Microbial Ecology

Bacterial Endosymbionts of *Pyrodinium bahamense* var. *compressum*

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Abstract

The study presents evidence in support of the bacterial theory associated with the toxicity of *Pyrodinium bahamense* var. *compressum*. Bacterial endosymbionts from Philippine *P. bahamense* var. *compressum* strain Pbc MZRVA 042595 were isolated and identified via 16S rDNA sequence analysis. Taxonomic diversity of the identified culturable intracellular microbiota associated with Philippine *P. bahamense* var. *compressum* was established to be limited to the Phyla Proteobacteria, Actinobacteria, and Firmicutes. Major endosymbionts identified included *Moraxella* spp., *Erythrobacter* spp., and *Bacillus* spp., whereas *Pseudomonas putida*, *Micrococcus* spp., and *Dietzia maris* were identified as minor isolates. All identified strains except *D. maris*, *P. putida*, and *Micrococcus* spp. were shown to contain either saxitoxin or neo saxitoxin or both at levels $\leq$73 ng/10^7 bacterial cells based on high-performance liquid chromatography analysis. Paralytic shellfish poisoning-like physiologic reactions in test animals used in the mouse assay were recorded for the endosymbionts except for *P. putida*. The study is the first to elucidate the possible contribution of bacterial endosymbionts in the toxicity of *P. bahamense* var. *compressum* isolated in the Philippines.

Introduction

Some studies on harmful algal bloom (HAB) phenomena emphasized the probable role of prokaryotes, primarily bacteria, on toxin production [11, 15, 23]. Proponents of these studies believed that there exists a symbiotic relationship between prokaryotes and marine organisms responsible for toxin production in HAB outbreaks. Among the early reports on the theory of bacterial origin of paralytic shellfish poisoning (PSP) toxins was suggested by Silva [41, 42]. Silva [43] cited the possibility that associated bacteria from toxic dinoflagellates might play a major role in the expression of toxicities of the dinoflagellates.

In the Philippines, toxic red tide outbreaks caused by the toxic dinoflagellate *P. bahamense* var. *compressum* has caused tremendous negative impact to the seafood industry of the country, particularly those involved in molluskan bivalves. The dinoflagellate *P. bahamense* var. *compressum* is known to produce PSP toxins, collectively termed saxitoxins. The presence of saxitoxins in the dinoflagellate associated with harmful algal outbreaks was first shown by Maclean [28]. The clinical effect of PSP toxins to humans involves the reversible and highly specific action on the ion transport by the sodium channels of excitable membranes such as nerve cells and fiber muscles [32].

Kodama *et al.* [23] first reported that a bacterium, belonging to the genus *Moraxella*, isolated from the toxic red tide dinoflagellate *Alexandrium tamarense*, demonstrated autonomous phycotoxin production. Thereafter, several studies have also reported the capability of other heterotrophic bacteria from toxic dinoflagellates in producing sodium channel-blocking toxins such as saxitoxins [8, 11, 13–15, 24, 33, 46].

At present, the only study on bacterial endosymbionts of *P. bahamense* var. *compressum* was reported by Sidharta [40], who cited the preliminary identification of *Alcaligenes* sp. as an endosymbiont from the dinoflagellate collected from Limay, Bataan, Manila Bay, on June 16, 1996. This study aimed to establish the saxitoxin production of culturable bacterial endosymbionts from the monoalgal culture of *P. bahamense* var. *compressum* (Pbc-MZ RVA 042595) isolated in Masinloc, Zambales, Philippines [4].

