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PSP toxin release from the cyanobacterium *Raphidiopsis brookii* D9 (Nostocales) can be induced by sodium and potassium ions

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ABSTRACT

Paralytic shellfish poisoning (PSP) toxins are a group of naturally occurring neurotoxic alkaloids produced among several genera of primarily freshwater cyanobacteria and marine dinoflagellates. Although saxitoxin (STX) and analogs are all potent Na^+ channel blockers in vertebrate cells, the functional role of these compounds for the toxigenic microorganisms is unknown. Based upon the known importance of monovalent cations (such as sodium) in the maintenance of cellular homeostasis and ion channel function, we examined the effect of high extracellular concentrations of these ions on growth, cellular integrity, toxin production and release to the external medium in the filamentous freshwater cyanobacterium, *Raphidiopsis brookii* D9; a gonyautoxins (GTX2/3) and STX producing toxigenic strain. We observed a toxin export in response to high (17 mM) NaCl and KCl concentrations in the growth medium that was not primarily related to osmotic stress effects, compared to the osmolyte mannitol. Addition of exogenous PSP toxins with the same compositional profile as the one produced by *R. brookii* D9 was able to partially mitigate this effect of high Na^+ (17 mM). The PSP toxin biosynthetic gene cluster (*sxt*) in D9 has two genes (*sxtF* and *sxtM*) that encode for a MATE (multidrug and toxic compound extrusion) transporter. This protein family, represented by NorM in the bacterium *Vibrio parahaemolyticus*, confers resistance to multiple cationic toxic agents through Na^+/drug antiporters. Conserved domains for Na^+ and drug recognition have been described in NorM. For the D9 *sxt* cluster, the Na^+ recognition domain is conserved in both SxtF and SxtM, but the drug recognition domain differs between them. These results suggest that PSP toxins are exported directly in response to the presence of monovalent cations (Na^+, K^+) at least at elevated concentrations. Thus, the presence of both genes in the *sxt* cluster from strain D9 can be explained as a selective recognition mechanism by the SxtF/M transporters for GTX2/3 and STX. We propose that these toxins in cyanobacteria could act extracellularly as a protective mechanism to ensure homeostasis against extreme salt variation in the environment.

Abbreviations: HAB, Harmful algal bloom; PSP, Paralytic shellfish poisoning; GTX, Gonyautoxin; STX, Saxitoxin; dc, Decarbamoyl; HPLC, High performance liquid chromatography; LC/MS/MS, Liquid Chromatography Coupled with Tandem Mass Spectrometry.

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1. Introduction

Paralytic shellfish poisoning (PSP) toxins comprise a group of about two dozen naturally occurring tetrahydropurine neurotoxins, of which saxitoxin (STX) and neosaxitoxin (NEO) are the most potent analogs (Llewellyn, 2006). The PSP toxins can be either non-sulfated, e.g. STX and neosaxitoxin (NEO), or sulfated, as in the case of gonyautoxins (GTXs) and C-toxins. The toxins can be further classified structurally as carbamoyl, decarbamoyl (dc) or N-sulfo-carbamoyl toxins, in decreasing order of potency in mammalian systems. All of these toxins are highly selective blockers of sodium (Na^+) channels in excitable cells, and belong to a larger class of Na^+ channel blocking toxins that affect nerve impulse generation in higher animals (Catterall, 1980; Cestele and Catterall, 2000), leading in extreme cases to paralysis and even death.

The PSP toxins have been associated with harmful algal blooms (HABs) of both cyanobacteria, primarily in freshwater and brackish waters, and marine dinoflagellates (Hallegraeff et al., 1988), representing a serious health and ecological concern worldwide. In freshwater and brackish water ecosystems, water supplies for humans and livestock may be contaminated by the presence of PSP toxins produced by cyanobacterial blooms (Carmichael et al., 1997).

For more than a decade certain filamentous cyanobacteria belonging to the genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya* and *Planktothrix* have been known to produce PSP toxins (Mahmood and Carmichael, 1986; Humpage et al., 1994; Carmichael et al., 1997; Lagos et al., 1999; Pomati et al., 2000). Recently, the genus *Raphidiopsis* has also been described as a PSP toxin producer (Plominsky et al., 2009; Yunes et al., 2009).

The functional role of PSP toxins in the ecology and evolution of toxigenic cyanobacteria and dinoflagellates remains unknown, although subject to continual speculation regarding chemical defense and/or ion transport and regulatory interactions (Cembella, 2003). Furthermore, the fact that toxigenic cyanobacteria are predominantly in freshwater, whereas PSP toxin-producing dinoflagellates are exclusively marine, opens the possibility for differences in functional roles in relation to ion regulation. Initially, PSP toxins in cyanobacteria were considered to be strictly endotoxins, released into the environment only after cell lysis (Negri et al., 1997). This tends to imply an exclusive intracellular role. However, later evidence that the increase in PSP toxins in the extracellular medium is concomitant with the decrease in the intracellular compartment, even in actively growing cell cultures of a PSP-toxin producing cyanobacterium (Castro et al., 2004), suggests an active export or leakage of these toxins into the extracellular medium, independent of cell lysis.

The biosynthetic genes for STX and sulfated analogs have now been elucidated in cyanobacteria, originally in *Cylindrospermopsis raciborskii* T3 (Kellmann et al., 2008). Later, the assessment of sulfotransferases function was also included (Soto-Liebe et al., 2010). The entire genome of the close phylogenetic relative, *Raphidiopsis brookii* D9, a PSP toxin-producing species and formerly considered congeneric with *C. raciborskii*, was sequenced (Stucken et al., 2010). The requisite biosynthetic genes for these toxins were found within the *R. brookii* D9 genome, representing the minimal genome thus far described for a multicellular filamentous cyanobacterium.

