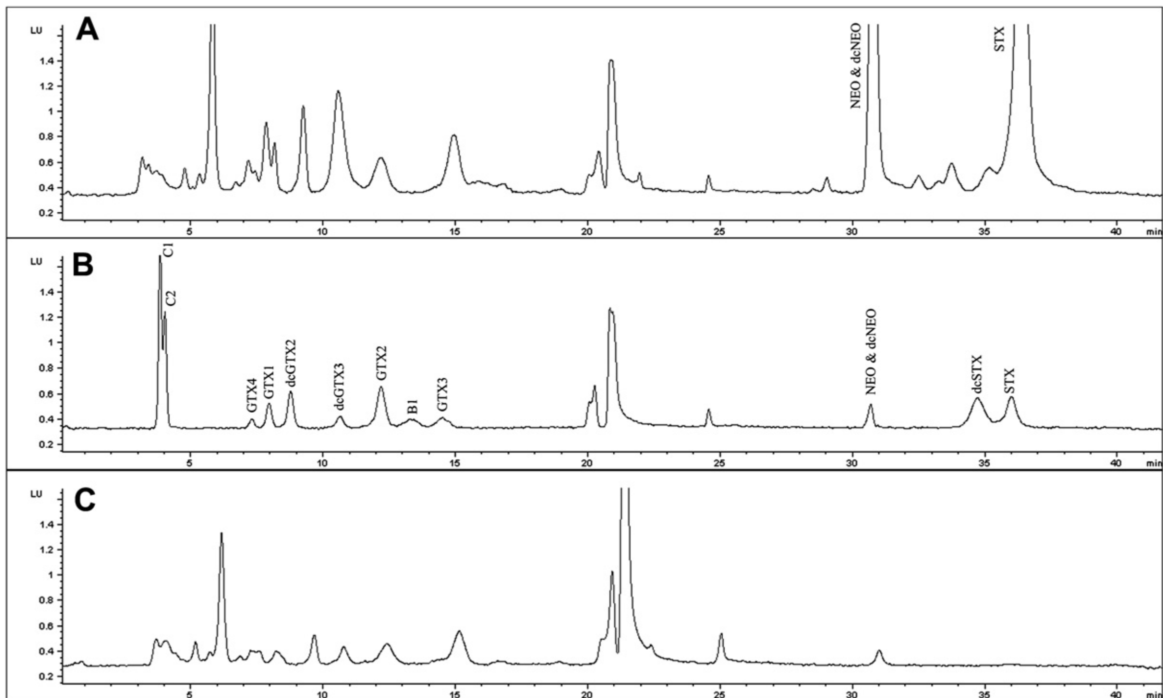

Fig. 3. High performance liquid chromatographic analysis with fluorescence detection (LC-FD) of PSP toxins. (A) Chromatogram of an extract of *C. raciborskii* T3 after post-column derivatization; (B) chromatogram of PSP toxin standards (C1/2, GTX1/4, dcGTX2/3, GTX2/3, B1, NEO, dcNEO, dcSTX, STX) after post-column derivatization; (C) chromatogram of an extract of *C. raciborskii* T3 without post-column derivatization.

We confirmed the T3 toxin profile by liquid chromatography with tandem mass spectrometry (LC–MS/MS) (Fig. 4A), as consisting of NEO, dcNEO and STX in decreasing order of relative abundance (Fig. 4B). The LC–MS/MS results were essentially consistent with the LC-FD analysis, but also provided mass spectral resolution of the co-eluting peaks of NEO and dcNEO. There was a faint peak in the T3 LC-MS/MS ion-trace for dcSTX (m/z 257 > 156, data not shown), however, this was below the limit of detection (signal to noise ratio <3). This means that also by LC-MS/MS we cannot unambiguously confirm the presence of dcSTX in T3, but if present, its abundance is <0.3 fmol mg^{-1} fresh weight (FW) of cyanobacteria, or <0.5% of the total PSP toxin content. Whereas Kellmann et al. (2008) employed LC–MS to identify key intermediates in the STX biosynthetic pathway, they only mention that the toxin profile of T3 comprises STX, NEO, dcSTX and B1, but did not provide chromatographic support for this profile. We found no evidence for the presence of B1 in this strain and as discussed here dcSTX is also questionable but cannot be ruled out.

