Toxicon 60 (2012) 1324–1334

Contents lists available at SciVerse ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

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

Katia Soto-Liebe [a], Marco A. Méndez [b,1], Loreto Fuenzalida [c,2], Bernd Krock [d], Allan Cembella [d], Mónica Vásquez [a,*]

[a] Pontificia Universidad Católica de Chile, Facultad de Ciencias Biológicas, Alameda 340, 8331150 Santiago, Chile
[b] Laboratorio de Genética y Evolución, Facultad de Ciencias, Universidad de Chile, 7800024 Santiago, Chile
[c] Laboratorio de Biotecnología, Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile, 7830490 Santiago, Chile
[d] Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

ARTICLE INFO

Article history:
Received 21 February 2012
Received in revised form 11 July 2012
Accepted 6 September 2012
Available online 13 September 2012

Keywords:
Cyanobacteria
Raphidiopsis brookii
PSP toxins
Ionic effect
Cyanotoxin export
MATE proteins

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.

* Corresponding author. Tel.: +56 2 6862847; fax: +56 2 6862185.
E-mail address: mvasquez@bio.puc.cl (M. Vásquez).
[1] Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Nunoa, 7800024 Santiago, Chile.
[2] Servei de Microbiologia, Hospital Universitari Germans Trias i Pujol, Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, 08916 Badalona, Spain.

0041-0101/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxicon.2012.09.001

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