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Methods

Isolation of Bacterial Endosymbionts. Axenic F/2 culture of *P. bahamense* var. *compressum* (Pbc-MZ RVA 042595) was prepared as described by Rausch de Traubenberg *et al.* [39]. Briefly, dinoflagellate in F/2 monoalagal culture was treated with a broad spectrum of antibiotic cocktail containing penicillin, streptomycin, kanamycin, and neomycin at final concentrations of 330, 160, 160, and 160 μg/mL, respectively. Bacterial endosymbionts of the antibiotic-treated F/2 cultures of *P. bahamense* var. *compressum* at the late log to midstationary phase were isolated by using procedures described by Sidharta [40]. Aliquot portions of axenic F/2 culture medium were sonicated by using Sanyo Soniprep 150 (Sanyo, Gallenkamp, England) set at 70 kHz for 90 s. Likewise, unsonicated aliquot portions of *P. bahamense* var. *compressum* control cultures in F/2 were sampled up to midstationary phase to continuously verify the effective inactivation of the extracellular bacteria from the antibiotic-treated *P. bahamense* var. *compressum*. The sonicated and unsonicated antibiotic-treated cultures were serially diluted up to 10^2 by using a sterile 3% NaCl solution. Aliquot portions of the diluent were spread plated on duplicate plates of sterile prepoured marine agar (MA) (Pronadisa, Spain). Colonial characteristics on MA plates were used as basis for primary selection of the isolates. Gram reaction and cellular morphology were also observed as part of the preliminary screening of the isolates. Pure isolates were maintained in MA slants at 28°C ± 2°C and subcultured monthly.

Identification of the Bacterial Isolates. Genotypic identification of the isolates were accomplished by extraction of the genomic DNA, polymerase chain reaction (PCR) amplification of the 16S rDNA, and subsequent direct sequencing and sequence analysis of the amplified gene for all isolates. Genomic DNA of the isolates was extracted by using Nucleospin® Nucleic Acid Purification Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) following the manufacturer's protocol. Forward primer 8FPL [47] and reverse primer 806R [48] were used to amplify the 16S rDNA fragment of the extracted genomic DNA per isolate. Components of the PCR mix per reaction were as follows: 4.0 μL of 5× PCR buffer with $MgCl_2$, 0.4 μL 10 mM dNTPs, 1.0 μL each of 10 μM 8FPL and 806R primers, 0.08pl 5 U/μL GoTag (Promega Corporation, Madison, USA) DNA Polymerase, 12.52 μL sterile distilled deionized water, and 1.0 μL template DNA. PCR conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles of 94°C, 55°C, 72°C at 1 min each, and final extension at 72°C for 7 min.

Amplified 16S rDNA fragments were purified following the NucleoTrap Gel Extraction and PCR Purification Protocol (Clontech Laboratories). Purified products of 16S rDNA gene fragment were sequenced under BigDye™ (Applied Biosystems, Foster City, CA, USA) terminator cycling conditions; the reacted products were purified by using ethanol precipitation, and run using Automatic Sequencer 3730xl (Applied Biosystems) at Macrogen Inc. (Seoul, South Korea). Sequences were checked using Chromas v. 1.45 (Conor McCarthy, Griffith University, Australia) and homology search was performed using BLAST [1]. Phenotypic characteristics of some representative strains were evaluated using API20E strips for Gram-negative enteric bacteria (Biomerieux, Marcy l'Etoile, France) to establish a range of biochemical reactions of the isolates within species or genus.

Culture of Bacterial Endosymbionts. Preparation of bacterial cultures for PSP toxin analysis was done by using the two-step scale-up microbial monoculture technique described by Gallacher *et al.* [14]. Briefly, the first step involved the inoculation of 30-mL portions of sterile marine broth (MB) (Pronadisa, Spain) from fresh working slant cultures of test bacterial isolates. The inoculated MB cultures were then incubated at 25°C for 18–24 h. The second step of the scale-up procedure involved the transfer of 10-mL aliquot portions of incubated MB cultures into eight 500-mL volumes of sterile MB in 2-L flasks. These MB flasks were then incubated at 25°C for 18–24 h. Cell density of the microbial cultures was determined by using a hemocytometer counting chamber (Neubauer, Germany) under 100× magnifications after the required incubation.

Cell Harvest and Toxin Extraction. Bacterial cells were harvested from the MB cultures and prepared for toxin analysis by using the modified technique described by Martins *et al.* [29]. Microbial cells from the MB cultures per test isolates were harvested via centrifugation at 3000 × *g* for 10 min using Survall Rotor Speed Centrifuge (Survall, Germany). Microbial pellets were washed with sterile seawater and collectively centrifuged per test isolate. Harvested cells were then extracted with 10 mL of 0.1 N acetic acid. Cell densities per test bacterial isolate in acetic acid medium were adjusted to a final concentration of about 10^7 bacterial cells/mL. The suspension was then sonicated (Sanyo Soniprep 150, UK) at 20 kCyc for 10 min [13]. The sonicated suspension was then centrifuged and passed through a 0.4-μm filter membrane to remove artifacts and obtain toxin acid extract. The toxin acid extracts were stored at −20°C for not more than a week before analysis.