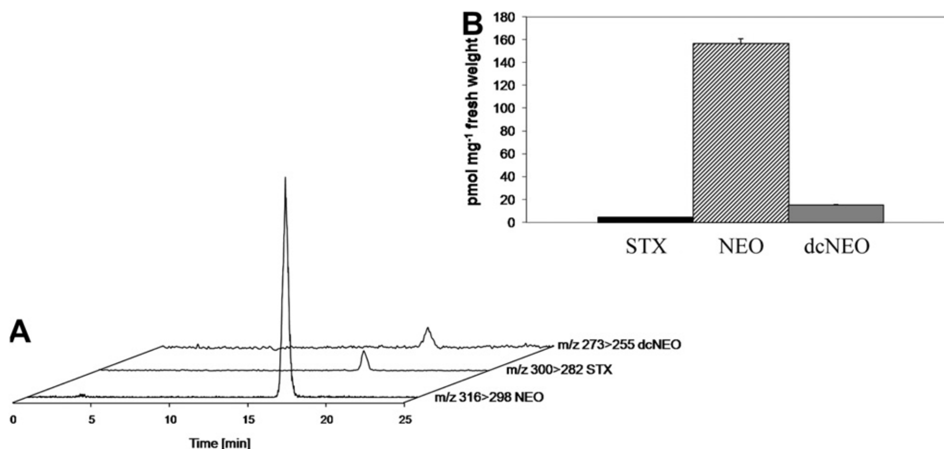

Fig. 4. LC–MS/MS analysis of PSP toxin content in *C. raciborskii* T3. (A) LC–MS/MS spectrum of PSP toxin components of a cellular extract. (B) Toxin content (pmol mg^{-1} of fresh weight).

According to our previous LC-MS/MS analysis, with respect to T3, *R. brookii* D9 produces a more complex toxin spectrum, characterized by the presence of STX and O-sulfated toxins GTX2/3, in addition to decarbamoyl analogues, dcSTX and dcGTX2/3 (Soto-Liebe et al., 2008).

Of course the validity of interpreting analysis of PSP toxin profiles in cyanobacteria is predicated upon the assumption that toxin composition is genetically determined and hence rather refractory to major changes over time in culture or in response to growth conditions. Empirical evidence suggests that whereas the cell toxin quota can markedly vary over the growth cycle in culture and is affected by external conditions, the toxin profile remains stable. For example, we have maintained *R. brookii* D9 for over five years under different growth regimes of temperature, nutrients and salinity, and in the case of *C. raciborskii* T3, for the past two years in either BG11 or MLA 1× culture media. None of these conditions led to significant alteration of the toxin profile of either strain, only to the total molar toxin concentration per cell or per until biomass.

3.3. Comparative analysis of the sxt gene cluster in C. raciborskii T3 with other cyanobacteria

Comparison of the gene cluster for biosynthesis of PSP toxins in *C. raciborskii* T3 with the *sxt* gene cluster of other cyanobacteria provides information on gene functions and also reflects the observed differences in toxin profile. For example, the published *sxt* cluster of *C. raciborskii* T3 comprises 35 Kb and 31 open reading frames (ORFs), whereas the smaller gene cluster of D9 covers only 25.7 Kb, and encodes for 25 ORFs (Fig. 5). Only 20 ORFs are shared between these clusters, but 19 ORFs share 100% similarity. Four genes in *C. raciborskii* T3: *sxtX*, *sxtN*, *sxtW* and *sxtV* are absent from the *sxt* gene cluster of *R. brookii* D9. The product of *sxtX* in T3 is putatively involved in the synthesis of NEO (Kellmann et al., 2008), and consistent with the lack of this gene in D9, this latter strain does not produce NEO. The *sxtV* and *sxtW* genes were described as part of a putative electron transport system to reposition electrons for the correct functioning of oxygenases. The *sxtN* gene was proposed to encode for a sulfotransferase that would produce the sulfated analogues of PSP toxins (Kellmann