The gene cluster for PSP toxin synthesis in *R. brookii* D9 (Stucken et al., 2010) shares a high number of genes with *C. raciborskii* T3 (Kellmann et al., 2008). In both strains, the gene cluster encodes for a multifunctional enzyme complex related to synthesis of STX and analogs. Both gene clusters include two genes (*sxtF* and *sxtM*) that encode for multidrug efflux pumps and belong to the MATE (multidrug and toxic compound extrusion) family of transporters. Among the MATE transporters the best characterized are the NorM proteins (McAleese et al., 2005). These proteins export norfloxacin and other cationic toxic compounds by means of an electrochemical gradient of Na^+ ions, acting as a Na^+/drug antiporter. The conserved regions $G^{184}KFGXP^{189}$ and $L^{381}RGYKD^{386}$ present in NorM of *Vibrio parahaemolyticus* and other bacteria have been characterized as recognition motifs for Na^+ and drugs, respectively (Singh et al., 2006).

The first studies performed on *C. raciborskii* T3 (Lagos et al., 1999) have shown that this strain produces STX and the N-sulfocarbamoyl derivatives C1/2. Pomati et al. (2003a,b; 2004) later proposed that these toxins can regulate the total cellular content of Na^+ and K^+ ions in this cyanobacterial strain. According to these authors, high NaCl concentration (10 mM) inhibited cyanobacterial growth and also promoted STX accumulation in a dose-dependent manner (Pomati et al., 2004). However, our further studies to reassess the T3 strain toxin profile by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) demonstrated that in fact T3 only produces NEO, as the main analog, with STX and dcNEO as minor components (Soto-Liebe et al., 2010). In summary, the role of Na^+ and/or K^+ ions and effect on PSP toxin production and either export or intracellular accumulation of toxins remains unresolved.

In this study, we explored the effect of monovalent cations (Na^+, K^+, Li^+) on growth and PSP toxin production in *R. brookii* D9. In particular, our focus was to evaluate whether or not high ion concentration can induce the release of PSP toxins to the extracellular medium in the toxigenic D9 strain. Furthermore, we attempted to establish whether this response is dependent on ionic or osmotic stress and if it is affected by the presence of exogenous PSP toxins. Finally, we considered the implication of the presence of the putative protein transporter (SxtF/M) and the role and effects of the toxins upon the cyanobacterial cells.

2. Materials and methods

2.1. Isolation and culture conditions

R. brookii D9 was obtained by sub-cloning from the mixed culture SPC338 (graciously provided by Maria Teresa de Paiva, Sao Paulo, Brazil), originally isolated from a branch of the Billings water reservoir Taquacetuba, Sao Paulo, Brazil. The growth kinetics for strain D9 were determined in MLA growth medium at pH 8.4 (Castro et al., 2004), with and without supplement of 17 mM NaCl in 6-multiwell plates with a final volume of 8 mL. Cultures

were grown at 23 °C under continuous cool-white fluorescent light at a photon flux density of 45 μmol m^{-2} s^{-1}.

In experiments on the effect of ionic and osmotic stressors on growth of *R. brookii* D9, cultures were incubated in MLA medium (control); MLA + NaCl (17 mM); MLA + KCl (17 mM); MLA + LiCl (17 mM); and MLA + mannitol (Sigma, St. Louis MO) (34 mM), for 24 h in 6-multiwell plates with 8 mL of final volume under the conditions described above. Treatment with the osmolyte mannitol at a concentration (34 mM) designed to generate the same level of osmotic stress as 17 mM NaCl or 17 mM KCl, served to differentiate between osmotic and ionic effects. Growth was determined with a Shimadzu UV mini 1240 CE spectrophotometer (Shimadzu, Kyoto, Japan), by measuring optical density (OD) at 750 nm as a biomass indicator, a wavelength at which cyanobacterial pigments exhibit negligible absorbance (Ernst et al., 2005).

Cyanobacterial cells were incubated in 24-multiwell plates with a total volume of 1.2 mL in each well to reduce the amount of PSP toxin required for incubations and to minimize the risk of toxin inactivation or metabolism in large volume cultures. Controls were prepared in MLA medium and incubated for 24 h under the following conditions: addition of NaCl (17 mM), or NaCl (17 mM) plus a mix of PSP toxins (GTX3 [117 nM]; GTX2 [354 nM]; STX [500 nM], final concentration).

With the aim of establishing whether the Na^+ ion gradient is necessary for PSP toxin release from the cells, cultures were incubated with monensin (Sigma, St. Louis, USA) (50 μM, dissolved in pure ethanol), a drug capable of dissipating a Na^+ ion gradient. Culture conditions for evaluating both ionic and osmotic stressor effects in 6-multiwell plates with 8 mL of final volume were as described above.

2.2. *Chlorophyll* a *quantification*

Chlorophyll *a* concentrations were determined with a Shimadzu UV mini 1240 CE spectrophotometer by the ethanol extraction method (ISO 10260:1992). Cell growth was estimated by chlorophyll *a* measurements, after the initial cell concentrations for incubation cultures were adjusted to a chlorophyll *a* concentration of 0.2–0.4 mg L^{-1}.

2.3. *Microscopic observations*

20 μL samples were diluted in 1 mL of MLA medium and filtered as described for bacterial epifluorescence analysis (Hobbie, 1977). Staining was performed with Acridine orange (0.01%) and cell integrity was visualized with a Nikon Labophot-2 (Nikon, Tokyo, Japan) epifluorescence microscope at 100× magnification. Samples of the cultures exposed to different treatments were taken during the course of the experiments and the cells were observed under both phase-contrast and epifluorescence microscopy.

2.4. *Extraction and analysis of PSP toxins by high performance liquid chromatography with fluorescence detection (HPLC–FD)*

Cyanobacteria were harvested at different time points of the culture cycle by centrifugation at 16,000 × *g* for 15 min to yield cell pellet and supernatant. Stock cultures were harvested for experiments in early exponential growth phase, and diluted 1:2 to obtain the experimental condition. To start the experiments, an OD_{750} of ~0.1 was reached (equivalent to 0.1 mg mL^{-1} cells in dry weight). A culture volume of 6 mL was harvested from each 6-multiwell plate and 1 mL was taken from each of the 24-multiwell plates. Supernatants were filtered through a 0.22 μm pore-size (to eliminate cyanobacterial cells) PVDF Millex™ membrane, 13 mm diameter syringe filters (Millipore, Sao Paulo, Brazil). Afterwards, supernatants were acidified with HCl (0.25 N) to pH 3 to maintain toxin stability. Cell pellets and supernatants were lyophilized, and extracted in either 100 or 300 μL of 0.05 M acetic acid, depending on the volume analyzed. Pellets were then disrupted with a Microson XL ultrasonic cell disruptor (Misonix, Farmingdale, USA) for 60 s.

