Journal of Microbiological Methods 117 (2015) 18–21

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

Note

Combining immunolabeling and catalyzed reporter deposition to detect intracellular saxitoxin in a cyanobacterium

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ARTICLE INFO

Article history:
Received 24 April 2015
Received in revised form 30 June 2015
Accepted 5 July 2015
Available online 9 July 2015

Keywords:
Immunolabeling
CARD
Saxitoxin
Cylindrospermopsis raciborskii

ABSTRACT

We combined the use of polyclonal antibodies against saxitoxin with catalyzed reporter deposition to detect production of saxitoxin by the cyanobacterium *Cylindrospermopsis raciborskii*. The procedure is simple, allows detection of intracellular saxitoxin in cyanobacteria filaments by confocal laser microscopy and is a promising tool to study toxin production and metabolism.

Saxitoxin (STX) is a harmful neurotoxin produced by freshwater cyanobacteria. It belongs to the paralytic shellfish toxins group (PSP), characterized by a common tricyclic backbone and different side group moieties. The list of PSP-producing species reported is increasing; generally associated to freshwater blooms, threaten water supplies. Generally, STX is produced by bloom-forming cyanobacteria of the genera *Anabaena*, *Cylindrospermopsis*, *Aphanizomenon*, *Raphidiopsis* and *Planktothrix* (Wiese et al., 2010). Although in situ toxin measurements provide information about bloom harmfulness, it is not known if toxins are constitutively produced by these cyanobacteria under different natural conditions, defying the prediction of toxic organism occurrence.

The genetic cluster putatively responsible for the synthesis of STX (*sxt* cluster) is known, and its biosynthetic pathway has been proposed (Kellmann et al., 2008) (Mihali et al., 2008) (Moustafa et al., 2009) (Stucken et al., 2010). Contrary, STX intracellular storage and export in cyanobacteria are still not well understood, partly due to the lack of appropriate methods for toxin location within the organisms. Available methods usually detect toxin concentration in a sample by ELISA, HPLC (Kaushik and Balasubramanian, 2013; D'Agostino et al., 2014), abundance of genes involved in STX synthesis (real-time qPCR) (Martínez De La Escalera et al., 2014) or toxic effect on target organisms (bioassays with crustaceans) (Ferrão-Filho et al., 2010; Abdul Keyon et al., 2014). However, there is no method to visually detect the location of the STX inside the cells, which could significantly contribute to studies in physiology and population dynamics.

Immunofluorescence techniques emerge as prominent new tools to underscore the location of toxins inside the cells. For instance, it has been shown that immunofluorescence-labeled microcystin in *Rivularia* sp. appeared as cytoplasmic granules in vegetative and differentiated cells (heterocytes and akinetes) and also in extracellular mucilage (Marco et al., 2012).

The aim of this work was to develop a simple immunofluorescence method for the detection of STX production within cyanobacterium cells, using *C. raciborskii* as a model species. Cultures of *C. raciborskii* MVCC19 (STX-producer, positive strain) and *C. raciborskii* LB2897 (non-toxic, negative control strain) were grown as previously described (Piccini et al., 2011). According to ELISA assays, average STX concentration in MVCC19 was 0.56 fg cell^{-1}, while no toxin could be detected in LB2897. Fifteen mL aliquots was harvested during the stationary growth phase (OD, 0.2) of each strain (MVCC: 5.25×10^5 fil ml^{-1} and LB2897: 4.75×10^5 fil ml^{-1}). Samples were fixed with 1% paraformaldehyde, kept in the dark during 1 h and then 500 μL aliquots of each strain was filtered through 0.2 μm Millipore polycarbonate filters.

The procedure is described in Fig. 1. Filters containing cyanobacteria filaments were embedded in 0.1% agarose, cut in sections and dried at 37 °C. Several permeabilization procedures were assayed including incubation with lysozyme or proteinase K and freeze thawing, but in all tested procedures the integrity of filaments was severely affected (data not shown). Therefore, we decided to exclude a permeabilization