Fig. 5. Comparative analysis of *sxt* gene clusters in five species of filamentous cyanobacteria. The ORFs forming the *sxt* gene cluster are indicated in white; those ORFs with unknown function are shown in black; the ORFs symbols filled with vertical straight lines code for transposases and those filled with slanted lines are pseudogenes. *sxtSUL* and *sxtDIOX* are presented with grid lines, the *sxtN* gene is depicted in dark grey and the truncated *sxt*/ACT gene is depicted in light grey. ORFs flanking the clusters, or without functions are shown in light grey bounded by dashed lines.

et al., 2008). Finally, in contrast to what was described for T3, genes which code for regulatory proteins located at one end of *sxt* gene cluster (*sxtY*, *sxtZ* and *ompR*), are not present in this position in the D9 *sxt* gene cluster and are located conserving the same organization in other place of D9 genome. The D9 cluster is flanked at the 3′ end by ORFs with translation products similar to the bifunctional protein GlmU, allophycocyanin β-subunit (ApcF), and glutamine synthetase (GS). Expression of *apcF* and *glnA* genes is regulated by nitrogen (Johnson et al., 1988; Herrero et al., 2001), therefore STX production may also be regulated by nitrogen availability in D9.

Since the description of the *sxt* gene cluster in *C. raciborskii* T3, corresponding clusters have also been described in three different genera of filamentous cyanobacteria. These clusters differ in size and gene content (Fig. 5). *A. circinalis* AWQC131C encodes a cluster of 30.9 Kb corresponding to 28 *sxt*-like ORFs, three transposase sequences, three disrupted and repeated fragments of *sxtN* and two disrupted fragments corresponding to *sxtW* and *sxtV* (Mihali et al., 2009). *Aphanizomenon* sp. NH-5 encodes a 29 Kb cluster comprising 24 *sxt*-like ORFs, where the most remarkable difference is the absence of *sxtO*, putatively involved in PAPS production, a fundamental precursor for the sulfation or sulfonation of STX to O-sulfated or N-sulfonated analogues, respectively (Mihali et al., 2009). Finally, *L. wollei* encodes a 35.6 Kb cluster formed by 26 *sxt*-like ORFs, including within these genes three copies of *sxtM* (MATE family efflux pump) and two copies of *sxtN* (Neilan and Mihali, submitted to the NCBI-database; accession number: EU603711).

3.4. Sulfotransferase-like genes and their role in PSP toxin synthesis

Although details of the complete synthesis of STX remain to be determined, recent studies showed 11,12,12-trideshydroxy-decarbamoyl STX (E′) as the last detectable intermediary to STX (Kellmann et al., 2008). Whereas our data essentially confirm the STX pathway in cyanobacteria as previously described by Kellmann et al. (2008), we propose an extension of the pathway from E′ to yield GTX2/3. Only three steps involving double hydroxylation of the C12, and the addition of the carbamoyl group to 11,12,12-trideshydroxy-decarbamoyl STX, would render STX (Fig. 6A, B).

Inferences based on the structure of sulfated and sulfonated PSP toxins in cyanobacteria indicate that two tailoring reactions, carried on by two different enzymes, are needed to form the different sulfated or sulfonated analogues. With STX as precursor, the first reaction would require an O-sulfotransferase for sulfation of the hydroxyl group at the C-11 position of STX, thereby rendering GTX2/3. The second reaction would involve an N-sulfotransferase that incorporates a sulfonyl group at the N-1 position of the STX carbamoyl group, yielding B1 (GTX5). The joint action of O- and N-sulfotransferases would produce C1/2 toxins (Fig. 6D, E). In the *sxt* cluster, two ORFs have been related with sulfotransferase activity, *sxtN* (Kellmann et al., 2008) and *sxtSUL*, from *L. wollei* (submitted by Neilan and Mihali, NCBI-database); neither of them, however, has been experimentally linked to the specific sulfotransferase functions.

