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Toxicity and Protein Expression of *Alexandrium* Species Collected in the Philippine Waters

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Isolates of *Alexandrium* species collected in the Philippine waters were examined during the exponential growth phase to compare their toxicities and protein expression profiles, and also to correlate protein expression with toxin production. Molecular methods showed that *Alexandrium* cf. *pacificum* was genetically divergent from *Alexandrium tamarense* complex Group IV/ *Alexandrium pacificum*. Toxin analyses using pre-oxidation method prior to HPLC purification were carried out to determine saxitoxin (STX), neosaxitoxin (neoSTX), and gonyautoxins 1-4 (GTX) levels. This study showed that cultured *Alexandrium affine* collected in Honda Bay, Palawan produced different STX analogs at various times of culture, which differed from other results showing that this species was non-toxic. The cultured *Alexandrium* cf. *pacificum* collected in Anda Channel (ATANDA) was two-fold more toxic than the cultured *Alexandrium* cf. *pacificum* collected in Bolinao Channel (ATBOL). Comparative protein expression analyses using 2-D gel electrophoresis were performed for the two *Alexandrium* cf. *pacificum* strains (ATANDA and ATBOL) during the exponential growth phase. A unique 2-DE protein spot in ATANDA showed sequence homology with bifunctional ornithine acetyltransferase/ N-acetylglutamate synthase (ArgJ) that has a role in the biosynthesis of arginine, a precursor in STX biosynthesis. The greater detectable expression of such enzyme in the ATANDA strain was correlated with the greater toxicity, suggesting the enzyme's major participation in toxin biosynthesis.

Key words: *Alexandrium* sp., harmful algal bloom, paralytic shellfish poisoning, paralytic shellfish toxin, proteomics, saxitoxin

INTRODUCTION

Research efforts have been geared towards the understanding of the major causative agents of paralytic shellfish poisoning (PSP) in the Philippines due to the numerous cases of PSP leading to human fatalities. It has been reported that majority of PSP cases in Southeast Asia occurred in the Philippines due mainly to blooms of dinoflagellates such as *Pyrodinium bahamense* and *Alexandrium* sp. (Yñiguez et al. 2012, Azanza & Benico 2013).

Paralytic shellfish toxins (PSTs) consist of saxitoxin (STX) and its analogs that differ in side group moieties and in their specific toxicities. The parent compound, STX, has a tetrahydropurine moiety with a five-membered

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ring bonded at an angular position, a ketone hydrate, and two guanidinium groups. Various STX derivatives are produced by the addition of hydroxyl or hydroxysulfate groups at C11, decarbamoylation to a hydroxyl function at C17, *N*-hydroxylation at N1, or *N*-sulfation at N21. Due to the substitutions on the STX structure resulting in variations in the charge state, the PSTs bind with different affinities to site 1 of Na^+ channels, leading to different toxicities, with the carbamoyl derivatives being the most toxic and the *N*-sulfocarbamoyl derivatives the least toxic (Dell'Aversano et al. 2008, Ballot et al. 2016).

Proteomic approach has been used to analyze the entire proteome of a cell in the study of dinoflagellates. The initial development of proteomics focused on 2-dimensional gel electrophoresis (2-DE) to separate protein constituents of cells. It is still in much use in conjunction with 2-D liquid chromatography-mass spectrometry. Applications of 2-DE proteomics in HAB research include differentiation between toxic and nontoxic HAB-causing species and the proteins involved in toxin biosynthesis (Chan et al. 2004, 2005, 2006, Lee & Lo 2008, Wang et al. 2013). Proteomics in tandem with toxin analysis have enabled the identification of potential biomarkers of toxicity. These biomarkers of toxicity have been utilized to detect toxic algae in routine monitoring programs and in the prediction of bloom development and movement (Chan et al. 2005, 2006).

The toxicity and protein expression analyses of *Alexandrium affine* and *Alexandrium* cf. *pacificum* isolates were performed. Various PSTs (STX, neoSTX, and GTX1-4) produced by *Alexandrium affine* and *Alexandrium* cf. *pacificum* grown under optimal laboratory conditions were determined. The protein profiles of the two *Alexandrium* cf. *pacificum* strains were compared and a uniquely expressed protein was identified. The strain of *Alexandrium affine* was originally collected in Honda Bay, Palawan in 2005 (AAHB; code name: AlexHBRVA102905), and the two strains of *Alexandrium* cf. *pacificum* were originally collected in Anda Channel, Pangasinan in 2010 (ATANDA; code name: ATANDARVA04210) and Bolinao Channel, Pangasinan in 2011 (ATBOL; code name: ATBOLRVA031711). This is a pioneering work in the use of 2-DE as a proteomic approach in the study of harmful algal bloom (HAB) species collected in the Philippines.