High-Performance Liquid Chromatography for Toxin Analysis. Toxin extracts obtained were analyzed via the high-performance liquid chromatography (HPLC) method described by Oshima [35]. The HPLC analysis

was conducted at the Biochemistry and Toxinology Laboratory at the Marine Science Institute, University of the Philippines, Diliman, Quezon City, Philippines. Separations were performed on C8-bonded reverse-phase Silicagel column using isocratic elution profiles. Toxins were converted to fluorescent derivatives by postcolumn oxidation and was detected at a wavelength of 390 nm following excitation at 330 nm. Profiling and quantification of extracted toxins were based on comparisons with reference standards saxitoxin (stx) and neo stx.

Mouse Bioassay. Mouse bioassay was carried out by using the standard technique described by the Association of Official Analytical Chemists [3]. Laboratory test mice weighing 18–21 g were intraperitoneally injected with 1 mL of the toxin acid extract. Control was run by using mice infected with 0.1 N acetic acid used as standard. All physiologic reactions of injected mice were recorded for 24 h. Mouse bioassay was performed for three trials.

Results

Bacterial Endosymbionts. Table 1 shows the 16S rDNA identification of the 25 bacterial endosymbionts that were isolated in the study. The sequencing of the 16S rDNA gene of the endosymbionts yielded 94–100% homology to known bacterial 16S rDNA sequences in the Genbank database using BLAST [1]. Identified microorganisms included *Moraxella* spp. (nine isolates), *Bacillus* spp. (seven isolates), *Erythrobacter* spp. (four isolates), *Micrococcus* spp. (three isolates), *Pseudomonas putida* (one isolate), and *Dietzia maris* (one isolate).

Phenotypic characteristics inclusive of Gram reactions, colonial characteristics, cell morphology, and biochemical reactions of major group of endosymbionts (with ≥4 isolates per species or genus) are shown in Table 2, whereas that of the minor isolates are shown in Table 3. Generally, Gram-negative rod bacteria were isolated as bacterial endosymbionts from the test dinoflagellate. Majority of the isolated Gram-negative rods were oxidase-positive. The few Gram-positive isolates included *Bacillus* spp. of the Phylum Firmicutes, and members of the of the Phylum Actinobacteria class *Actinobacteria–Micrococcus* spp. and *D. maris*. *D. maris* uniquely exhibited distinct orange coloration on the MA plate. The range of biochemical reactions of the isolates is presented in Tables 2 and 3.

PSP Toxin Production of Bacterial Endosymbionts. *Moraxella* spp., *Erythrobacter* spp., and *Bacillus* spp. were shown to contain stx, neo stx, or both at levels ≤73 ng toxin/10^7 bacterial cells (Table 4). All the representative strains of the three genera elicited PSP-like poisoning

Table 1. 16S rDNA identification of bacterial endosymbionts from axenic culture of *Pyrodinium bahamense* var. *compressum* (MZ RVA 042495) using BLAST

		16S rDNA	
Trial	*Isolate code*	*Identification*	*% Identity (base pair alignment)*
Trial 1	0301	*Moraxella* sp.	98% (703/717)
	0302	*Moraxella* sp.	98% (703/717)
	0303	*Moraxella* sp.	98% (703/717)
	0304	*Pseudomonas putida*	100% (767/767)
	0305	*Erythrobacter citreus*	98% (535/542)
	0306	*Erythrobacter* sp.	98% (481/490)
	0307	*Micrococcus luteus*	99% (744/750)
	0308	*Micrococcus* sp.	99% (692/696)
	0309	*Bacillus flexus*	97% (724/740)
	0310	*Bacillus flexus*	98% (581/589)
	0311	*Bacillus cereus*	97% (682/697)
	0312	*Bacillus* sp.	98% (768/783)
	0313	*Bacillus* sp.	96% (724/747)
	0314	*Bacillus* sp.	96% (524/555)
Trial 2	0501	*Moraxella* sp.	98% (706/732)
	0502	*Moraxella* sp.	98% (706/732)
	0503	*Moraxella* sp.	98% (706/732)
	0504	*Moraxella* sp.	98% (706/732)
	0505	*Moraxella* sp.	98% (644/655)
	0506	*Moraxella* sp.	98% (646/658)
	0507	*Erythrobacter flavus*	99% (719/722)
	0508	*Erythrobacter flavus*	99% (719/722)
	0509	*Micrococcus* sp.	99% (744/750)
	0510	*Dietzia maris*	97% (635/648)
	0511	*Bacillus cereus*	99% (724/740)