Analysis of PSP toxins was conducted by high performance liquid chromatography with fluorescence detection (HPLC–FD) after post-column oxidation, according to the protocol detailed by Oshima (1995) with slight changes. The LC–FD analysis was carried out on an LC-2000 Plus Series HPLC (Jasco, Tokyo, Japan) consisting of a quaternary pump (PU-2089) and a fluorescence detector (FP2020 Plus).

Chromatographic conditions: mobile phase for the gonyautoxins (GTXs), 2 mM 1-heptanesulfonic acid in 10 mM ammonium phosphate buffer (pH 7.1); mobile phase for the non-sulfated carbamoyl toxins (STX), 2 mM 1-heptanesulfonic acid in 30 mM ammonium phosphate buffer (pH 7.1) and 5% (v/v) acetonitrile. The flow rate was 1 mL min^{-1}. The separation of analytes was performed on a 100 × 4.6 mm i.d. × 3.5 μm, Kromasil C8 reversed-phase column (Kromasil, Bohus, Sweden). The column eluate was continuously oxidized with 7 mM of periodic acid in 10 mM potassium phosphate buffer (pH 9.0) at a flow rate of 0.7 mL min^{-1} in a reaction coil set at 65 °C. Subsequently, the eluate was continuously acidified with 0.5 M acetic acid at a flow rate of 0.7 mL min^{-1} and the toxins were detected by a dual monochromator fluorescence detector (λ_{ex} 330 nm; λ_{em} 390 nm). Data acquisition and processing was performed with the Chrompass data system (Jasco, Tokyo, Japan). PSP toxin concentrations were determined by external calibration. Toxin concentrations were normalized at an OD of 750 nm.

All extracts were centrifuged at 5000 × *g* for 10 min and filtered with a 0.22 μm pore-size PVDF membrane, 13 mm diameter syringe filters PVDF Millex™ membrane (Millipore, Sao Paulo, Brazil) prior to HPLC separations.

Standard solutions of PSP toxins (STX and GTX2/3) were purchased from the Certified Reference Materials Programme of the Institute for Marine Biosciences (National Research Council, Halifax, NS, Canada).

2.5. *Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)*

To confirm the identity of the PSP toxin components, mass spectral experiments were performed on an API 4000 QTrap, triple quadrupole mass spectrometer equipped with a TurboSpray® interface (Applied Biosystems, Darmstadt, Germany), coupled to a liquid chromatography (LC) Agilent model 1100 (Agilent, Waldbronn, Germany). The Agilent LC

equipment included a solvent reservoir, in-line degasser (G1379A), binary pump (G1311A), refrigerated autosampler (G1329A/G1330B), and temperature-controlled column oven (G1316A). Mass spectrometric analyses for PSP toxins were performed according to the hydrophilic interaction liquid ion-chromatography (HILIC) method (Diener et al., 2007) with slight modifications.

The analytical column (150 × 4.6 mm) was packed with 5 μm ZIC-HILIC stationary phase (SeQuant, Haltern, Germany) and maintained at 35 °C. The flow rate was 0.7 mL min^{-1} and gradient elution was performed with two eluents: eluent A, consisting of 2 mM formic acid and 5 mM ammonium formate in acetonitrile/water (80:20 v/v); and eluent B, consisting of 10 mM formic acid and 10 mM ammonium formate in water. The gradient process was as follows: 20 min column equilibration using an 80% eluent A solution, linear gradient for 5 min reaching 65% A, 10 min for a 60% A concentration, then 20 min using 55% eluent A, subsequent isocratic elution with 55% A for 24 min, and finally returning to an initial 80% concentration of eluent A for 25 min (total run time: 45 min).

Selected reaction monitoring (SRM) experiments were carried out in positive ion mode by selecting the following transitions (precursor ion > fragment ion): 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), 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: 5000 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.

Reagents were purchased from Merck (Darmstadt, Germany). The certified standard calibration solution of PSP toxins was obtained from the Institute of Marine Bioscience (IMB), CRM Program, National Research Council of Canada, Halifax, Nova Scotia, Canada.

2.6. Bioinformatic analysis

For *in silico* protein analysis, we accessed PSORTb v.2.0 (http://www.psort.org/psortb/) to predict cellular location. To predict protein structure, we used the programs PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/), Predict Protein (http://www.predictprotein.org/) and the I-TASSER Servers (Wu et al., 2007; Zhang, 2007, 2008) (http://zhang.bioinformatics.ku.edu/).

GenBank ID: ZP_06305235 (SxtF, *R. brookii* D9), ZP_06305227 (SxtM, *R. brookii* D9).

2.7. Statistical analyses

Statistical analyses were performed with SYSTAT 11.0 (Wilkinson, 2006) and Origin 8.0. Data were analyzed by ANOVA and *post-hoc* analysis of means by Tukey's test. All data were transformed to log10, in order to meet the requirements of homoscedasticity and normality of ANOVA.