METHODS

Culture of dinoflagellates

The *Alexandrium* spp. isolates (AlexHBRVA102905, ATANDARVA04210 and ATBOLRVA031711) have been continuously maintained in the laboratory using modified F/2 medium (Guillard & Ryther 1962). From these cultures, subcultures of *Alexandrium affine* (AAHB) and *Alexandrium* cf. *pacificum* strains (ATANDA and ATBOL) were maintained in F/2 culture medium (Azanza-Corrales & Hall 1993) at 24° C (±2). *Alexandrium affine* was maintained at 80-130 $\mu Em^{-2}s^{-1}$ (Nguyen-Noc 2004), while the two *Alexandrium* cf. *pacificum* strains were maintained at 200 ± 50 $\mu Em^{-2}s^{-1}$ (Hamasaki et al. 2001). Cell counts were taken every 4-5 days to monitor growth. For both toxin and proteome analyses, cells were harvested during the exponential growth phases at 12th to 14th day. Approximately 2.0 x 10^6 cells at exponential phase were obtained. The cells were centrifuged at 500 x g twice at 4° C for 30 min. Cells were washed with sterile filtered seawater and stored in cryovials at -80° C until further use.

Morphological characterization of dinoflagellates

Cells were preserved in 2% glutaraldehyde and harvested by centrifugation at 2,095 x g for 5 min. They were stained with 1% calcofluor white (Fritz & Triemer 1985) and examined under a confocal laser scanning microscope capturing stack scans which were reconstructed into 3D images. Thecal configuration and other taxonomic features of *Alexandrium* species were identified based on the taxonomic keys of Balech (1995), Tomas (1997), and Fukuyo (2001).

Molecular characterization of dinoflagelates

Extraction of total genomic DNA from dinoflagellate samples, PCR amplification, and purification of amplicons were performed as described in Onda et al. (2013). The universal primer pairs DinFi (5'-GCATATAAG TAMGYGGWGG-3') and DinRi (5'-CCGTGTTTCAAGACGGGTC-3') were used for the amplification of 28S rDNA (LSU) genes following the conditions described by Logares et al. (2007). Purified amplicons were sent to 1st Base Laboratories SDN BHD (Malaysia) for single pass Sanger sequencing.

Contigs were built from the generated sequences using DNA Baser Sequence Assembler and subjected to NCBI database search for most similar sequences using BLAST (Altschul et al. 1990). The reference and related sequences were downloaded from GenBank and aligned with the generated contigs using the ClustalW in MEGA v. 5.0. The alignment was used to build a phylogenetic tree using Maximum Likelihood (ML) method (Tamura et al. 2011, http://www.megasoftware.net/).

Analysis of toxins

Triplicates of toxin analysis were performed for each of two replicates of biological samples. The toxicity values were determined based on the toxin pre-oxidation method (Lawrence & Ménard 1991, Lawrence & Niedzwiadek 2001). Periodate analysis was performed for the detection

of STX, neoSTX, and GTX1,4, while peroxide analysis was performed for the detection of STX and GTX2,3. Each mixture was applied on C18 reverse phase HPLC column (5 µm, 4.6 x 250 mm) with 1.0 M ammonium formate (pH 6.0) and 5% acetonitrile (ACN) at a flow rate of 0.8 mL min^{-1}. Toxin standards were similarly tested. Oxidation products were monitored using fluorescence detector set at wavelengths 330 nm (excitation) and 400 nm (emission).

Extraction and quantitation of proteins

Proteins were extracted by using urea-triton X-100 buffer with TCA/acetone precipitation (Wang et al. 2009), and with protease inhibitor cocktail (1% v/v). The pellet was lysed on ice with ultrasonic probe at 60 Hz for 3 min in 5 s bursts, and sample aliquot was observed under a microscope to confirm lysis. The sample was centrifuged at 15000 x g for 30 min at 4° C, and the supernatant was transferred to a microcentrifuge tube.

Proteins were precipitated by 500 µL of freshly prepared 20% (w/v) TCA/acetone at 4° C for 30 min. The pellet was collected by centrifugation at 15000 x g for 30 minutes at 4° C, washed in 1 mL of cold acetone with 20 mM DTT, and then air-dried. Twenty microliters of 0.2 M NaOH was added for fast solubilization (Nandakumar et al. 2002). Rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.4% (w/v) ampholytes (pH 3-10), and 0.2% DTT (Lee & Lo 2008) was added up to 500 µL. A modified Bradford assay (Kruger 2009) was used to quantify the proteins, with ovalbumin (5 mg mL^{-1}) or bovine serum albumin (1 mg mL^{-1}) as standard.