The D9 *sxt* gene cluster contains two ORFs (CRD_02149 and CRD_02148) that are absent from the T3 cluster. Our bioinformatic analysis of the gene product of CRD_02149 shows clear similarities to a sulfotransferase, and has an 89% amino acid identity with SxtSUL of *L. wollei*, whereas the gene product of CRD_02148 shows 85% amino acid identity with SxtDIOX of *L. wollei*, corresponding with a hydroxylase. Both SxtSUL and SxtDIOX likely modify STX to form the C11-sulfated derivatives GTX2/3 (Fig. 6C, D).

Despite the lack of experimental or bioinformatic support, Mihali et al. (2009) proposed a dual function for SxtN in *A. circinalis* AWQC131C, a producer of STX, GTX2/3 and C1/2. The O-sulfotransferase function was attributed to the synthesis of GTX2/3, whereas the N-sulfotransferase function would yield C1/2.

In the *sxt* gene cluster of T3, *sxtN* is the only ORF described with a putative sulfotransferase function, assigned by a profile library search from which the two best hits in the database were with proteins with sulfotransferase functions and the next best hit was with a putative branched-chain amino acid aminotransferase (Kellmann et al., 2008). The function assigned to SxtN was supported by structural relatedness to an estrogen sulfotranferase 1AQU, and by similarity of the conserved N-terminal sequence to a PAPS binding domain (Kakuta et al., 1997). Our re-analysis of the sequence, however, found that there are several features in the 1AQU sequence related to PAPS binding that are not conserved in the SxtN sequence and may not allow SxtN to bind the sulfate donor (PAPS) (Fig. S2). Moreover, our analysis of SxtN showed no significant hit with any sulfotransferase after PSI-blastp. This is consistent with the absence of sulfated and sulfonated toxins in the T3 toxin profile (STX, NEO and dcNEO) as we confirmed by LC-MS/MS (Fig. 4). Alternative explanations would be that SxtN is not functional as a sulfotransferase in T3 or that the proper precursor for the enzyme function is not available. We argue that the classification of the *sxtN* gene was forced to explain the toxin profile of T3.

The confirmed PSP toxin profile of *R. brookii* D9 includes STX and dcSTX, as well as the sulfated analogues GTX2/3 and dcGTX2/3. We postulate that differences in the sequence of *sxtN* and *sxtSUL* can explain the synthesis of GTX2/3 in D9 and the lack of function of the *sxtN* gene in T3, which is not acting as a sulfotransferase in T3, as proposed by Kellmann et al. (2008).

With exception of *L. wollei*, all cyanobacterial strains which contain *sxtX* are able to synthesize the carbamoylated NEO analogue (Table 2). In the case of *L. wollei* the absence is probably due to loss of function of *sxtI* preventing R4-carbamoylation and subsequent hydroxylation at the N-1 position. Furthermore, the two NEO-producing cyanobacteria are incapable of synthesizing the O- or N-sulfated analogues, whereas *L. wollei* can produce O-sulfated dcGTX2/3. This correlates with the presence of *sxtSUL* in the *sxt* gene cluster of this cyanobacterium.

The presence of the *sxtN* gene may correlate with synthesis of N-sulfonated analogues, but only *A. circinalis* AWQC131C that contains *sxtN* is able to produce the N-

