Screening Assay Establishment for Toxigenic Bacteria from PSP-producing Dinoflagellate *Alexandrium tamarense* Cultures

Xiaoling Zhang [a]

Key Laboratory of East China Sea & Oceanic Fishery Resources Exploitation and Utilization of Ministry of Agriculture (MOA), East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Shanghai, 200090, China

[a] *zhxl@ecfs.ac.cn*

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Abstract. Paralytic shellfish poisoning (PSP) toxins are potent neurotoxins mainly produced by marine dinoflagellates. However, the increasing number of findings of toxin-producing bacteria in dinoflagellate intracellular cells supports the hypothesis of the bacterial origin of PSP toxins. Although toxigenic bacteria could be isolated from toxic dinoflagellates, it was not clearly proven whether the isolated bacterial strains based on culture-dependent manner and the corresponding intracellular bacteria were the same because of microbial unculturability and shorting of the genetic evidence. This paper aims to demonstrate the development of a screening assay for toxigenic bacteria from marine dinoflagellate *A. tamarense* based on molecular analysis of the possible *sxt*A gene for the toxic PSP biosynthesis.

Introduction

Paralytic shellfish poisoning (PSP) toxins are potent neurotoxins comprising of saxitoxin (STX) which are blocking the voltage-gated sodium channels on excitable cells, and thus leading to severe neurological symptoms and even death [1]. Up to now, totally 58 STX analogs has been found [2]. The main producers of PSP are marine dinoflagellates, in particular, *Alexandrium* spp., *Pyrodinum bahamense* var. compressum and *Gymnodinium catenatum* [3], but some freshwater cyanobacteria such as *Aphanizomenon flos-aquae, Anabaena circinalis* and *Lyngbya wollei* [4]. Interestingly, PSP toxins have been also detected in intracellular bacteria isolated from toxin-producing dinoflagellates [5]. Silva first suggested the theory of a bacterial origin of PST in 1979, with the first reported PST-producing intracellular bacterium marine *Moraxella* sp. (PTB-1) isolated from the dinoflagellate nucleus of *Alexandrium tamarense* [6]. The subsequent increasing number of finding of toxin-producing bacteria in the cells of dinoflagellate supports Silva's hypothesis [7]. There is also increasing evidence that there are specific symbiosis bacterial taxa associated with phytoplankton, indicating the presence of specific selective mechanisms, and implying that the symbiosis bacteria have some function to the benefit of the alga, and these interactions could be the product of co-evolution between bacteria and algae over millions of years [8]. Studies on the role of toxin-producing symbiosis bacteria in the marine ecosystem are considered to be becoming more important. The evolutionary history that resulted in the curious cross-kingdom distribution of STX-synthesis remained unclear. Increasing number of studies have reported the direct observation of intracellular bacteria in toxic species of dinoflagellate [9-12].

Although toxigenic bacteria could be isolated from toxic dinoflagellates, it was not clearly proven whether the isolated bacterial strains and the corresponding intracellular bacteria were the same because of microbial unculturability and shorting of the genetic evidence. The purpose of this paper is

to demonstrate the development of a screening assay for toxic bacteria from marine dinoflagellate *Alexandrium tamarense* based on molecular analysis of the possible *sxt*A gene, the initial starting gene during the toxic PSP biosynthesis pathway.

Materials and Methods

Sample Culture

The toxic dinoflagellate *Alexandrium tamarense* (880#) was collected and kindly provided by Prof. Hung-Non Chou at National Taiwan University. An axenic culture of Alexandrium tamarense was cultured in f/2 medium. Cultures were kept at 28℃ and a 12 h light:12 h dark cycle. With the light intensity of ca. 200 umol photons m-2·S-1.

Bacterial Isolation

Cultivable bacteria were isolated and maintained on a marine Zobell 2216 prepared with aged filtered natural seawater, supplemented with GSe trace elements and vitamins were used as necessary [13]. acterial isolation was performed by harvesting 1 ml of a late-logarithmic phase *A. tamarense* culture by brief centrifugation (4 000 g for 20 min). The spent medium was removed and the algal cell pellet resuspended in 100 μL of sterile seawater and vortexed. The cell suspension was diluted 10-fold and 100 μL of each dilution spread onto 2216 agar plates and incubated in the dark at 25°C for 6-10 days. Bacterial colonies with distinct colony morphology were picked . Bacterial isolates were grown in 2216 broth, glycerol was added (25% v/v), and the cells were stored at -80℃.