Two-dimensional polyacrylamide gel electrophoresis

For 2-DE, two replicates for each strain were performed with triplicate 2-DE runs for each biological replicate. Isoelectric focusing (IEF) was performed (Lee & Lo 2008) with a linear ramp on an IEF system. Protein (100 µg) was loaded onto the immobilized pH gradient (IPG) strips of pH 4-7 (17 cm), which were actively rehydrated at 50 V for 16 h. Electrode wicks were inserted between the strips and the IEF cell electrodes. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using 12.5% polyacrylamide gels. The 2-DE standards were run along with the samples at 12-15 mA per gel for 5-8 h, or until the bromophenol dye indicator was ~1 cm from the gel bottom. The gels were silver stained (Gromova & Celis 2006), and gel images were captured using GS-800 densitometer and analyzed using PDQuest software v.8.0.1 with Gaussian fitting and spot identification threshold of 95%.

Extraction and in-gel digestion with trypsin

The destained gel pieces were rinsed with water, 50% ACN, 10 mM ammonium bicarbonate, and dehydrated with 100% ACN. The gel plugs were rehydrated with 12.5 ng µl $^{-1}$ trypsin in 10 mM ammonium bicarbonate (~50 µl). After incubation for 16 h at 37° C, the peptides were extracted twice using 5% formic acid (FA)/50% ACN and once with 100% ACN. After drying in a vacuum concentrator, the peptides were resuspended in 0.1% FA, and purified using µC-18 Ziptips. One microliter of the peptide was spotted before applying 1 µL of 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid matrix dissolved in 0.1% FA/50% ACN (Shevchenko et al. 2006).

Mass spectrometry

Determination of peptide mass was outsourced at the Centre for Genomic Sciences, University of Hong Kong. The 4800 MALDI-TOF/TOF Analyzer equipped with a Nd:YAG laser at 355 nm was used. Mass spectra were acquired in positive ion reflector mode using the 4000 Series Explorer version 3.5.28193 software. The MS and tandem mass spectrometry (MS/MS) were calibrated with the peptide calibration standard, 4700 Cal-Mix. The sample was analyzed to create peptide mass fingerprint (PMF) data (scanning range 900-4000 m/z).

Homology-driven identification of protein

A modified multi-layered bioinformatics approach (Wang et al. 2011) was adapted for protein identification. The combined PMF and MS/MS search was done using GPS Explorer algorithm version 3.6 against the non-redundant NCBI database and the in-house MASCOT search engine version 2.2 at 95% confidence interval. Low confidence hits using MASCOT analysis were subjected to automated de novo peptide sequencing using PEAKS 7.0.2 software. Candidate sequences from the MS/MS spectra were submitted for MS BLAST search following the automated interpretation of MS/MS spectra (Shevchencko et al. 2003). MS BLAST searches were then queried at the online website http://genetics.bwh.harvard.edu/msblast/ (Shevchenko et al. 2001, 2003). The MS BLAST search results with high confidence and high-scoring segment pairs, a high score of at least 60 and total score of 100 were considered for protein identification.

Protein interaction network analysis of the identified protein was generated using STRING database analysis from the online website: http://string-db.org/ (Szklarczy et al. 2014).

RESULTS

Growth curves of cultures

Alexandrium affine (AAHB) and the two strains of *Alexandrium* cf. *pacificum* (ATANDA and ATBOL) are healthy cultures with exponential growth rates of 0.3-0.5/d and similar growth curve patterns. The data are consistent

with the growth rate of other healthy cultures, such as that of *Alexandrium tamarense* that had exponential growth rate of ~0.5/d (Wang & Hsieh 2005).

Identification of *Alexandrium* species

The *Alexandrium* spp. isolates were identified as *Alexandrium affine* (AAHB) and *Alexandrium* cf. *pacificum* (ATANDA and ATBOL) based on their morphological and molecular characteristics. *Alexandrium* cf. *pacificum* (Figure 1A-C) is a pentagonal shaped cell with a mean length of 24.15 ± 2.76 μm (n=10) and a mean transdiameter of 21.62 ± 3.17 μm (n=10). The first apical (1') plate is connected to the apical pore complex and does not have a ventral pore. The connecting pore when present is located on the left side of the foramen. *Alexandrium affine* (Figure 1D-F) is solitary and has a pentagonal shape with a mean length of 24.96 ± 2.18 μm (n=10) and a mean transdiameter of 21.46 ± 2.17 μm (n=10). The 1' plate is connected to the apical pore complex and has a ventral pore on the right margin of the 1' plate. It is easily distinguishable from other *Alexandrium* species by the presence of a connecting pore on top of the pore plate.

Three 28s rDNA (D1-D2 domains) sequences were obtained from *Alexandrium affine* (AAHB) and *Alexandrium* cf. *pacificum* (ATANDA and ATBOL). A matrix of 44 aligned sequences were 581 bp long, with 233 conserved sites, 348 variable sites, and 159 parsimonous informative sites. The phylogenetic tree inferred by ML analysis showed that both ATANDA and ATBOL fall within *Alexandrium* cf. *pacificum* of *Alexandrium tamarense* complex while AAHB was clustered with *Alexandrium affine* with strong bootstrap support (Figure 2).