3. Results

3.1. PSP toxin profile of R. brookii D9

Chromatograms of the PSP toxin profiles produced by *R. brookii* D9 are shown in Fig. 1B and D, compared to a standard mixture of PSP toxins (Fig. 1A and C). In early stages of culture growth, the D9 strain mainly produces GTX2/3 and STX and low amounts of dcGTX2/3. Ratios of 10:1 and 5:1 (GTX2/3: STX) were observed in the intracellular and extracellular fraction, respectively. Additional analysis

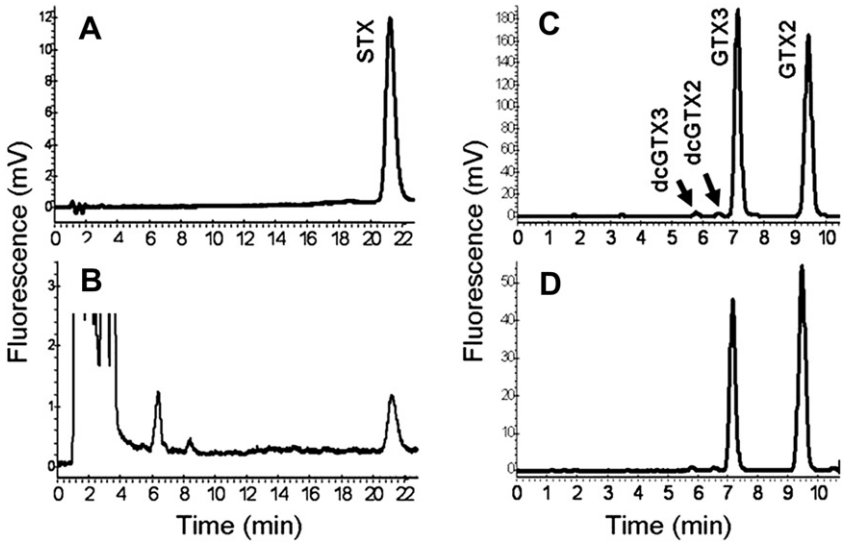

Fig. 1. HPLC chromatograms with fluorescence detection of STX (A) and GTX2/3 (C) in analytical standards, and in *R. brookii* D9 extracts containing STX (B) and GTX2/3 (D).

performed by LC-MS/MS confirmed this general profile (Fig. S1A and B).

3.2. Effect of cationic ions on growth and cell integrity

We observed that *R. brookii* D9 can grow and even increase both its growth rate and the yield evaluated by amount of chlorophyll *a* in the presence of high (17 mM) NaCl in comparison to MLA controls where the Na^+ concentration was only 4 mM (Fig. 2A). Lysis of cells was not observed by microscopic examination during the experimental period in either controls or high Na^+ treatments. Growth analysis of between 0 and 24 h, measured as changes in OD_{750} nm as an estimation of biomass, revealed no significant differences (ANOVA, $p < 0.05$) among the cultures with MLA plus 17 mM NaCl, 17 mM KCl or 34 mM mannitol, with respect to MLA controls. Incubation with 17 mM LiCl had toxic effects on the cells in culture, causing lysis of the filaments within the first 4 h of exposure (data not shown), and therefore showed significant differences (ANOVA, $p < 0.05$) in growth yield with respect to the other treatments (Fig. 2B).

3.3. Effect of cationic ions on intra- and extra-cellular levels of PSP toxins

Measurements of GTX2/3 and STX in the intra- and extra-cellular fractions of cultures grown in MLA medium, showed substantial differences over 24 h depending on the ionic and osmotic regime (i.e. 17 mM NaCl; 17 mM KCl or 34 mM mannitol). The treatment with LiCl was not considered in this analysis due to its cell lytic effect.

The intracellular and extracellular GTX2/3 and STX levels were significantly different (ANOVA, $p < 0.05$) in the presence of 17 mM NaCl, 17 mM KCl and 34 mM of mannitol (Fig. 3). Initially, at 1 h after the initiation of incubation, 17 mM NaCl appears to negatively affect GTX2/3 and STX synthesis because both intra- and extra-cellular levels are diminished with respect to the control (Fig. 3A and B). However at 3 and 6 h we observed an increase in extracellular STX levels and at 3 h in the GTX2/3 levels (Fig. 3B and D). Clearly, the high NaCl concentration had an effect on the initial synthesis inhibition or rate of toxin degradation, followed by active toxin release. At 6 h we did not observe significant differences in the intracellular levels of STX and GTX2/3 between control and 17 mM NaCl treatment. Finally, at 12 and 24 h we again observed a decrease in intracellular levels of STX and GTX2/3 but without significant differences in the extracellular toxin levels (Fig. 3B and C). A high KCl (17 mM) concentration had similar effects compared to those of high NaCl on the intracellular toxin levels (Fig. 3A and C).

The effect of mannitol on the intracellular toxin levels of both STX and GTX2/3 was comparable to that observed with NaCl and KCl over the entire exposure period (Fig. 3A and C). However, extracellular toxin levels with mannitol were, most of the time, significantly lower (ANOVA, $p < 0.05$) than those observed for the other two treatments, and were dramatically lower than the control except at 6 h (Fig. 3B and D). Therefore, it appears that mannitol acts as a true osmolyte for toxin interactions, whereas NaCl and KCl seem to be primarily ionic rather than osmotic stressors.

3.4. Effects of the simultaneous addition of PSP toxins and sodium chloride on intracellular GTX2/3 levels

Exposure of the cells to 17 mM NaCl versus cells exposed to 17 mM NaCl plus PSP toxins showed statistical differences among the treatments. The intracellular GTX2/3 levels of cultures exposed to high NaCl (17 mM) were significantly lower (ANOVA, $F = 5.782$; g.l. $= 2$, $p < 0.009$; *post-hoc* Tukey's test) than those for cultures grown in MLA (control) after 6 h, although the differences were reduced over time and were not significant after 24 h (Fig. 4). Exposure to an exogenous mixture of PSP toxins containing only STX and GTX2/3 (similar to the toxin profile synthesized by strain D9) appears to somewhat mitigate the ionic effect of high (17 mM) NaCl on intracellular GTX2/3 toxin concentrations because no statistical differences were found compared to the control cultures. In other experiments, we assayed by adding 1 μM STX but were not able to compensate the effect of 17 mM NaCl on the intracellular toxin decrease (data not shown).

