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Amplification of Genes Associated with Saxitoxin Biosynthesis in Bacteria Associated with *Pyrodinium bahamense* var. *compressum* Using Primers Designed from Other Saxitoxin-Producing Organisms

Sylvia Yahumin, Grace Joy Wei Lie Chin* and Kenneth Francis Rodrigues

Biotechnology Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Sabah
*Corresponding author's email: gracejoy@usm.my

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ABSTRACT

Pyrodinium bahamense var. *compressum*, the primary producer of saxitoxin (STX) in tropical water, poses a palpable threat to public health and safety. Seafood and aquaculture products contaminated with lethal concentrations of the principal neurotoxin, saxitoxin, have been implicated in mortality and morbidity. Previous research revealed that the relationship between bacteria and algae may contribute directly or indirectly to the synthesis of saxitoxin. This study investigates the potential relationship between the dinoflagellate and its associated bacteria; hence the initial step is to determine whether the genes responsible for the STX production in *P. bahamense* can be found in the associated bacteria. A total of six bacterial species associated with *P. bahamense* were successfully identified in a previous study. The presence of the *sxt* genes in the associated bacterial genome was determined using primers that have previously been utilized in other saxitoxin-producing species, such as in dinoflagellates (*Alexandrium fundyense*) and cyanobacteria (*Anabaena* and *Aphanizomenon gracile*). This study showed that the utilized primers were unsuitable as the primers produced non-specific amplification in the bacterial genome. We suggest that specific primers targeting the *sxt* homolog genes in bacterial species should be designed to obtain the desired genes from the associated bacteria in a future study.

Keywords: saxitoxin, bacteria, dinoflagellate, *sxt* genes

INTRODUCTION

Saxitoxins (STXs), also known as paralytic shellfish toxins (PSTs), are potent water-soluble neurotoxins that block sodium from moving across nerve cell membranes and impede the passage of nerve impulses. Human ingestion of the potent toxin through consumption of toxin-contaminated shellfish can cause the illness known as paralytic shellfish poisoning (PSP). Symptoms of PSP include numbness, paralysis, disorientation, and death through respiratory paralysis (Grattan et al., 2016). Saxitoxins are produced naturally by certain dinoflagellate species in marine waters and cyanobacteria in freshwater (Cusick & Sayler, 2013). PSP has placed a significant economic burden on the world as outbreaks brought on by toxic algal blooms frequently result in the death of marine life and livestock and the closure of contaminated fisheries while necessitating ongoing expenses for the upkeep and operation of monitoring programmes (Hallegraeff et al., 2021).

During a harmful algal bloom (HAB), the bacterial community structure is complex and changes as the bloom grows depending on the algal species, physiological status, environmental circumstances, and bloom phase (Zheng, 2011). The bacterial community's structure and metabolic characteristics influence their ecological activities, which include the provision of nutrients, the release of organic compounds, and their role as an adversary of the algae in a particular environmental niche (Amin et al., 2015). The relationship between dinoflagellates and bacteria establishes an extensive regulatory network that may have an impact on the bloom dynamics (Doucette, 1995; Tan et al., 2015) and the synthesis of biotoxins (Lee et al., 2016).

One of the earliest reports of PST production in bacteria was made in 1988 when Kodama et al. (1988) used HPLC-fluorometric analysis to detect the presence of STX in a cultured bacterial broth isolated from *Protogonyaulax tamarensis* (now known as *Alexandrium tamarense*) homogenate. They hypothesized that the dinoflagellate inside was where the saxitoxin-producing bacteria originated. Gallacher et al. (1997) also revealed how the associated bacteria in several *Alexandrium* strains produce PST. The associated bacterial cultures from the *Alexandrium* strains were subjected to HPLC analysis, which revealed the presence of saxitoxin and several of its analogues, including gonyautoxin 1 to 4 and neo-saxitoxin. The summary of studies that demonstrated prokaryotic organisms could produce saxitoxins is shown in Table 1. Due to these investigations, the role of prokaryotes in toxic algal bloom events has been given more attention, and the perception of toxin producers has altered.