Table 2. Phenotypic characteristics of major isolated bacterial endosymbionts of *Pyrodinium bahamense* var. *compressum* (MZ RVA 042495)

	Moraxella spp.				*Erythrobacter spp.*			*Bacillus spp.*		
Characteristics	*0301*	*0501*	*0502*	*0503*	*0306*	*0507*	*0508*	*0314*	*0315*	*0511*
Color[a]	YO	C	C	Y	YO	YO	YO	C	W	C
Gram reaction	−	−	−	−	−	−	−	+	+	−
Cell morphology[b]	R	R	R	R	R	R	R	R	R	R
Biochemical reactions[c]										
Oxidase	+	+	+	+	+	+	+	+	−	+
O-Nitrophenyl-phenyl-β-D-galactopyranoside	+	−	−	−	+	−	+	−	+	+
Arginine dihydrolase	−	−	+	−	+	−	+	−	−	+
Lysine decarboxylase	−	−	−	−	−	−	−	−	−	−
Ornithine decarboxylase	−	−	−	−	−	−	−	−	−	+
Citrate utilization	+	+	−	+	+	−	+	+	+	+
H_2S production	−	−	−	−	−	−	−	−	−	−
Urease	−	−	+	−	−	−	−	−	−	−
Tryptophane deaminase	−	−	+	−	−	+	+	−	−	−
Indole production	−	−	−	−	−	−	−	−	−	−
Voges-Proskauer	+	+	−	+	+	+	+	+	+	+
Gelatin hyrolysis	+	+	−	+	+	+	−	−	+	+
Glucose fermentation	−	−	−	−	+	−	−	−	+	−
Mannitol fermentation	−	−	−	−	−	−	−	−	−	−
Inositol fermentation	−	−	−	−	−	−	−	−	−	−
Sorbitol fermentation	−	−	−	−	−	−	−	−	−	−
Rhamnose fermentation	−	−	−	−	−	−	−	−	−	−
Sucrose fermentation	−	−	−	−	+	−	−	−	−	−
Melibiose fermentation	−	−	−	−	−	−	−	−	−	+
Amygdalin fermentation	−	−	−	−	−	−	−	−	−	−
Arabinose fermentation	−	−	−	−	−	−	−	−	−	−

[a]YO: yellow orange; Y: yellow; C: cream; W: white.
[b]R: rods.
[c]Biochemical reaction: positive (+) or negative (−) using API20E strips for Gram-negative enteric bacteria.

reactions in test animals used in the mouse assay. However, the HPLC analysis of *Micrococcus* spp., *D. maris*, and *P. putida* did not record any stx nor neo stx content per 10^7 bacterial cells. *Micrococcus* spp., meanwhile, elicited lethal reactions in mouse assay although no stx and neo stx was recorded in the HPLC analysis.

Discussion

The exact role or contribution of bacterial symbionts in toxin production and bloom formation of toxic dinoflagellates is still not fully understood considering that several studies have already been conducted to address these issues. Although a number of reports have cited the autonomous toxin production from bacterial symbionts of toxic dinoflagellates [8, 11, 14, 25, 26], Martins *et al.* [29] have nonetheless reported inconsistent findings using reported analytical and biological assays to determine PSP toxin production from bacterial strains isolated from toxic dinoflagellates. Córdova *et al.* [9] also reported a phenomenon that was termed *a bloom inside a bloom* involving uncontrolled growth of bacterial endosymbionts in dinoflagellates during blooms, starting from the stationary phase up to the eventual demise of the bloom.