3.5. Effects of monensin on intra- and extra-cellular levels of PSP toxins

The differential effect of monensin, a disruptor of Na^+ gradients, on intracellular GTX2/3 and STX levels was shown over a time-series experiment. At the concentration

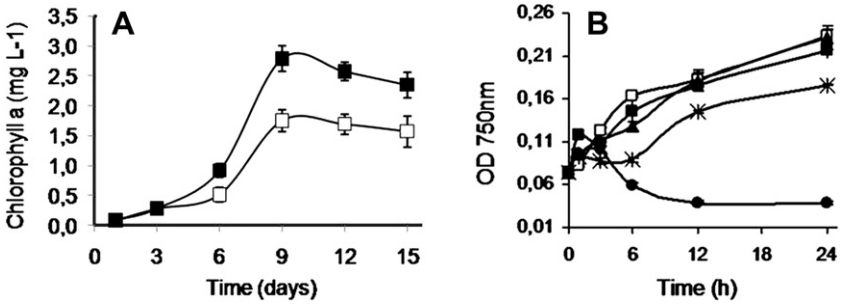

Fig. 2. Effect of ionic and osmotic stressors on *R. brookii* D9 growth. (A) Cultures grown in MLA -□- and MLA + NaCl (17 mM) -■-; (B) Cultures grown in MLA -□-, MLA + NaCl (17 mM) -■-, MLA + KCl (17 mM) -▲-, MLA + LiCl (17 mM) -●- and MLA + mannitol (34 mM) -*-. Data points represent the mean ± S.E of three experimental samples.

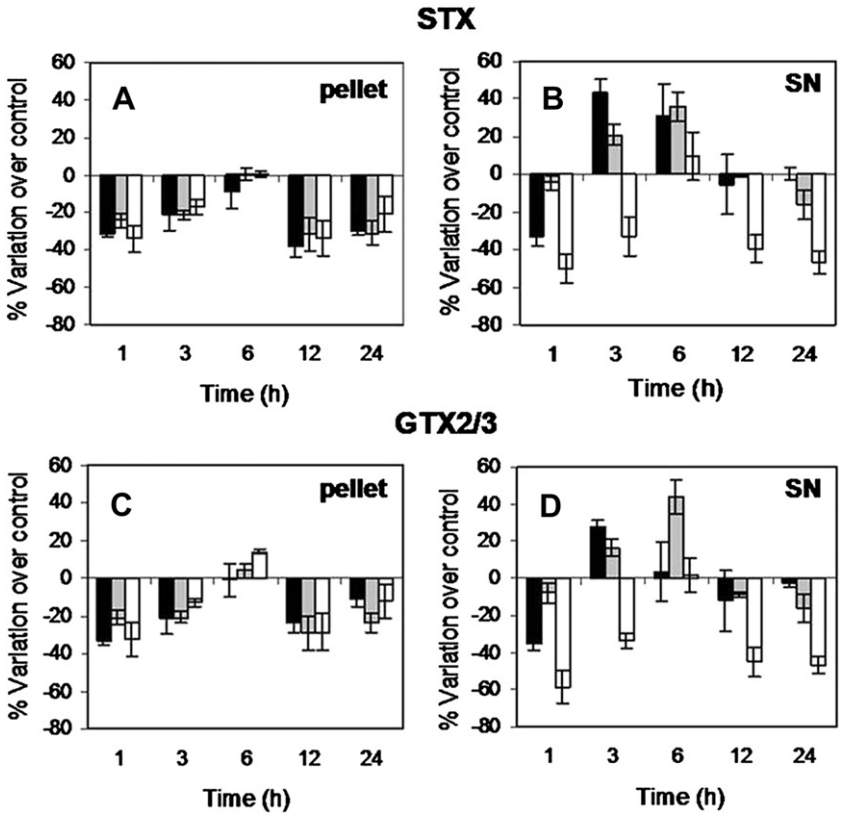

Fig. 3. Effect of ionic and osmotic stressors on PSP toxin levels in *R. brookii* D9. The cultures were grown in MLA (control) or MLA plus NaCl (17 mM) (black bars), KCl (17 mM) (gray bars) or mannitol (34 mM) (white bars). The intracellular (A and C) and extracellular (B and D) levels of STX and GTX2/3 are expressed as variation (%) versus the control. Error bars represent the mean ± S.E. of three experimental samples. SN: supernatant.

tested (50 μM), monensin did affect intracellular levels of STX, causing a marked decrease during the first few hours of incubation (Fig. 5A). In contrast, intracellular levels of GTX2/3 appeared to be relatively unaffected over the first 5 h (Fig. 5B). Over longer periods (at 24 h), a lethal effect of monensin on the cells was observed (data not shown).

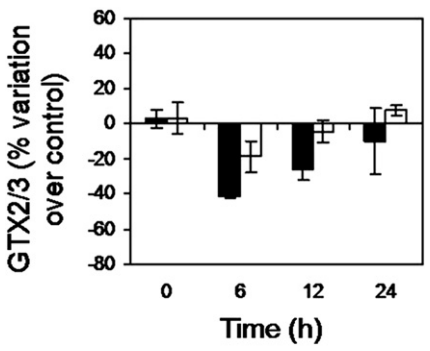

Fig. 4. Effect of exogenous PSP toxins added to the culture media on intracellular GTX2/3 levels. The cultures of *R. brookii* D9 were grown in MLA (control); MLA + 17 mM NaCl (black bars) and MLA + 17 mM NaCl + PSP toxins [117 nM GTX3, 354 nM GTX2, 500 nM STX] (white bars). Data (mean ± S.E.) represent the variation (%) of three experimental samples over the control. Only the effect on intracellular levels of GTX2/3 was measured due to the similar variations observed for STX and GTX2/3 levels (shown in Fig. 3).

3.6. SxtF/M amino acid sequence analysis

Bioinformatic analysis allowed to predict that at least six proteins could be located in the membrane: SxtA (loading of ACP, methylation, ACP Claisen condensation), SxtD (sterol desaturase-like protein), SxtF and SxtM (multidrug efflux proteins), SxtJ (unknown protein) and SxtU (short-chain dehydrogenase/reductase SDR). Within this group, SxtF and SxtM are the only one that can be involved in the secondary metabolites exportation, such as PSP toxins. In SxtF and SxtM, we identified 12 transmembrane segments (TMS) using the Predict Protein Server to analyze the secondary structure (Fig. 6).