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http://dx.doi.org/10.1016/j.mimet.2015.07.004
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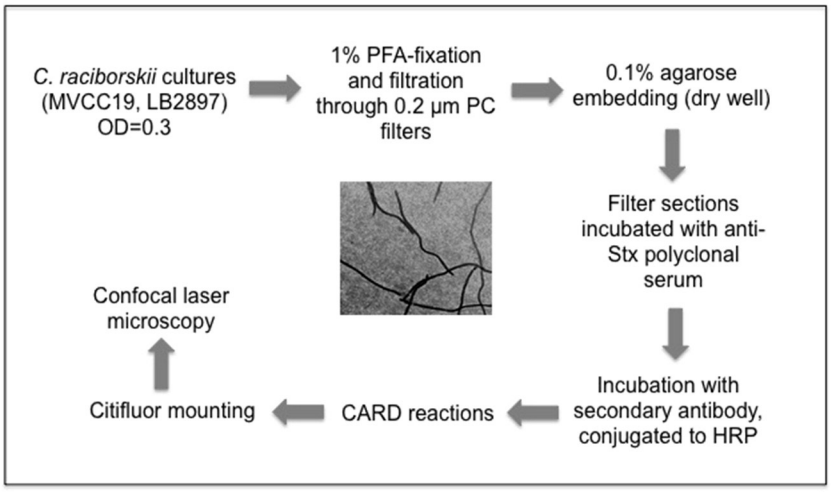

Fig. 1. Scheme of the protocol applied for the immunostaining + CARD method.

step. Since catalyzed reporter deposition (CARD) is based on peroxidase reaction, filter sections containing the cyanobacteria were incubated in 0.01 M HCl for 15 min at room temperature to kill endogenous peroxidases (Pernthaler et al., 2002). After washing in ultrapure water, filters were incubated with the polyclonal IgG solution (rabbit antiserum) included in an ELISA commercial kit for STX determination (Abraxis). Filter sections (we analyzed five different filter sections from each strain) were submerged in undiluted antibody solution and incubated overnight at 37 °C in a wet chamber. After that, filter sections were washed with 2% BSA–PBS on an orbital shaker for 15 min at room temperature. Then, secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) (Invitrogen) was added to each filter

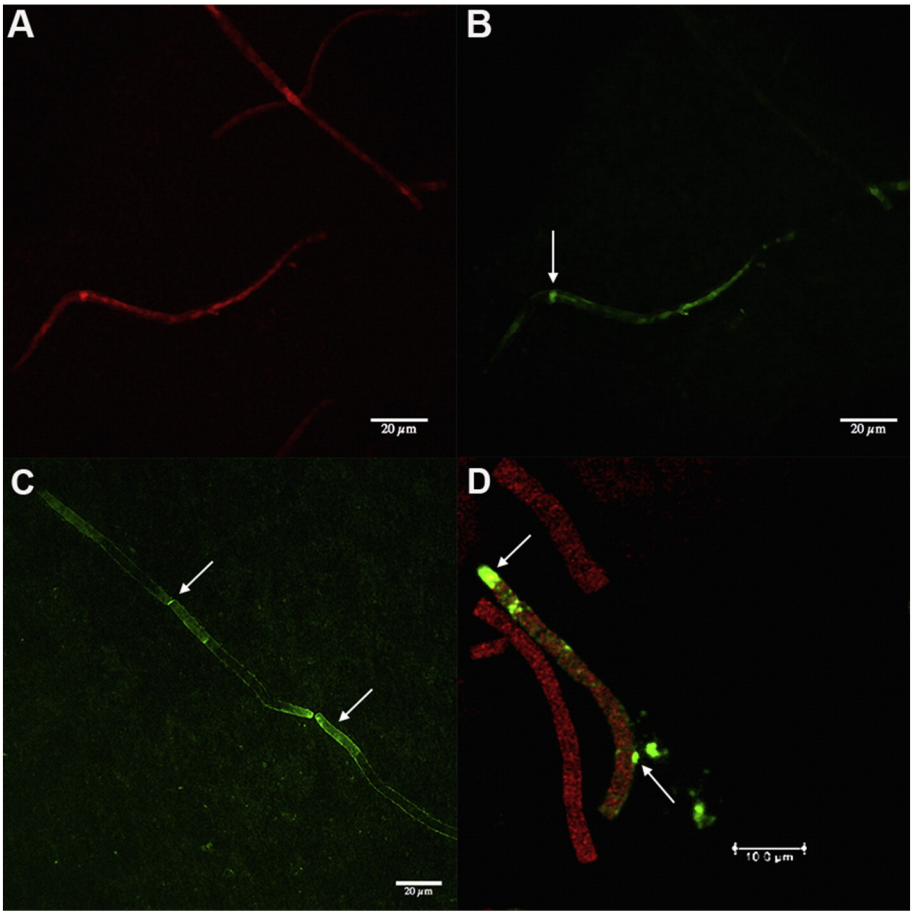

Fig. 2. CLM images from *C. raciborskii* MVCC19 taken at 60× magnification. (A) Image showing the chlorophyll-*a* fluorescence (red channel); (B) FITC signal obtained from the immunostaining using CARD amplification (green channel); (C) FITC signal in filaments showing the apical location of STX-label; (D) composite image (red and green channels) of a representative FITC-labeled cell. Arrows indicate the zones where the STX signal was stronger.