Toxicity values

The differential toxicities of STX analogs were reported as STX eq based on conversion factors as follows: STX = 1, neoSTX = 0.92, GTX 1= 0.99, GTX2 = 0.36, GTX3 = 0.64, and GTX4 = 0.73 (Oshima 1995). A culture of *Alexandrium affine* (AAHB1) had STX and neoSTX as the major toxins with toxicity values of 7.08 and 12.41 fmol STX eq cell^{-1}, respectively, and a total toxicity value of 19.49 fmol STX eq cell^{-1}. Another culture (AAHB2) was found to have a different toxin profile showing only GTX1,4 as its major toxins with a toxicity value of 9.32 fmol STX eq cell^{-1}.

Both *Alexandrium* cf. *pacificum* (ATANDA and ATBOL) strains produced GTX1-4. Based on the identified toxins, ATANDA strain was two-fold more toxic than ATBOL strain, with total GTX1,4 and GTX2,3 contents of 118.75 and 60.16 fmol STX eq cell^{-1} for ATANDA and ATBOL, respectively. The ATANDA strain produced GTX1,4 and GTX2,3 with toxicity values of 77.58 and 41.17 fmol STX

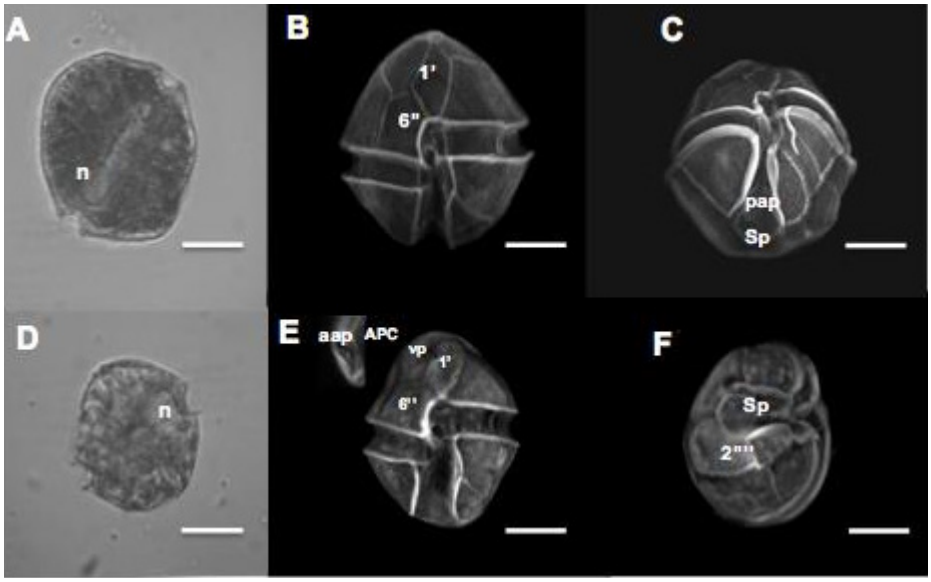

Figure 1. (A-C) *Alexandrium* cf. *pacificum*: (A) Differential Interference Contrast (DIC) image showing the roundish to pentagonal shape and centrally located nucleus (n); (B) Laser micrograph in ventral view showing the first apical plate (1') without ventral pore (vp) and wider than longer sixth precingular plate (6''); (C) Antapical view showing isodiametric and rhomboidal posterior sulcal plate (Sp) and posterior attachment pore (pap). (D-F) *Alexandrium affine*: (D) DIC image showing the centrally located nucleus (n) (E) Laser micrograph in ventral view showing the first apical plate (1') with ventral pore (vp) and wider than longer sixth precingular plate (6''). Apical pore complex (APC) with anterior attachment pore (aap) located directly above the foramen; (F) Antapical view showing the isodiametric posterior sulcal plate (Sp) and second antapical plate (2''''). Bar = 10 μm.

Figure 2. Phylogenetic tree inferred from maximum likelihood (ML) analysis of *Alexandrium* cf. *pacificum* and *Alexandrium affine* based on 28s rDNA (D1-D2 domains, LSU) sequences. Numbers near the branches correspond to ML bootstrap per cent values. ATANDARVA04210 (ATANDA), *Alexandrium* cf. *pacificum* from coastal waters of Anda, Pangasinan; ATBOLRVA031711 (ATBOL), *Alexandrium* cf. *pacificum* from coastal waters of Bolinao, Pangasinan; AlexHBRVA102905 (AAHB), *Alexandrium affine* from Honda Bay.

eq cell^{-1}, respectively, whereas ATBOL produced GTX1,4 and GTX2,3 with toxicity values of 53.34 and 6.82 fmol STX eq cell^{-1}, respectively.