Shimizu et al. (1984) were the first to elucidate the STX biosynthesis pathway based on precursor-feeding experiments with ^{13}C- and ^{15}N-labelled amino acids and acetic acid using the marine dinoflagellate *Gonyaulax tamarensis* and the freshwater cyanobacterium *Aphanizomenon flosaquae*. They proposed a biosynthetic pathway towards STX, including the Claisen condensation of arginine and acetic acid, and suggested that acetic acid, arginine, and S-adenosyl methionine are the components required to build up PST molecules. Numerous enzyme isolation and characterization experiments, which putatively participate in the STX pathway,

further expand the understanding of cyanobacteria's STX biosynthesis. The enzymes are s-adenosylhomocysteine hydrolase, methionine aminopeptidase (Taroncher-Oldenburg, 2000), sulfotransferase (Sako et al., 2001), Na (+)-dependent transporter (Pomati et al., 2004), aminotransferase, and O-carbamoyltransferase (Kellmann & Neilan, 2007). The saxitoxin gene cluster (*sxt*) has previously been identified and sequenced in *Anabaena circinalis* AWQC131C (NCBI accession no.: DQ787201) (Mihali et al., 2009), *Aphanizomenon* sp. NH-5 (NCBI accession no.: EU603710) (Mihali et al., 2009), *Raphidiopsis brookii* D9 (NCBI accession no.: ACYB00000000) (Stucken et al., 2010) and *Lyngbya wollei* (NCBI accession no.: EU603711) (Murray et al., 2011).

A potential STX biosynthesis pathway has been proposed based on the STX biosynthetic gene cluster discovered in the STX-producing cyanobacteria strain *Cylindrospermopsis raciborskii* T3. Kellmann et al. (2008) used functional silico homology analysis and an intermediate biosynthesis LC-MS analysis to screen the possible biosynthetic intermediates from concentrated cell extracts of *C. raciborskii* T3. According to their study, the STX biosynthesis pathway is encoded by more than 35 kb, and up to 26 genes (*sxtA – sxtZ*) are responsible for 30 catalytic actions. A recent study used a gene derived from a bacterium to modify the enzymes necessary for the biosynthesis pathway of saxitoxin. The work converted the less lethal PST into a more toxic compound by including the pure *sxtN* and *sxtSUL* PST biosynthesis genes in the bacteria *Microseira wollei* and *Aphanizomenon* sp. NH-5 (Lukowski et al., 2019). According to this study, the molecule can be changed into a more toxic derivative or vice versa during toxin biosynthesis by integrating specific enzymes involved in saxitoxin production.

In contrast to cyanobacteria, the genetic basis for STX-biosynthesis in dinoflagellate remains unclear as lacks data can be obtained from the NCBI databank regarding the *sxt* genes. Saxitoxin biosynthesis genes have recently been identified in multiple species of dinoflagellates via high-throughput sequencing technologies (Stüken et al., 2011; Hackett et al., 2013). Despite technological advancement, the genetic basis for STX production and genes responsible for dinoflagellates remained elusive. This is most likely due to the particularly large (3-245 GB of DNA) and complex (highly redundant, with high gene copy number) (Lin, 2011) genomes of dinoflagellates which have posed significant challenges in identifying toxin-related genes.

In an earlier study, Yahumin et al. (2022a) used the 16S rRNA region to explore the bacterial communities associated with the same *Pyrodinium bahamense* var. *compressum* strain used in this study. A variety of interesting bacterial species were discovered, where some bacteria had been previously reported to be linked to the synthesis of saxitoxin. In addition, the identification and phylogeny analysis of the two domains (*sxtA1* and *sxtA4*) of the starting genes, *sxtA*, of the saxitoxin biosynthesis pathway of the same *P. bahamense* var. *compressum* strain was also carried out by Yahumin et al. (2022b), where the two domains were identified in the harmful alga and found to have a close relationship with other well-known STX-producing organisms. To complement the previous findings, studies on the characterization of the *sxt* genes in *P. bahamense* (ongoing) and its associated bacteria (this study) were conducted. The present study reports the findings of the PCR amplification of the *sxt* genes in bacteria associated with toxic algal blooms in Sabah.