Majority of identified bacteria from marine environments belong to the Phylum Proteobacteria [16]. Similarly, among the culturable microorganisms identified in this present study are members of the Phylum Proteobacteria, which included *Moraxella* spp. (nine isolates), *Erythrobacter* spp. (four isolates), and *P. putida* (one isolate). Other isolates belonged to the Phylum Firmicutes, *Bacillus* spp. (seven isolates), and some members of the Phylum Actinobacteria, *Micrococcus* spp. (three isolates) and *D. maris* (one isolate). The Phylum Proteobacteria include organisms that are evolutionarily related to each other, but have so evolved that among the few characteristic common to them is their Gram-negative cell wall [36]. There are five subgroups of Proteobacteria based on gene sequences: α, β, γ, δ, and ε [36].

The genera *Moraxella* and *Pseudomonas* belong to the γ-Proteobacteria class, which was reported to form majority of the identified culturable marine bacteria [16]. *Erythrobacter* spp., together with *Roseobacter* spp. belonging to the α-Proteobacteria class, has been more recently described to form part of the microbial ecology of the marine environment via molecular identification techniques that are not based on classical bacterial cultural

Table 3. Phenotypic characteristics of minor isolated bacterial endosymbionts of *Pyrodinium bahamense* var. *compressum* (MZ RVA 042495)

	Micrococcus spp.			*Pseudomonas putida*	*Dietzia maris*
Characteristics	*0307*	*0509*	*0308*	*0304*	*0510*
Color[a]	Y	LY	C	C	O
Gram reaction	+	+	+	−	+
Cell morphology[b]	C	C	C	R	R
Biochemical reactions[c]					
Oxidase	+	+	+	+	+
O-Nitrophenyl-phenyl-β-D-galactopyranoside	−	−	−	+	+
Arginine dihydrolase	−	−	−	+	−
Lysine decarboxylase	−	−	−	−	−
Ornithine decarboxylase	−	−	−	+	−
Citrate utilization	+	−	+	+	−
H_2S production	−	−	−	−	−
Urease	−	−	−	−	+
Tryptophane deaminase	−	+	−	−	−
Indole production	−	−	−	−	−
Voges-Proskauer	+	+	+	−	+
Gelatin hyrolysis	+	+	−	+	−
Glucose fermentation	−	+	−	+	−
Mannitol fermentation	−	+	−	−	−
Inositol fermentation	−	+	−	−	−
Sorbitol fermentation	−	+	−	−	−
Rhamnose fermentation	−	+	−	−	−
Sucrose fermentation	−	−	−	−	−
Melibiose fermentation	−	+	−	+	−
Amygdalin fermentation	−	+	−	−	−
Arabinose fermentation	−	+	−	+	−

[a]Y: yellow; LY: light yellow; C: cream; O: orange.
[b]R: rods; C: cocci.
[c]Biochemical reaction: positive (+) or negative (−) using API20E strips for Gram-negative enteric bacteria.

procedures [31]. Hold *et al.* [19] stated that some dinoflagellates have their own distinctive associated bacterial communities and these are mostly α- and β-Proteobacteria. Jasti *et al.* [22] provided evidence for the specificity of bacterium–phytoplankton associations, especially between toxic dinoflagellates and members of the Roseobacter and Erythrobacter clades.

Using BLAST, we noted that nine isolates in this study yielded 98–99% 16S rDNA sequence homologies to *Moraxella* spp. In fact, these endosymbionts exhibited high sequence homology with the bacterial strain isolated by Kodama [25] in 1990 from *Protogonyaulax tamarensis*, and a bacterial strain identified as member of the α-Proteobacteria by Groben *et al.* [18]. Kodama [25] and Cordova *et al.* [8] have also previously reported the isolation of *Moraxella* spp. as bacterial endosymbionts from toxigenic dinoflagellates *P. tamarensis* and *Alexandrium catenellalatum*, respectively. Kodama [25] reported the ability of a strain of *Moraxella* sp. to produce GTX 1 and 4, which are in fact the major toxins normally associated with *P. tamarensis*.