Based upon the model bacteria *Vibrio cholerae* (Vc) and *V. parahaemolyticus* (Vp), we also identified the presence of a described conserved motif implicated in sodium and drug recognition in NorM protein (Singh et al., 2006). The motif characterized as the recognition site for Na^+ is located in the periplasmic loop between TMS V and VI and is conserved in SxtF and SxtM. The conserved region implicated in drug recognition in Vc/Vp is located in the cytoplasmic loop between TMS X and XI in *R. brookii* D9, but the

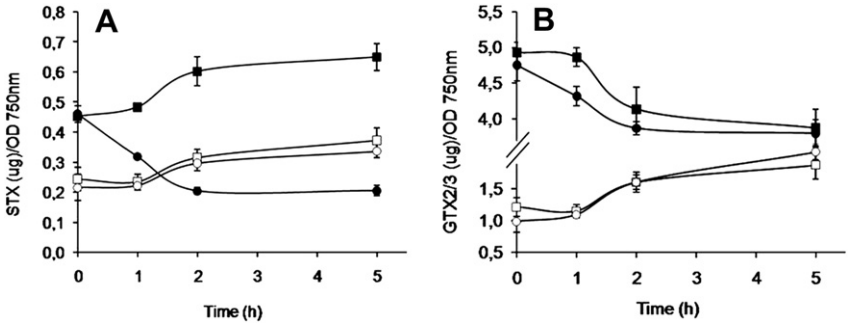

Fig. 5. Effect of monensin on PSP toxin levels in *R. brookii* D9. (A) STX in cultures grown in MLA: intracellular (-■-) and extracellular (-□-) levels; and in MLA + monensin (50 μM): intracellular (-●-) and extracellular (-○-) levels. (B) GTX2/3 in cultures grown in MLA: intracellular (-■-) and extracellular (-□-) levels; and in MLA + monensin (50 μM): intracellular (-●-) and extracellular (-○-) levels. Data points represent the mean ± S.E. of three experimental samples.

amino acid sequences are not completely conserved between both SxtF and SxtM, and also differ when compared to Vc and Vp (Fig. 6).

Finally, we used the I-TASSER server to predict the protein structure of the SxtF protein of *R. brookii* D9 (Fig. S2) (C-score = −2.01), in the range of [−5, 2], where a C-score of higher value means a model with a higher confidence level. The best model was obtained with the crystal structure of the glycerol-3-phosphate transporter (GlpT) from *Escherichia coli*. GlpT is a member of the major facilitator superfamily (MFS), which represents a large and diverse group of secondary transporters that includes uniporters, symporters, and antiporters.

4. Discussion

The ecophysiological function and evolutionary role of PSP toxins produced by cyanobacteria remains an enigma. Why should a freshwater microorganism produce Na^+ channel blocking molecules that are highly toxic for vertebrates? It now appears likely that the focus on the toxic aspects may have obscured the search for other adaptive mechanisms to enhance the survival of toxigenic cyanobacteria. Bioinformatic analysis of the *sxt* gene cluster in all cyanobacterial species containing the cluster could shed light upon this issue.

Among the genes conserved in all the *sxt* clusters, but not directly related with toxin synthesis, there is at least one copy of a gene that encodes multidrug efflux pumps that belong to the MATE (multidrug and toxic compound extrusion) family of transporters (Stucken et al., 2010). MATE proteins comprise the most recently designated family of multidrug transporter proteins, and are widely distributed in all kingdoms of extant organisms. The bacterial MATE-type transporters that have been characterized are exporters of cationic drugs such as norfloxacin and ethidium through an H^+ or Na^+ exchange mechanism (Morita et al., 1998; Chen et al., 2002; Omote et al., 2006; Singh et al., 2006). In higher plants, MATE-type transporters are involved in the detoxification of secondary metabolites, including alkaloids (Gomez et al., 2009). For instance, in the *sxt* cluster of *R. brookii* D9, we previously identified two genes *sxtF* and *sxtM*, which encode for these transporters. In both SxtF and SxtM in *R. brookii* D9, we identified 12 transmembrane segments typically described for MATE-type transporters (Omote et al., 2006), and which also contain the conserved motif implicated in Na^+ recognition. However, the domain involved in recognition of the exported drug is not completely conserved, and differs between SxtF and SxtM in D9 strain (Fig. 6). These facts indicate that these proteins require further analysis.

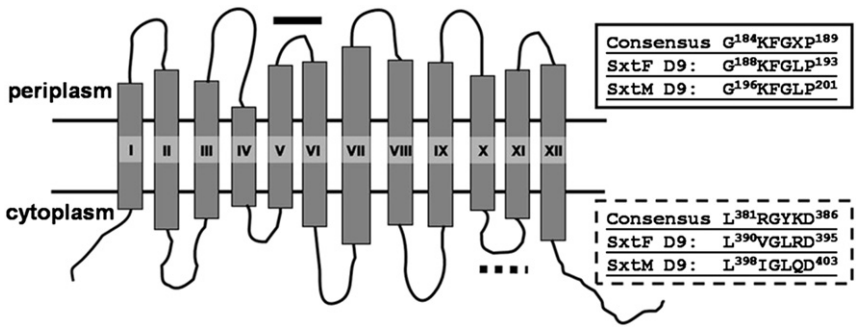

Fig. 6. Schematic representation of the predicted secondary structure of SxtF/M based on *R. brookii* D9 sequences. All 12 transmembrane helixes are shown in gray rectangles. The solid line shows the domain related to cation recognition and the dashed line shows the putative domain implicated in toxin recognition. The consensus region used as reference corresponds to *Vibrio parahaemolyticus* and *V. cholerae* (Singh et al., 2006). The topology was designed based on Predict Protein analysis.