PSP Analysis by LC-MS

LC-MS analysis of the STX toxin production of the isolated microbial strain was performed according to the method reoprted previously LC-MS analysis of the STX toxin production of the isolated microbial strain was performed according to the method reoprted previously [8,14].

Assay Development

Genomic DNA of the samples was extracted using Wizard® DNA Kit (Promega, Madison, USA) following the manufacturer's instruction. The quality of extracted DNA was checked by 0.8% agarose gel electrophoresis and spectrophotometry (optical density at 260 nm/280 nm ratio). The *sxt*A1 region of bacterial DNA were amplified by PCR [13]. The *sxt*A gene A1 region of bacteria was amplified using the designed primers as shown in Table 1. PCR amplifications of the *sxt*A gene A1 region were performed. The amplicon mixture was subject to sequencing (Illumina, San Diego, CA, USA). Alignment of the obtainded sequences were performed by blast using GenBank database.

Results and Discussion

Total 3 positive DNA samples of intracellular bacteria strains isolated from *A. tamarense* culture were tested as for selectivity of the primers for PCR amplification. Table 1 shows the total 10 primers based on the conserved region of the gene of *sxt*A1 region. Based on the agarose gel electrophoresis analysis for PCR amplification products (Fig. 1), the of sxt 3-F and 4-R was selected as the primer for PCR amplification.

Table 1 The tested primers for PCR in this study

Primers	Sequences (5'→3')
0-F	CCGTAGGTGAACCTGCG
1-R	CCTCCGCTTATTGATAT
2-F	ATGCTAACATGGGAGTCA
3-R	GGGTCCAGTAGATGTTGAC
4-F	TAGTAGGAGTAGCKACG
5-R	TCCTTCCTRGACCACGA
6-F	GCAAGTATCTCCGCAGGCT
7-R	GTGGAGGAGCATGTTGACAG
8-F	TGCAGCGMTGCTACTCCTACT
9-R	GGTCGTGGTCYAGGAAGGAG

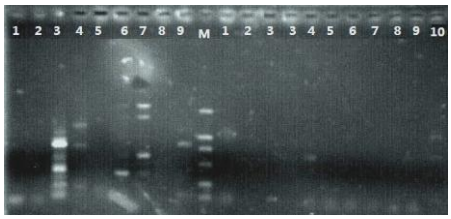

Fig. 1 Primers selection based on PCR amplification of *sxt*A1 region of positive bacteria DNA

It has been reported that, *Sxt*A, the unique starting gene for STX synthesis in cyanobacteria, possiblely origins from an actinobacterial specie or a *Proteobacterium* by independent horizontal gene transfers (HGTs) and gene fusion [5-7]. In order to elucidate the relationship of the intracellular bacteria with the host, the *sxt*A biosynthesis gene analysis of six isolated strains for *A. tamarense* culture was performed. The result is shown Fig 2. It indicated that, five isolated intracellular bacteria strains from toxic *A. tamarense* have the sxtA1 gene for the biosynthetic intermediate of PSP toxins, STX. Addtionly, this bacteria stain has possible toxic biosynthetic gene as its gene sequence obtained in this study has high similarity with dinoflagellate *sxt*A gene. This finding implyes that the development of the rapid method such as *in situ* DNA hybridication using specific DNA-targeted probes for the the study of the interaction relationship between the intracellular bacteria and the host toxic dinoflagellate.

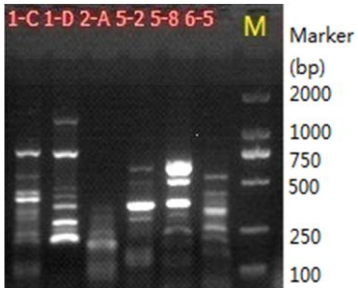

Fig. 2 PCR amplification of *sxt*A1 region of the intracellular bacteria DNA isolated from *a. tamarense* cultures

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