Table 1 List of studies that found PST production in bacteria associated with harmful algae

Year	Bacterial strain	Toxin ($\times 10^{-4}$ pg STX equiv. cell^{-1})	Toxin Profile	Origin	Reference
1990	Ten unidentified strains	0.06-0.004	STX	*A.tamarense* OF 84423D-3	(Kodama et al., 1998, 1990)
	Moraxella sp.		Neo-STX, GTX1-4		
1992	*Vibrio* sp.	1.47	STX, neo-STX	*Perna perna* (Mussels)	(Freitas et al., 1992)
	Pseudomonas sp.	3.71	GTX1–4		
1996	Pseudomonas stutzeri	Not available	GTX4, C4	A.lusitanicum	(Franca et al., 1996)
	Pseudomonas diminuta		GTX1, 3, 4, C2-4	G.catenatum	
	Sp. 1 and 2	1.00	STX, neo-STX	*A.tamarense* Ipswitch strain	(Shimizu et al., 1996)
	Pseudomonas sp.	0.005-0.18	C1/GTX2	Gulf of St. Lawrence, Canada	(Levasseur et al., 1996)
	DCM 10		C2/GTX3/B2/ neo-STX		
	Alteromonas sp. 6SM1	0.05-0.59	C4/GTX4		
	Acinetobacter sp. 6SN9	0.02-0.98	C3/GTX1, C4/ GTX4		
	Acinetobacter sp. 5Ms5	0.01-0.78	C3/GTX1, C4/ GTX4		
1997	667-2	0.87-2.94	STX, neo-STX, GTX1-4, B2, C toxins	*A.affine* NEPCC 667	(Gallacher et al., 1996 and 1997)
	407-2			*A.tamarense* NEPCC 407	
	UW4-1			A.tamarense	
	Uw2c-6			*A.tamarense* UW2c	
	253-19			*A.lusitanicum* NEPCC 253	
2002	*Moraxella* sp. like	Not available	STX	*A.catanella* clone ACC01	(Córdova et al., 2002)
	Pseudomonas diminuta				
2006	*Moxarella* spp.	5.6×10^{-4}	STX	*Pyrodinium bahamense* var. *compressum* strain Pbc MZRVA 042595	(Azanza et al., 2006)
	Erythrobacter spp.	$(51\text{-}60)\ 10^{-4}$	STX, neo-STX		
	Bacillus spp.	$7.3\ 10^{-4}$	STX		

MATERIALS AND METHODS

Details of the Associated Bacteria Strains

A total of six marine bacteria associated with the toxin-producing harmful alga, *P. bahamense*, were used in the study, namely *Mameliella atlantica*, *Muricauda lutimaris*, *Roseibium denhamense*, *Roseibium hamelinense*, *Marinobacter salsuginis* and *Micrococcus luteus*. The associated bacteria were isolated in a previous study by Yahumin et al. (2022a). The bacteria were cultured and maintained on sterile marine agar (Difco). The bacterial isolates were cultured overnight at 37°C in 10 ml marine broth (Difco) prior to DNA extraction.

DNA Extraction

Genomic DNA from each isolate was extracted using the DNeasy Blood and Tissue DNA Isolation Kit (Qiagen Biotechnology), following the manufacturer's instructions. The DNA samples were stored at −20°C prior to PCR amplification.

Polymerase Chain Reaction (PCR) Amplification of the sxt Genes

The amplification of the *sxt* genes was carried out using the Phusion Flash PCR Master Mix (Thermo Fisher Scientific). For each reaction, 1 µL of DNA template was added into 19 µL of PCR reaction mixture consisting of 1× Phusion Flash PCR Master Mix and 0.5 µM of each of the forward and reverse primers. The PCR *sxt* primers used in this study are shown in Table 2. In addition, a 464-bp long 16S rDNA bacterial identification primer pair (S-D-Bact-0785-a-A-21 and S-D-Bact-0341-b-S-17) (Klindworth et al., 2013) amplifying the associated bacteria DNA template was used as a positive control. The PCR amplification was performed on a PTC-200 Thermal Cycler (MJ Research) according to the following thermal cycling conditions: 10 s of an initial denaturation step at 98°C followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 8 s, and lastly, a final extension at 72°C for 1 min. The expected size of each targeted gene is stated in Table 2. PCR products were then analyzed for size verification on a 2% (w/v) agarose gel electrophoresis with a 100 bp DNA ladder (Thermo Fisher Scientific) as the molecular weight-size standard. The PCR products were stored at −20°C until further use.