In summary, the known biological activity of PSP toxins and the linkage to genes included in the *sxt* cluster in cyanobacteria justified our deeper analysis of PSP toxin release to the extracellular medium, as dependent on the concentration of monovalent cations (Na^+, K^+, Li^+). Our results indicated that the effects of the ionic compounds NaCl and KCl on toxin release are similar, but very different from those of high concentrations of LiCl and the osmotic stressor mannitol (Fig. 1B). Although high levels of NaCl, KCl and mannitol did not affect growth or cell integrity (even after 7 days exposure), a high concentration of LiCl was toxic for D9 and caused cell lysis, correlated with an increase in extracellular PSP toxin levels. Other authors have observed that Li^+ can inhibit the activity of HalA, a protein closely related to 3′-phosphoadenosine-5′-phosphatase in the cyanobacterium *Arthrospira platensis* (50% inhibitory concentration [IC_{50}] = 3.6 mM) and ultimately causes cell lysis (Zhang et al., 2006).

Although previous studies showed that the freshwater cyanobacterium *C. raciborskii* T3 did not tolerate concentrations >10 mM NaCl (Pomati et al., 2004), we found that in MLA culture medium, the growth tolerance at high NaCl concentrations was similar to that of *R. brookii* D9 (Soto-Liebe, unpublished results). Interestingly, when we analyzed the effect of high NaCl (17 mM) on chlorophyll *a* synthesis, we identified an enhanced synthesis with high Na^+ compared to the control (growth in MLA culture medium) (Fig. 2A). The dependence of photosynthetic activity on Na^+ ion concentration in cyanobacteria has been demonstrated in *Anabaena* sp. PCC 7119 and it is particularly relevant under low CO_2 tensions (Maeso et al., 1987). Furthermore, the Na^+/HCO_3^- co-transporters BicA and SbtA have been described in cyanobacteria (Price et al., 2011), but they have not been identified in *R. brookii* D9.

Exposure to high concentrations of monovalent cations does appear to have a rather important short-term effect on toxin production and sequestration in strain D9, where the addition of either NaCl or KCl to the culture medium at a final concentration of 17 mM produced an initial intracellular decrease in STX and GTX2/3 levels. This effect may be due either to a direct inhibition of toxin synthesis or to an increase in the rate of toxin catabolism or intracellular transformation to other analogs. Although we cannot distinguish among these possibilities, we can reject the hypothesis that this decrease is related to the effect of release into the culture medium, since after 1 h we did not observe an increase in the extracellular toxin levels.

In addition to the direct ionic effects, one of the causes of the decrease in intracellular levels of toxins may be due to the increase in osmotic pressure generated by high NaCl or KCl concentrations, since a similar albeit not identical effect was observed in cultures incubated with the osmotic stressor mannitol. Studies in other freshwater cyanobacteria such as *Anabaena* have shown that salinity could be more detrimental than pure osmotic stress in cyanobacteria, especially in the ones that fix nitrogen (Torrecilla et al., 2001). Recent studies have indicated that osmotic stressors in *Synechocystis* sp. PCC 6803 cause a 50% reduction in the Na^+-stimulated phosphate (Pi) uptake system, Pst1 (Burut-Archanai et al., 2011). Our results showed that the mannitol exerts a negative effect on extracellular toxin levels compared with the control, suggesting that its effect on toxin release could be inhibit the export system (Na^+-stimulated).

In cultures exposed to high NaCl and KCl concentrations after 3 and 6 h the initial inhibition/degradation effect upon PSP toxins was reversed because extracellular toxin levels increased compared to the control culture. Therefore, we conclude that several hours after the initial exposure, toxin export can occur in response to high concentrations of monovalent cations, but is not exclusively related to the osmotic effect.

Additionally, the cyclic variation of the intra- and extracellular toxin levels observed in the NaCl and KCl treatments could imply rhythmicity in the response. Rhythmicity is not unusual in cyanobacteria, but further analysis should be done to address this issue. The observed cell integrity allows us to suggest that the toxin measured in the extracellular medium was due to the salt effect (NaCl and KCl) on release and not to simple cell breakage.

The fact that the addition of exogenous PSP toxins in the presence of high NaCl concentration was able to mitigate the decrease in intracellular toxin levels compared to treatment with high NaCl alone suggests that PSP toxins interfere with the Na^+ ion effect, thereby inhibiting toxin export. PSP toxins are apparently released directly in response to the presence of NaCl and thus could act extracellularly upon strain D9 as a feedback mechanism.

The presence of the ionophore monensin, which causes a loss of the Na^+ gradient, does not affect GTX2/3 levels, but after 1 h exposure, the intracellular levels of STX decreased. We previously proposed that STX is the precursor of GTX2/3 in cyanobacteria (Soto-Liebe et al., 2010), and these results could indicate either that STX synthesis is sensitive to the loss of a Na^+ gradient, or that the presence of high Na^+ concentrations may inhibit the enzymes involved in STX synthesis. Note that by accessing the Predict Protein bioinformatic server, we identified that the SxtA enzyme, responsible for the early stages of STX synthesis (Kellmann et al., 2008), presents a membrane binding domain. Furthermore, via *in vitro* PSP toxin synthesis assays, Kellmann and Neilan (2007) showed that STX synthesis requires the presence of a cofactor present in the membrane fraction of *C. raciborskii* T3. If this "complex" responsible for the synthesis of PSP toxin is anchored to the plasma membrane, the presence of the ionophore should affect PSP toxin synthesis.

It is well established that salt stress induces many biologically adaptive and acclimation processes that assist organisms to survive in environments with high salt concentrations. In cyanobacteria, a sodium ion gradient can play a dominant role in solute transport (bicarbonate uptake coupled to sodium influx) under photoautotrophic conditions and through pH regulation by sodium-proton antiporters (Carrieri et al., 2011). The maintenance of a Na^+ gradient turns out to be highly efficient in freshwater cyanobacteria, when taking into account that in *Anabaena torulosa* the intracellular Na^+ concentration remains between 20 and 30 times lower in the cytoplasm than in the culture medium ($\Delta_{\psi Na^+}$ = +81.4–+90.5 mV), within an extracellular concentration range between 1 and 60 mM Na^+ (Apte and Thomas, 1986).