Four isolates in the study also showed a 98–99% sequence homology to members of *Erythrobacter*. These isolated strains included *Erythrobacter flavus* (two isolates), *E. citreus* (one isolate), and *Erythrobacter* spp. (one isolate). All reported *Erythrobacter* spp. with validly published names have been isolated in marine environments [10, 21, 50–52]. In a study by Jasti *et al.* [22], some bacterial strains having a high homology with *Erythrobacter* spp. were also shown to be isolated from vegetative cells of the dinoflagellates *Skeletonema costatum* and *Chaetoceros* cf. *tortissimus*. The genus *Erythrobacter* is a member of the aerobic anoxygenic phototrophic α-Proteobacteria [53].

One isolate in the study yielded 100% 16S rDNA sequence homology with *P. putida*. *Pseudomonas* spp. has been reported to be to be closely associated with HAB causing organisms [8, 12, 17]. A strain of *P. putida*, in particular, was reported by Cordova *et al.* [8] to have been isolated from *A. catenella*. Plumley *et al.* [37] were able to isolate a strain of *Pseudomonas stutzeri* that could be transformed to accumulate PSP toxins and increase toxin production when added to axenic cultures of *A. lusitanicum* when grown under suitable laboratory conditions.

Among the Gram-positive rods isolated in the study, seven strains exhibited a 94–98% sequence homology with *Bacillus* spp. Heterotrophic *Bacillus* strains are hardly considered to be species of any distinct habitats because of their ubiquity in diverse environments and their ability to survive under adverse culture con-

Table 4. PSP toxin content and clinical manifestations in mouse bioassay of isolated bacterial endosymbionts from axenic culture of *Pyrodinium bahamense* var. *compressum* (MZRVA 042495)

Endosymbionts	*Isolate code*	*PSP toxin content (ng toxin/ 10^7 bacterial cells)*		*Clinical manifestation of mouse bioassay (17–21 g ICR male mouse; 10^7 bacterial cells per acid extract injected)*
		STX	*Neo STX*	
Moraxella sp.	0502	56	ND	Lethargy Abdominal contractions Death ≥5 h 100% mortality
	0504	71	29	Lethargy Abdominal contractions Paralysis of hind limbs Death ≥1.5 h 100% mortality
Erythrobacter flavus	0507	60	ND	Lethargy Abdominal contractions Paralysis of hind limbs Death ≤1 h 100% mortality
	0508	ND	51	Lethargy Abdominal contractions Paralysis of hind limbs Death ≤1.5 h 100% mortality
Dietzia maris	0510	ND	ND	Lethargy Recovery within 2 h
Micrococcus sp.	0509	ND	ND	Lethargy Abdominal contractions Death ≥5 h 100% mortality
Bacillus sp.	0511	73	ND	Lethargy Abdominal contractions Death ≥5 h 100% mortality
Pseudomonas putida	0304	ND	ND	Uneventful

ND: not detectable; PSP: paralytic shellfish poisoning.

ditions [6, 20]. Most common species of aquatic *Bacillus* spp. include *B. marinus*, *B. subtilis*, and *B. cereus*, which were reported to be in the Pacific Ocean [20]. Zheng *et al.* [54] isolated a marine *Bacillus* strain (S_{10}) from sediments in the Western Xiamen Sea, which was established to significantly impact the growth of *A. tamarense* and its PSP production under controlled experimental conditions.

The possible association of *P. bahamense* var. *compressum* with *Bacillus* spp. could theoretically be related to the dinoflagellates' existence in sediments during their dormant cysts state, and likewise related with resuspension of the cyst by turbulence during the primary stages of bloom formation. The life cycle of *P. bahamense* var. *compressum* includes a dormant cyst stage that is characteristic of most bloom-forming dinoflagellates [2, 44]. This signifies that the cells spend some part of their lives in a resting state in the sediments [2] where *Bacillus* spp. might be found. During resuspension by turbulence, possible close interfacing of upwelled dinoflagellate cysts with marine sediments containing *Bacillus* sp. is a strong possibility.

In this study, by using BLAST, we were able to isolate three bacterial strains that exhibited a 96–99% sequence homology with *Micrococcus* spp. *Micrococcus* spp., another Gram-positive, aerobic bacterium, is a member of the Micrococcaceae family. In a similar study by Hold *et al.* [19], a previously isolated bacterial strain from *Alexandrium* spp. [14] was found to be closely related to *Micrococcus luteus* as identified by 16S rDNA sequence analysis.