Table 2 Primers used for PCR amplification of the sxt genes

Genes	Domain (Kellmann et al., 2008)	Primer	Sequence (5'-3')	Size (bp)	Reference
sxtA1	Class I SAM-dependent methyltransferase	Forward	TCGAGCGMTGCTACTCCTACTAC	550	Stüken et al. (2011)
		Reverse	GGTCGTGGTCYAGGAAGGAG		
sxtA4	Aons (8-amino-7-oxononanosynthase)	Forward	ATGCTCAACATGGGAGTCATCC	750	
		Reverse	GGGTCCAGTAGATGTTGACGATG		
sxtB	Cytidine deaminase	Forward	TGTTGTGCTTGCTGCTCTATCAG	957	Savela et al. (2015)
		Reverse	CAGCGTTTTCAGCGTAYCGAC		
sxtG	Amidinotransferase	Forward	AGGAATTCCCTATCCACCGGAG	893	Casero et al. (2014)
		Reverse	CGGCGAACATCTAACGTTGCAC		
sxtH	Phenylpropionate dioxygenase	Forward	AAGACCACTGTCCCCACCGAGG	812	
		Reverse	CTGTGCAGCGATCTGATGGCAC		
sxtI	Carbamoyltransferase	Forward	AGCGCTGCCGCTATGGTTGTCG	910	
		Reverse	ACGCAATTGAGGGCGACACCAC		
sxtX	Cephalosporin hydroxylase	Forward	GATGCAACCCATAAACTCGCAC	812	
		Reverse	AAGGTACTCGTTTTCGTGGAGC		
sxtD	Sterole desaturase-like protein	Forward	TCTTCAAGGAATTGGGATGG	759	Primers were self-designed based on the DNA sequence of STX genes from *C. raciborskii* (NCBI accession no.: DQ787200).
		Reverse	TGCGTCAACCAAAGAAACTG		
sxtU	Alcohol dehydrogenase	Forward	GGTGGTCAAGCATTGCCTAT	750	
		Reverse	TCTGCGGGATTTCCAGTATC		
sxtS	Phytanoyl-CoA dioxygenase	Forward	GGATGCAAGTGCGTTCAATA	720	
		Reverse	GGAGTATTGGCGGGTGACTA		

Sequence Determination of sxt Genes

The raw sequences of the *sxt* genes of the different bacterial isolates were pre-analyzed using the GeneStudio™ Professional (http://genestudio.com). The sequences were viewed and analyzed with the chromatogram file as references to trim and edit the sequences by removing high noise at the 5' or 3' end, which was then compiled into FASTA format. The trimmed sequences from bacterial isolates were then identified by aligning the sequences against reference sequences in the BLAST Nucleotide collection (nr/nt) of the National Centre for Biotechnology Information (NCBI) GenBank (https://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

The PCR amplification results of the *sxt* genes of all six bacterial isolates are shown in Figure 1. Based on the presence of the positive control band at Lane 11, indicated that the DNA templates used in the PCR amplifications are suitable for the experiment. Based on Figure 1, it appeared that not all the *sxt* genes were present in all the associated bacteria. Only *M. atlantica* showed a positive amplification of the essential starting gene domain, *sxtA4*.

In a study by Yahumin et al. (2022b), the starting genes *sxtA1* and *sxtA4* were present in a similar strain of *P. bahamense,* where the associated bacteria were isolated. The phylogenetic analysis of the starting genes in the *P. bahamense* showed a close relationship with other well-known saxitoxin-producing harmful algae, such as *Alexandrium* spp. and *Gymnodinium catenatum,* with a bootstrap value of 66 – 100%. Furthermore, it was shown that the genes also formed sister clades with other STX-producing species, including cyanobacteria (Yahumin et al., 2022b). The genes, *sxtA1* and *sxtA4,* are the domains of the starting genes and are essential for the synthesis of STX. According to Stüken et al. (2011), the presence of the *sxtA1* and *sxtA4* genes is both necessary for the synthesis of STX and a reliable indicator of the synthesis of STX itself. It has been shown that most STX-producing dinoflagellate strains have genomes that encode both the *sxtA1* and *sxtA4* domains; however, in non-STX-producing dinoflagellate strains, either the last domain *sxtA4* or both domains may be missing. The lack of *sxtA4* motifs in the dinoflagellate strain makes it impossible for it to produce the toxin (Suikkanen et al., 2013). Additionally, Murray et al. (2015) revealed that the *sxtA4* domain was absent in non-producing species but present in numerous strains of STX-producing dinoflagellates, including *Alexandrium*, *Pyrodinium*, and *Gymnodinium*.