In the cyanobacterium *Synechocystis* sp. PCC 6803, the addition of NaCl to the medium enhanced the expression of genes related to salt tolerance, such as those that express glucosylglycerol–phosphate synthase (Kanesaki et al., 2002; Marin et al., 2002). In this case, there is an apparent signal transduction pathway related to salt stress. A signal transduction system based on cAMP has been proposed to mediate the response to NaCl in *Anabaena* sp. PCC7120 (Imashimizu et al., 2005). These authors demonstrated that genes related to heterocyst development in *Anabaena* sp. PCC7120 have NaCl-dependent transcription start sites. In the case of *R. brookii* D9, we did not use shock-stress NaCl concentrations, as evidenced by the fact that growth evaluated by chlorophyll *a* content was slightly better at 17 and 25 mM NaCl than in the absence of salt. Future researches are required to determine which is the of gene expression response in strain D9 as consequence of high Na^+ in the medium.

Our hypothesis on the role of Na^+ ions in toxin synthesis, export and gene regulation is illustrated in an integrated summary (Fig. 7). A selective recognition mechanism for the analogs GTX2/3 and STX by both NorM-like transporters could explain why the domains maintain their differences. We have putatively located the SxtF/M protein, which likely resides in the inner membrane according to the NorM protein description. Such proteins could use the K^+ and Na^+ gradients to export STX and GTX2/3 (Fig. 7A). Na^+/H^+ antiporters are part of a mechanism with a defined role in the maintenance of the Na^+ gradient between the intracellular and extracellular compartments. The presence of monensin affected STX synthesis starting from the first hour (Fig. 5A), and the effect could be caused by the disruption of the Na^+ gradient and/or a direct effect on enzyme function, primarily upon proteins involved in PSP toxin synthesis, which are predicted to be associated with membranes (Fig. 7B and E). Finally, we propose that an increase in Na^+ and K^+ levels in the culture media causes active release of STX and GTX2/3 (Fig. 7C), but the presence of exogenous STX and GTX2/3 mitigates that effect (Fig. 7D).

Further analysis is required to confirm the relevance of the presence of one or two NorM proteins in the *sxt* cluster, and to experimentally evaluate the detailed role of monovalent cations (Na^+, K^+) in toxin export. It is also important to establish whether or not the enzymes involved in the PSP toxin synthesis are organized as a protein complex that is anchored to the membrane or if they exist as independent units.

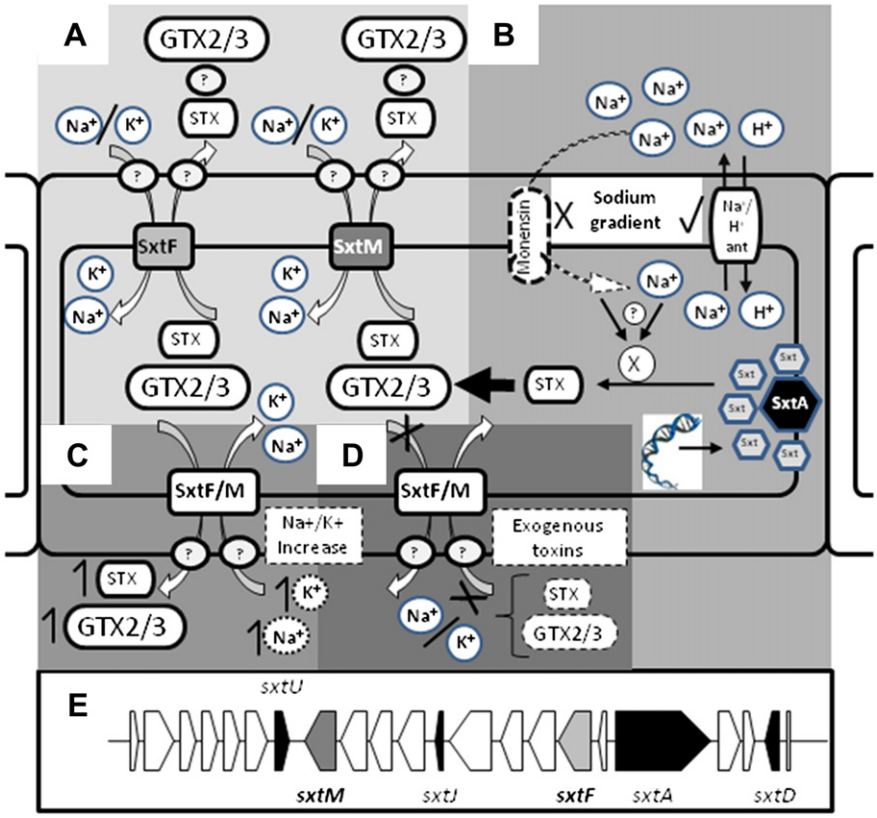

Fig. 7. Diagram representing the main results. (A) Putative location of the SxtF/M transporters in the cytoplasmic membrane in *R. brookii* D9 and the probable function of Na^+/K^+ in toxin export. (B) The Na^+/H^+ antiporter maintains the sodium gradient in cyanobacteria; the drug monensin (exogenously added) disrupts this gradient. We propose that monensin and/or an increase in intracellular Na^+ levels, negatively affects STX synthesis and probably part of the "sxt-enzymes" anchored to the membrane. The increase in Na^+/K^+ levels induces toxin expulsion (C) and the presence of exogenous toxins (STX and GTX2/3) revert this effect (D). Bioinformatic analysis shows that other four proteins encoded by *sxt* genes could be located or anchored to the membrane. These genes are marked in black in the *sxt* gene cluster (E).

5. Conclusions

In summary, our results indicate that *R. brookii* D9 is capable of releasing PSP toxins into the extracellular medium in response to the presence of high, but not deleterious for growth, Na^+ and K^+ ion concentrations. These results correlate with the presence of conserved motifs that are implicated in Na^+ ion recognition of NorM proteins. If PSP toxins can be exported as a protective mechanism against salt variation in the environment, this trend could enhance the fitness and adaptability of cells and explain the invasive capacity of PSP-toxin producing cyanobacteria within a broad range of aquatic ecosystems.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2012.09.001.

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