Another Gram-positive bacterial strain isolated in the study exhibited a 98% sequence homology with *D. maris*. Colquhoun *et al.* [7] isolated bacteria species from deep-sea sediments collected from both the Izu Bonin Trench and from the Japan Trench, which also showed close homology with *D. maris*. Strains of *D. maris* were reported to be commonly isolated from soil and from skin of intestinal tracts of carps (*Cyprinus carpio*) [38]. It was also reported that *D. maris* exhibits orange colony growth on various nutrient media including nutrient, glycerol, and wort agar substrates [38]. Similarly, an orange growth of *D. maris* on MA was observed in the

study. The reported association of *D. maris* with *P. bahamense* var. *compressum* can similarly be explained by the residence of dormant cysts of the dinoflagellate in marine sediments and also the reported close association of *D. maris* to the same environments.

PSP Toxin Production of Bacterial Endosymbionts. The study reported detectable levels of stx and neo stx for *Moraxella* spp., *Erythrobacter* spp., and *Bacillus* spp. Azanza *et al.* [5] indicated that PSP toxin profiles of naturally contaminated *Perna viridis* from the Philippines collected during *P. bahamense* var. *compressum* toxic blooms were shown to contain stx and neo stx in addition to B1 and dcstx. Similarly, Oshima [34] reported that the toxin profiles of *P. bahamense* var. *compressum* from Palau, some PSP-contaminated Philippine green mussels, and a Borneo planktinous fish included stx, neo stx, gtx5, and dcstx. Usup *et al.* [45] reported that *P. bahamense* var. *compressum* isolated from Malaysia differed from *Alexandrium* and *Gymnodium* in that it produced only neo stx, stx, gtx5-6, and dcstx as compared to the more diverse toxin profile of the other dinoflgellates. Oshima's report [34] supported the fact that *P. bahamense* var. *compressum* appears to have less diversity in toxin profile compared to *Alexandrium* sp.

Interestingly, *Micrococcus* sp. indicated toxicity in the mouse assay, although no detectable amount of saxitoxin in the HPLC analysis was established. Although *Micrococcus* is rarely known to cause morbidity and or mortality in humans, Monodane *et al.* [30] noted that this organism was recently recognized as an opportunistic pathogen and has been implicated in recurrent human bacteremia, septic shock, septic arthritis, endocarditis, meningitis, intracranial suppuration, and cavitating pneumonia in immunosuppressed patients. *M. luteus* cells and cell walls were found capable of inducing anaphylactoid reactions leading to death in C3H/HeN mice primed with muramyl dipeptide [30]. Subsequent studies attributed the cytokine-inducing activity of *M. luteus* cell walls to its teichuronic acid component [49].

Conclusion

The established taxonomic diversity of culturable intracellular microbiota associated with Philippine *P. bahamense* var. *compressum* strain MZ RVA 042595 was shown to be limited to the phyla Proteobacteria, Actinobacteria, and Firmicutes. Majority of the isolates were Gram-negative rods that were oxidase-positive belonging to known organisms mostly associated with marine environments including *Moraxella* spp., *Erythrobacter* spp., and *P. putida*. Some of the endosymbionts inclusive of *Moraxella* spp., *E. flavus*, and *Bacillus* spp. were shown to produce stx and neo stx. Isolates from these three genera, in addition to *Micrococcus* spp., elicited PSP-like physiologic reactions in test mice in the mouse assay. The study for the first time provided a possible explanation on the contribution of *P. bahamense* var. *compressum* bacterial endosymbionts in bloom toxicity and coloration. The study also suggested that the presence of bacterial endosymbionts normally associated with sediments might be explained by the exposure of dinoflagellate's cysts to sediments during their dormant cyst state and its resuspension during turbulence.

Based on these results, it is recommended that the profile of gene expression during toxin production of *P. bahamense* var. *compressum* be compared to that of bacterial endosymbionts shown to produce toxins in this study by using differential display [27] and/or microarray procedures. Further studies using culture-independent molecular techniques can be used in the future to characterize the complete microbiota of *P. bahamense* var. *compressum* by removing bias toward culturable isolates only.

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