All PCR products were subjected to direct sequencing. However, only those PCR bands highlighted in red boxes in Figure 1 produced similar results in the BLASTx search. Table 3 shows the results of the BLASTx search with the highest similarity acquired from the NCBI GenBank protein database. According to the results of the BLASTx search, the desired genes have not been found. The primers used in this study were obtained from previous studies of *sxt* genes in other saxitoxin-producing organisms, which are *Alexandrium fundyense* dinoflagellate (Stüken et al., 2011), *Anabaena* cyanobacteria

(Savela et al., 2015), and *Aphanizomenon gracile* cyanobacteria (Casero et al., 2014). As a result, the initial conclusion was that the primers utilized in this study were not suitable since the primer annealing site is non-specific. This may be due to the vast complexity of eukaryote's genome as compared to the genome of cyanobacteria and bacteria. For example, eukaryotic transcripts generally contain longer untranslated leader and terminal sequences (untranslated regions) than those of prokaryotes (Lynch, 2006). The primers designed based on the DNA sequences of cyanobacteria and dinoflagellate may be solely specific to their species and do not function on other species. Consequently, to address the non-specific and lack of amplifications of the *sxt* genes in the associated bacteria, a follow-up study will be carried out to design primers based on *sxt* protein homologs that can be found in the NCBI GenBank database.