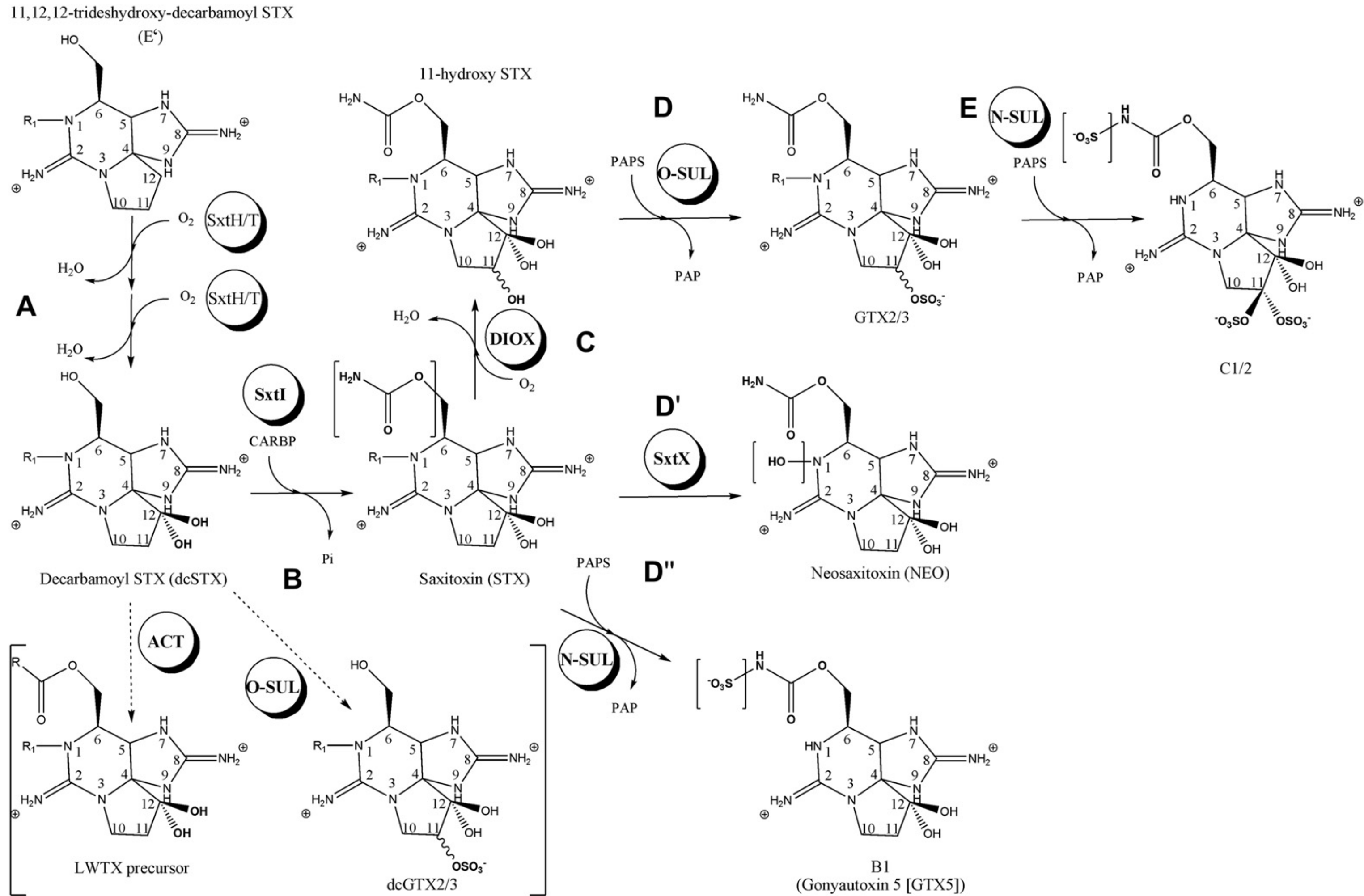

Fig. 6. Biosynthetic pathway of PSP toxin, including new proposed pathway extension from E′ to GTX2/3. (A, B) Synthesis of STX from E′ (Kellmann et al., 2008). (C) Dioxygenase activity of SxtDIOX for the hydroxylation of C-11 to form 11-hydroxy STX. (D) Specific O-sulfotransferase activity of SxtSUL to produce GTX2/3 analogues. STX as precursor for the synthesis of other PSP toxin analogues; the specific enzymes (gene products) modifying STX are shown in circles. (D′) Hydroxylation of N1 by SxtX (cephalosporin hydroxylase) to produce NEO. (D″) N-sulfation at carbamoyl group nitrogen to form B1 (GTX5). (E) N-sulfation to produce C1/2 analogues from GTX2/3 precursors. In square brackets are shown the activity of *sxtACT* and *sxtSUL* to form the LWTX precursor and dcGTX2/3, respectively in *L. wollei*.