Proteome profiles and protein characterization

The higher toxicity value of ATANDA led us to compare its proteome profile with that of ATBOL. Based on PDQuest-assisted analyses of protein spots generated by 2-DE, there were 1,285 and 1,006 protein spots for ATANDA and ATBOL, respectively. Among these protein spots, 616 were common in both strains, 669 and 390 spots were unique in ATBOL and ATANDA, respectively. At the regions below 20 kDa, similar protein spots were present in both strains except for a unique At01 (Figure 3) that has an apparent molecular weight of 16.5 kDa and pI of 6.4.

MALDI-TOF analysis and subsequent MS/MS analysis of precursor ions of At01 were performed. Five precursor ions have masses of 957.6197 m/z, 969.6241 m/z, 1734.0612 m/z, 1889.1364 m/z, and 1902.2542 m/z. The MS/MS data were subjected to de novo peptide sequencing using PEAKS 7.0.2 software, generating peptide sequences that were then queried using MS BLAST. This led to the identification of two internal peptide sequences from precursor ions 969.6241 m/z and 1734.0612 m/z with 100% (amino acid sequence: KGAGMLAPG) and 72% (amino acid sequence: EYNRALRAARK) identities with bifunctional ornithine acetyltransferase/N-acetylglutamate synthase based on the genome of *Streptomyces collinus* (http://www.ncbi.nlm.nih.gov/protein/WP_020938939.1).

DISCUSSION

Alexandrium sp. is a widely dispersed dinoflagellate in many coastal areas in the world, and many species produce potent toxins that can cause PSP (Anderson et al. 2012). The morphological and molecular characterization of cultured *Alexandrium affine* (AAHB) has been earlier reported (Onda et al. 2013). ATANDA and ATBOL were initially identified as strains of *Alexandrium catenella* that has now been renamed as *Alexandrium pacificum*, under the *Alexandrium tamarense* complex comprised of *A. pacificum, A. australiense, A. tamarense, A. mediterraneum*, and *A. fundyense* (John et al. 2014). Moreover, *Alexandrium pacificum* was formerly known as *Alexandrium tamarense* complex Group IV (Lilly et al. 2007) comprised mostly of *Alexandrium catenella* morphotypes from Japan, Korea, China, and Hong Kong. The sampled strains have close morphological resemblance to this group but still form significant genetic divergence; hence they are referred to as *Alexandrium* cf. *pacificum* strains. More detailed morphological and phylogenetic analyses of *Alexandrium* cf. *pacificum* will be reported in another paper, documenting that it is an in-clade of *Alexandrium pacificum* Litaker but significantly divergent from this group. Hence, a novel clade – *Alexandrium* cf. *pacificum* Group VI (Tropical Asian Clade) – is proposed.

The toxin profiles of two separate cultures of *Alexandrium affine* (AAHB1 and AAHB2) were determined because of the ambiguity of previous reports with regard to the toxicity of this species. The initial culture (AAHB1) produced STX

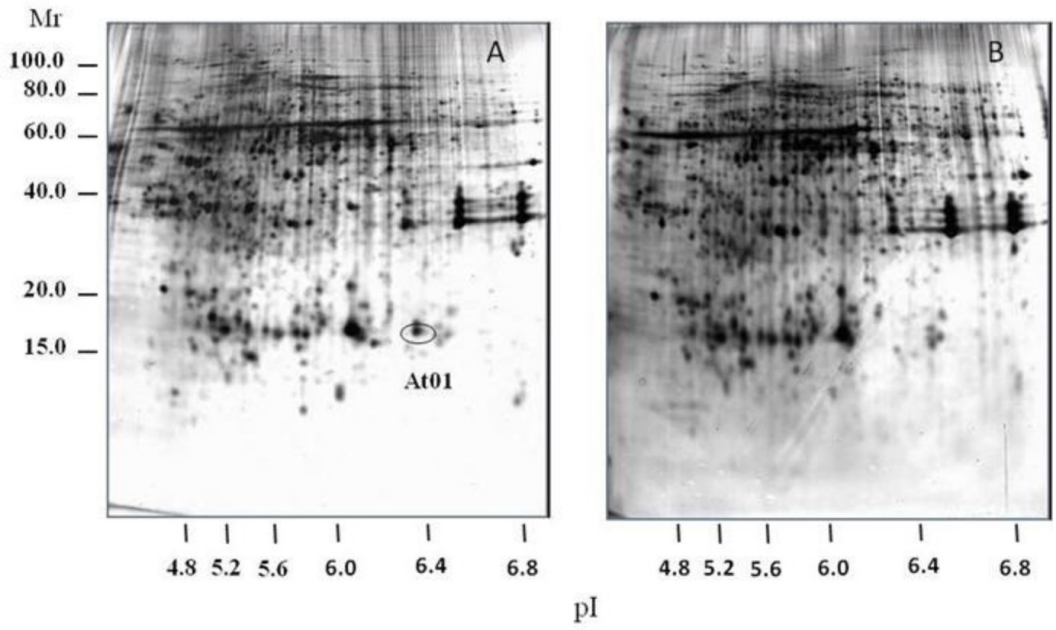

Figure 3. Proteome profiles of cultured *Alexandrium* cf. *pacificum* collected in (A) Anda Channel (ATANDA) and (B) Bolinao Channel (ATBOL), obtained by 2-D gel electrophoresis, showing the unique protein spot (At01) in ATANDA strain.