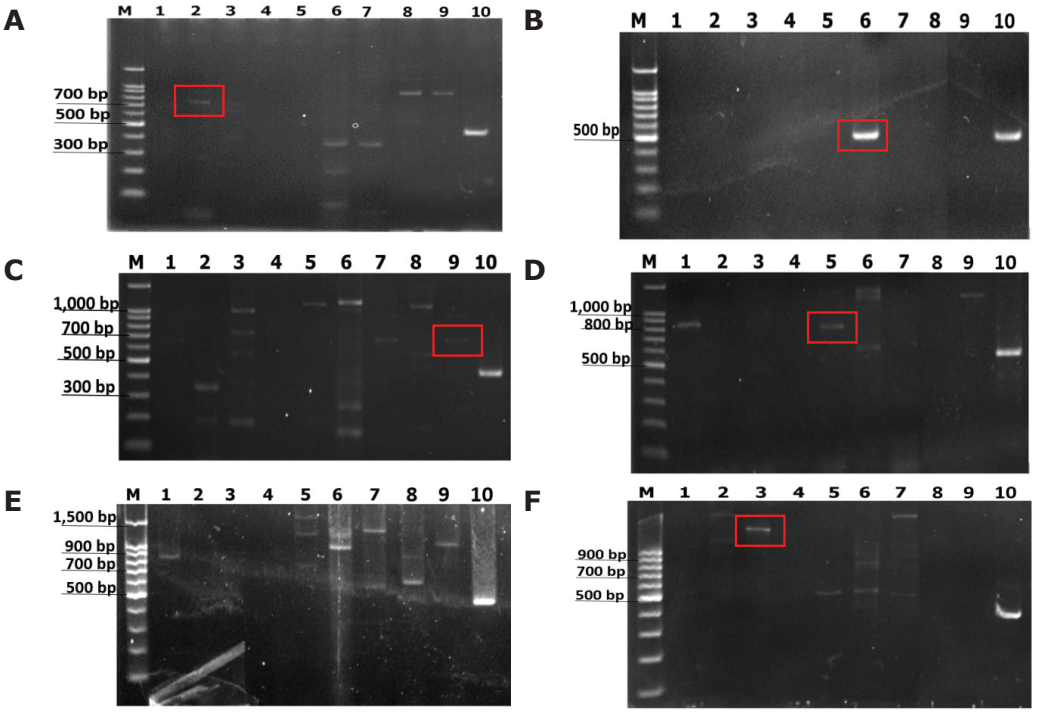

Figure 1 PCR amplification of the *sxt* genes in the six bacterial isolates, *Mameliella atlantica* (A), *Muricauda lutimaris* (B), *Roseibium denhamense* (C), *Roseibium hamelinense* (D), *Marinobacter salsuginis* (E), *Micrococcus luteus* (F). Lane M: 100 bp DNA ladder marker; Lane 1: *sxtA1*; Lane 2: *sxtA4*; Lane 3: *sxtB*; Lane 4: *sxtD*; Lane 5: *sxtG*; Lane 6: *sxtI*; Lane 7: *sxtH*; Lane 8: *sxtS*; Lane 9: *sxtU*; Lane 10: positive control. The PCR products highlighted in red rectangle were the ones that produced similar results in the BLASTx search.

Since bacteria and algae co-evolved millions of years ago, they have formed a wide variety of interactions, some of which involve the production of saxitoxin by the respective dinoflagellates (Ashen & Goff 2000). Despite multiple reports on the influence of bacteria on the synthesis of toxins in dinoflagellates, the exact mechanisms of interaction remain unknown (Córdova et al., 2002; Doucette, 1995; Franca et al., 1996; Gallacher et al., 1997; Gallacher & Smith, 1999; Kodama et al., 1988). In a study by Singh

et al., 1982, axenic cultures were reported to produce less toxicity (measured in mouse units) per cell than non-axenic cells. Removing the majority of the symbionts may have reduced the production of the toxin, indicating that the symbionts may have co-produced some of the *sxt* genes together. Low, but not absent, toxicities in axenic culture suggested that bacteria played a substantial indirect role in saxitoxin biosynthesis. The information in Figure 1 and Table 3 may imply that a mechanism of interaction between the dinoflagellate and its associated bacteria exists. From a molecular standpoint, the related bacteria may also play a role in the saxitoxin pathway by producing enzymes necessary for the manufacture of saxitoxin alongside the dinoflagellate. We suggest that the two organisms co-produce the required enzymes for saxitoxin production as the interaction mechanism between the bacteria and the dinoflagellate. Although the differences in the gene structure have led to non-specific amplification, this may be resolved by designing primers targeting the *sxt* gene homologs specifically to the bacterial species.

Table 3 BLASTx similarity analysis of the selected band (Figure 1)

Refer (Fig. 1)	Template	Target gene	Protein	BLASTx similarity	Per. Ident. (%)	E-value
A	*Mameliella atlantica*	*sxtA4*	AONS (8-amino-7-oxononanoatesynthase)	MULTISPECIES: ATP-dependent helicase [*Rhodobacteraceae*] (WP_043143649)	59	$7e^{-46}$
B	*Muricauda lutimaris*	*sxtI*	carbamoyltransferase	DUF349 domain-containing protein [*Muricauda* sp. ARW1Y1] (WP_179384164)	97.24	$9e^{-95}$
C	*Roseibium denhamense*	*sxtU*	Alcohol dehydrogenase	Lytic murein transglycosylase [*Labrenzia* sp. OB1) (WP_068409032)	56	$7e^{-28}$
D	*Roseibium hamelinense*	*sxtG*	Amidinotransferase	Peptidylprolyl isomerase [*Labrenzia* sp.VG12] (WP_094070848)	78	$2e^{-160}$
F	*Micrococcus luteus*	*sxtB*	Cytidine deaminase	Transposase of ISAar43, IS3 family, IS407 group, orfB [*Micrococcus luteus*] (EZP62592)	73	6^{e-11}

CONCLUSION

The study failed to characterize the genes associated with the saxitoxin biosynthesis in the bacteria associated with the harmful microalga, *Pyrodinium bahamense* var. *compressum*. Nonetheless, the acquired data in this study have taken the lead in showing insights into the potential bacterial-algal interaction in saxitoxin biosynthesis.

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