Table 2
PSP toxin profiles and *sxt* gene content in cyanobacterial species.

Strain	PSP toxin profile (molar %)										*sxt* genes			Geographical origin	Ref.
	STX	dcSTX	GTX2/3	dcGTX2/3	NEO	dcNEO	C1/2	B1 (GTX5)	B2 (GTX6)	LW1-6	*sxtX*	*sxtN*	*sxtSUL*		
R. brookii D9	4.5	2.9	**94.6**	0.7							[d]	[d]	[e]	Brazil	This work
A. circinalis ACMB13	0.8	0.1	6.9				**86.6**	5.7			[d]	[e]	[e]	Australia	This work
A. circinalis AWQC131C	2.0	11	16	7			64				[d]	[e]	[b]	Australia	Llewellyn et al. (2001)
C. raciborskii T3	2.8				**87**	10.1					[e]	[e]	[d]	Brazil	This work
Aphanizomenon sp. NH-5	[a]				[a]						[e]	[e]	[d]	USA	Mahmood and Carmichael (1986)
L. wollei		1.4		5.4						**(1) 24.5** (2) 13.9 **(3) 32.7** (4) 0.7 (5) 17.8 (6) 3.4	[e]	[e]	[e]	USA	Onodera et al. (1997) Carmichael (1997)
Aph. gracile AB2008/31	10.5	6.3			2.9			**80.2**			[c]	[c]	[c]	Germany	Ballot et al. (2010)
Aph. gracile AB2008/65	12.2	1.6			**69.4**			16.9			[c]	[c]	[c]	Germany	Ballot et al. (2010)
Aph. issatschenkoii LMEYA 31	5.4	6.1			23			**64.5**	1.1		[c]	[c]	[c]	Portugal	Pereira et al. (2000)
A. circinalis AWQC323B	8.0							**93**			[c]	[c]	[c]	Australia	Llewellyn et al. (2001)

Bold numbers are terminal analogues, typically present in high molar percentage.
[a] The analogue is present but the accurate PSP toxin profile has not been determined by LC/MS.
[b] The presence could be checked.
[c] The sequences are not available.
[d] The gene is not present.
[e] The gene is present.

sulfonated analogues C1/2. However, in order to synthesize C1/2, it is necessary to synthesize GTX2/3 as precursors for the C1/2 N-sulfonation (Fig. 6E); therefore an O-sulfotransferase function is needed. As *A. circinalis* AWQC131C produces GTX2/3, we postulate that the *sxtSUL* gene is located in another region of the genome.

We could not access ACWQ131C for direct comparison, but we sequenced and analyzed the *sxt* gene cluster of the closely related *A. circinalis* ACMB13. This strain was also isolated from Australia and produces a toxin profile (STX, dcSTX, GTX2/3, dcGTX2/3, C1/2 and B1) similar to that of ACWQ131C. Most importantly, the *sxt* clusters of these strains are identical (100%). Moreover, the genome of ACMB13 contains *sxtSUL*, as detected by positive PCR amplification with specific primers against the *sxtSUL* sequence, with 91.6% and 90.1% identity to SxtSUL of *L. wollei* and *R. brookii* D9, respectively.

Analysis of the *sxt* gene clusters and toxin profile of *A. circinalis* AWQC131C, *Aphanizomenon* sp. NH-5, *L. wollei* and the other four strains (whose sequences have been not described) allowed us to conclude that the toxin profile reflects an equilibrium between STX and production of terminal analogues, typically present in high molar percentage (bold numbers in Table 2). Terminal analogues could be sulfated and/or sulfonated (GTX2/3, B1, C1/2, LW1-3) or N-1-hydroxylated (NEO) derivatives (Table 2, Fig. 6), indicating very efficient and specific enzymatic activity to utilize the available substrate (STX). Conversion of STX to NEO is completely favored in T3, even though the presence of SxtN could also allow conversion to B1, as in *Aphanizomenon issatschenkoi* LMEYA 31 (Table 2). This is more clearly illustrated by comparing the profiles of *Aphanizomenon gracile* AB2008/31 and AB2008/65, conspecific isolates from lakes in Germany, but indicating that in the former strain B1 synthesis is strongly favored versus NEO synthesis in the latter (Table 2).