and neoSTX with toxicity value of 19.49 fmol STX eq cell^{-1}, while a subsequent culture (AAHB2) produced GTX1,4 with toxicity value of 9.32 fmol STX eq cell^{-1}. In contrast, it was reported that *Alexandrium affine* could be non-toxic most of the time (Band-Schmidt et al. 2003). Moreover, *Alexandrium affine* originally collected in Vietnam waters produced STX and neoSTX with toxin concentration of 2.28 fmol STX eq cell^{-1} in one culture, GTX1-4 with toxin concentration of <1.0 fmol STX eq cell^{-1} in another culture, or no toxin at all (Nguyen-Ngoc 2004). Thus, the toxicity of *Alexandrium affine* that we had in cultures was about ten-fold higher than the sample collected in Vietnam waters (Table 1). The phenomenon in which toxin profiles change for the same strain has been attributed to changes in nutrient supply or genetic mutation. A study on the effect of nitrate enrichment on *Alexandrium pacificum* has shown changes in toxin profiles from C1 to GTX3 after 12 h (Han et al. 2016).

Alexandrium cf. *pacificum* was examined to compare two PST-producing strains originally obtained from different localities during different blooming periods. Both strains produced GTX1-4, with about two-fold greater toxicity in the ATANDA strain (118.75 fmol STX eq cell^{-1}) than in the ATBOL strain (60.16 fmol STX eq cell^{-1}). A comparison with *Alexandrium tamarense* and *Alexandrium catenella* (renamed *Alexandrium pacificum*) strains of the *Alexandrium tamarense* complex – to which *Alexandrium* cf. *pacificum* belongs – showed variations in toxicity levels, with *Alexandrium* cf. *pacificum* (ATANDA strain) showing a relatively high toxicity level (Table 1). *Alexandrium tamarense* strains ATDY03 and ATDY04 isolated along the coast of China produced GTX1/4, GTX2/3, dcGTX2/3, neoSTX, and STX with toxicity value of 16.37 fmol STX eq cell^{-1}, and C1/2, GTX1/4 and dcGTX2/3 with toxicity value of 3.54 fmol STX eq cell^{-1}, respectively (Zou et al. 2014). Cultured *Alexandrium tamarense* collected in southern Brazil produced STX, neoSTX, GTX1-4, decarbamoyl gonyautoxin 3 (dcGTX3), C1, and C2 with toxicity values ranging from 42-199 fmol STX eq cell^{-1}. Most strains produced C1 and C2 as the major toxins, while a single strain had GTX4 as its major toxin (Persich et al. 2006). The *Alexandrium tamarense* obtained in western Japan produced GTX1-4 and C1-4, with toxicity value of 1.35 fmol eq cell^{-1} (Hamasaki et al. 2001). During an outbreak in southern Chile, seven strains of *Alexandrium catenella* were detected, four of which had only GTX1-4 with total toxin contents ranging from 8.5-18.5 fmol STX eq cell^{-1}, whereas the most toxic strain had a total toxin content of 96.9 fmol STX eq cell^{-1} consisting of GTX1-6, neoSTX, STX, and C1,2,4 (Aguilera-Belmonte et al. 2011) (Table 1).

The researchers focused on the proteomic study of interstrain analysis of *Alexandrium cf. pacificum* (ATANDA and ATBOL), since it is one of the most common etiologic HAB agents in the Philippines. It has been found that proteome expression can vary between ATANDA and ATBOL strains. Transcriptomic method revealed that species-specific and inter-individual genetic expression variations could occur among dinoflagellates. It was suggested that genetic expression variations related to photosynthesis, fatty acid metabolism, and biosynthetic pathways reflect selection pressures that could drive niche diversification (Parkinson et al. 2016). The variations are being explored as potential biomarkers of toxicity and biomarkers to distinguish different species and strains (Chan et al. 2006).

A uniquely expressed protein (At01) in ATANDA strain shared homologous internal sequences with bifunctional ornithine acetyltransferase/ N-acetylglutamate synthase

Table 1. Comparison of toxin profiles.