In *R. brookii* D9, which does not possesses either SxtX or SxtN, conversion of STX to GTX2/3 is completely favored, supporting the proposed function of SxtSUL (CRD_02149) as a specific O-sulfotransferase. The case of *A. circinalis* AWQC131C is quite remarkable, because this cyanobacterium produces C1/2 analogues in higher proportion than all other analogues (as does strain ACMB13) (Table 2), but only encodes for SxtN inside the *sxt* gene cluster. As we propose, an O-sulfotransferase function is needed in the first place to form GTX2/3 as the precursors of C1/2. The presence of an *sxtSUL* sequence (91.6% identity to SxtSUL of *L. wollei*) in the genome of ACMB13, however, suggests the presence of an O-sulfotransferase function encoded outside of the *sxt* gene cluster in *A. circinalis*. Our hypothesis of the requirement for a specific precursor for a specific enzymatic function to produce the sulfated and sulfonated analogues from STX has no experimental support, but is highly correlated with the toxin profile of *L. wollei* and consistent with its *sxt* gene cluster composition.

L. wollei provides another remarkable example of the plasticity of the *sxt* gene cluster in cyanobacteria. The *L. wollei* cluster encodes the *sxtX* gene, two copies of *sxtN* gene and also the *sxtSUL* gene, but only produces dcSTX, dcGTX2/3 and LWTX 1-6. The latter toxins are result of *sxtI* gene disruption by *sxtACT* (predicted acyltransferases) that would transfer an acyl group to R4 and render the base of the LWTX structure (Fig. 6, square brackets). *L. wollei* is therefore unable to carbamoylate dcSTX (Fig. 6B), such that the proper precursor for SxtX and SxtN is not present and only SxtSUL can act to O-sulfate dcSTX to dcGTX2/3 (Fig. 6, square brackets).

4. Conclusions

Analysis of the *sxt* gene cluster sequences and gene expression levels, coupled with confirmation of toxin profiles, provides a valuable approach to understanding the genetic and enzymatic basis of differences in toxin profiles among cyanobacteria. We propose that STX is the necessary substrate for GTX2/3, B1 and NEO biosynthesis, corresponding to O-sulfotransferase (*sxtSUL*), N-sulfotransferase (*sxtN*) and N1-hydroxylation (*sxtX*) activities, respectively. Furthermore, GTX2/3 are precursors for C1/2 biosynthesis by N-sulfonation of the carbamoyl group. The absence of sulfated and sulfonated PSP toxin in *C. raciborskii* T3 and *Aphanizomenon sp.* NH-5, despite the presence of *sxtN* in the *sxt* cluster of both strains, could be explained by the absence of substrate (no GTX2/3 production), and a complete synthesis favored to produce NEO, leaving no free substrate to produce B1.

The accurate identification of the toxin profiles in this group of cyanobacteria was a primary task, in order to identify the function of the genes present in the cluster. It has not yet been possible to perform mutagenesis experiments or heterologous expression of this kind of cluster. A strong bioinformatic basis could help to assign correct hypothetical functions to given genes for the synthesis of STX and analogues, until appropriate experimental protocols are developed to test these functions. Application of this "gene profile analysis" to the prediction of toxin profiles in new toxic isolates could also assist in the assessment of toxicity risk due to cyanobacterial blooms in natural water systems.

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Conflict of interest statement

None declared.

Appendix. Supplementary material

Supplementary data associated with this article can be found in the on-line version, at doi:10.1016/j.toxicon.2010.07.022.

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