Algal strain/ species	Toxicity value (fmol STX eq cell^{-1})	Toxins detected	Reference
AAHB1	19.49	STX, neoSTX	This Work
AAHB2	9.32	GTX1,4	This Work
AAV1	2.28	STX, neoSTX	Nguyen-Ngoc 2004
AAV2	<1	GTX1-4	Nguyen-Ngoc 2004
AAV3	0	None	Nguyen-Ngoc 2004
ATANDA	118.75	GTX1-4	This Work
ATBOL	60.16	GTX1-4	This Work
ATDY03	16.37	GTX1/4, GTX2/3, dcGTX2/3, neoSTX, STX	Zou et al. 2014
ATDY04	3.54	C1/2, GTX1/4, dcGTX2/3	Zou et al. 2014
ATSB	42-199	STX, neoSTX, GTX1-4, dcGTX3, C1, C2	Persich et al. 2006
ATWJ	1.35	GTX1-4, C1-4	Hamasaki et al. 2001
ACSC1-4	8.5-18.5	GTX1-4	Aguilera-Belmonte et al. 2011
ACSC5	96.9	GTX1-6, neoSTX, STX, C1,2,4	Aguilera-Belmonte et al. 2011

AAHB1 and AAHB2, *Alexandrium affine* strains from Honda Bay; AAV1, AAV2 and AAV3, *Alexandrium affine* strains from Vietnam; ATANDA and ATBOL, *Alexandrium* cf. *pacificum* strains from Anda Channel and Bolinao Channel, respectively; ATDY03 ATDY04, *Alexandrium tamarense* strains along the coast of China; ATSB and ATWJ, *Alexandrium tamarense* from southern Brazil and western Japan, respectively; ACSC1-4 and ACSC5, *Alexandrium catenella* strains from southern Chile; STX, saxitoxin; neoSTX, neosaxitoxin; GTX, gonyautoxin; dcGTX, decarbamoyl gonyautoxin; C1, epimeric GTX8; C2, GTX8; eq, equivalent.

(ArgJ) produced by *Streptomyces collinus* (NCBI reference sequence: WP_020938939.1). It was earlier reported that STX-biosynthesis proteins (e.g., SxtH and SxtT) have closest homology with capreomycidine hydroxylase produced by *Streptomyces vinaceus* (Kellmann et al. 2008). In both studies, *Streptomyces* sp. was used as a model organism with all of its protein-interaction networks already mapped. The ArgJ enzyme has a role in the biosynthesis of arginine that is a precursor in STX biosynthesis; it has been found in bacteria and yeast (Sakanyan et al. 1993, Crabeel et al. 1997, Marc et al. 2000) but its presence in a dinoflagellate species has not been documented. The enzyme ArgJ in bacteria is a bifunctional enzyme that is involved in the conversion of N-acetylornithine and L-glutamate to L-ornithine and N-acetyl-L-glutamate, and the conversion of acetyl-CoA and L-glutamate to CoA and N-acetyl-L-glutamate. Arginine, along with acetate (acetyl-coenzyme A) and methionine methyl (via S-adenosylmethionine), is an important reactant for STX biosynthesis. The first reaction in the biosynthetic pathway involves the Claisen condensation of Arg and acetic acid. The second reaction requires amidino transfer from a second Arg to the α-amino group of the product in the first reaction step (Shimizu et al. 1984, Shimizu 1993, Kellmann 2008).

A STRING database analysis revealed that the enzyme ArgJ interacts with enzymes that function as aminotransferase class III (pink ovals) and those that function as arginosuccinate synthase (white ovals) (Figure 4). The aminotransferase class III enzymes include ArgD (acetylornithine aminotransferase), SSMG_02935 and SSMG_06289 (adenosylmethionine-8-amino-7-oxononanoate transaminases), and

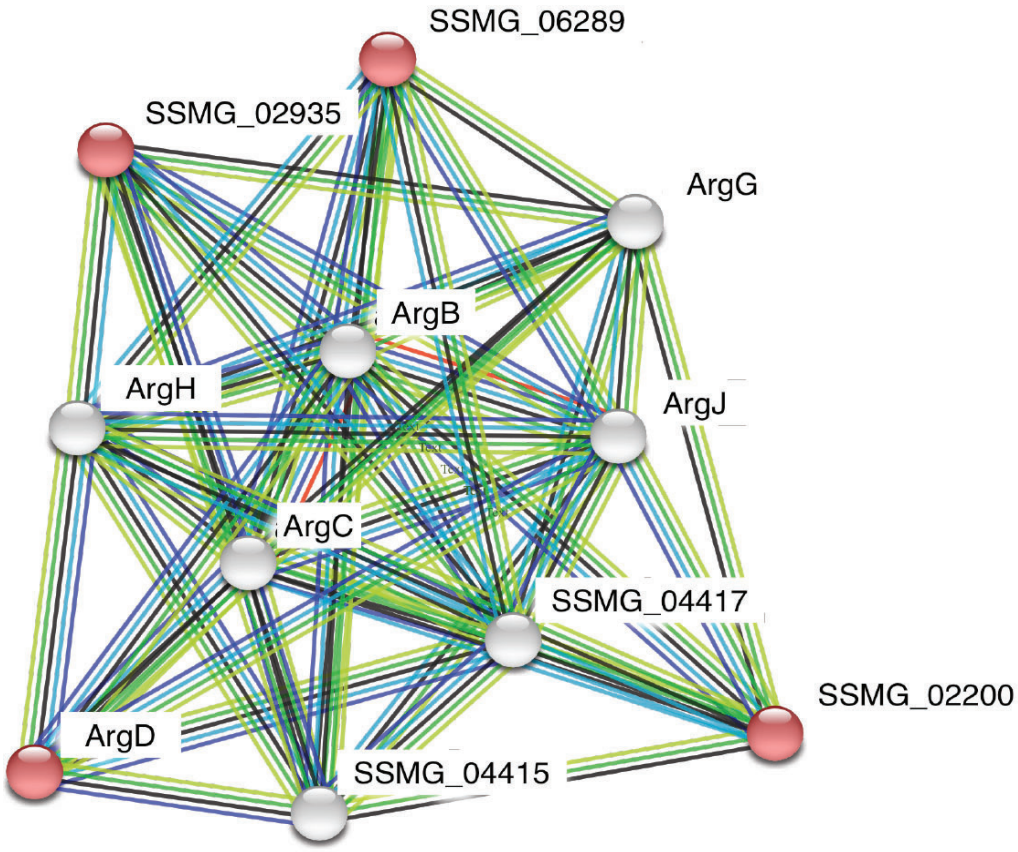

Figure 4. Protein interaction network based on STRING database analysis with *Streptomyces* sp. AA4 as reference organism shows interaction of the ArgJ enzyme with other enzymes which function as aminotransferases class III (pink ovals) and those which function as arginosuccinate synthase (white ovals). ArgD (acetylornithine aminotransferase), SSMG_02935 and SSMG_06289 (adenosylmethionine-8-amino-7-oxononanoate transaminases), SSMG_02200 (ornithine-oxo-acid transaminase), ArgB (N-acetylglutamte kinase), ArgH (arginosuccinase), ArgC (N-acetylglutamate semialdehyde dehyrodgenase), ArgG (citrulline-aspartate ligase), SSMG_04417 (ornithine carbamoyltransferase), and SSMG_04415 (argininosuccinate synthase). Network statistics shows 11 nodes, 48 edges, average node degree of 8.73, and clustering coefficient of 0.882.

SSMG_02200 (ornithine-oxo-acid transaminase). The arginosuccinate synthase enzymes include ArgJ, ArgB (N-acetylglutamte kinase), ArgH (arginosuccinase), ArgC (N-acetylglutamate semialdehyde dehyrodgenase), ArgG (citrulline-aspartate ligase), SSMG_04417 (ornithine carbamoyltransferase), and SSMG_04415 (argininosuccinate synthase).

Wang and co-workers (2013) documented the possible occurrence in *Alexandrium catenella* of nine proteins that are involved in PST biosynthesis. Jiang and co-workers (2015) discovered seven proteins that are related to PST biosynthesis in *Alexandrium tamarense*. Among those proteins, ornithine carbamoyltransferase and argininosuccinate synthase are found in the protein interaction network generated from our STRING database analysis of ArgJ. To the researchers' knowledge, this study is the first to document that the bifunctional ornithine acetyltransferase/ N-acetylglutamate synthase could be present in *Alexandrium* cf. *pacificum*.

The toxicity, as well as the composition of the major toxins produced by dinoflagellates, varied depending on the geographical locations from which the specimens were isolated, collection period, and subsequent culturing at laboratory conditions. These factors contribute to the difficulty in predicting the specific conditions for the occurrence of HABs based on toxicity studies alone. This study of different strains collected in different localities at different blooming times showing differences in toxicity and proteome profiles indicate that different strains possibly have some adaptive strategies unique to their specific habitats and ecological conditions. Toxin profiles and toxicity often change in response to the nutrients available in the environment (Han et al. 2016). Studies have shown that dinoflagellates grown in ammonium have higher toxin production than those grown with nitrate as nitrogen source. Moreover, P-depleted, nitrate grown cultures tend to produce more toxins (Hii et al. 2016).

The unique protein spots may be useful in elucidating the roles of certain enzymes in the molecular mechanism of the algal blooming process. By investigating the protein expression of dinoflagellates, enzymes initially and principally involved in toxin biosynthesis – which are potential biomarkers of toxicity – could possibly be found.

Studies of other proteins that are uniquely or differentially expressed in different algal strains will hopefully identify major enzymes involved in toxin biosynthesis that could become useful in early detection of HAB event. Identification of these proteins could further elucidate the life cycle of dinoflagellates resulting in toxin production. Knowledge derived from studies of both toxicity and protein expression of dinoflagellates will be ultimately helpful in understanding the blooming process and in the prevention and/or monitoring of HABs and in subsequently undertaking effective contingency measures during a HAB occurrence.

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