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# Gene expression and molecular evolution of *sxtA4* in a saxitoxin producing dinoflagellate *Alexandrium catenella*

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ABSTRACT

Dinoflagellates of the genus *Alexandrium* produce the neurotoxin saxitoxin (STX), responsible for paralytic shellfish poisoning (PSP) and accumulates in marine invertebrates. The recent identification of STX biosynthesis genes allowed us to investigate the expression of *sxtA4* at different growth stages in *Alexandrium catenella* Group IV. We found no significant differences in expression of *sxtA4*, despite significant differences in STX levels at different growth stages ($P < 0.023$). Three reference genes were tested for normalisation: actin, cytochrome b (*cob*), and the large subunit ribosomal RNA (LSU rDNA). *cob* was most stably expressed but the combination of two reference genes, actin and *cob*, resulted in the best stability factor. Most genomic sequences of *sxtA4* from *A. catenella* were in a clade that included sequences from *Alexandrium fundyense* Group I, however, one paralogue was not related to the others, suggesting recombination or lateral transfer. A comparison of the *sxtA4* cDNA sequences with genomic DNA sequences indicated the possibility of transcript editing and the preferential transcription of certain genomic DNA loci. The results show that, in dinoflagellates, post-transcriptional mechanisms play a major role in the regulation of saxitoxin biosynthesis.



## 1. Introduction

STX is one of the most potent marine biotoxins, and the parent compound of 57 analogs, which are commonly known as the paralytic shellfish toxins (PSTs) (reviewed in Wiese et al., 2010). The toxicity of STX is mediated mainly via the inhibition of the flow of sodium ions through voltage-gated sodium channels (Ritchie and Rogart, 1977; Su et al., 2004; Wang et al., 2003). PSTs are the causative agents of paralytic shellfish poisoning (PSP) and saxitoxin pufferfish poisoning (SPFP) (Kao, 1966; Anderson et al., 1996; García et al., 2004; Rodrigue et al., 1990). In marine waters, PSTs are produced by dinoflagellates of the genus *Alexandrium*, and a single species each of *Gymnodinium* and *Pyrodinium* (Anderson et al., 1996; Shimizu, 1979; Oshima et al., 1993; Usup et al.,1994). Due to the high potency of PSTs, PSP outbreaks can occur when cell densities are very low, as few as 100–200 cells $L^{-1}$ (Anderson, 1997; Townsend et al., 2001; Anderson et al., 2002).

The enormous genome size of dinoflagellates (Lin, 2011) initially hampered the identification of genes putatively associated with STX production, despite many attempts and their successful identification in cyanobacterial PST producers (Kellmann et al., 2008). The sequencing of large libraries of Expressed Sequence Tags (EST) facilitated the detection of saxitoxin biosynthesis associated genes and transcripts in the dinoflagellates *Alexandrium fundyense* Group I, *Alexandrium minutum*, *Alexandrium tamarense* Group IV, *Gymnodinium catenatum* and *Pyrodinium bahamense*, including *sxtA*, which encodes the enzyme proposed

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to initiate the synthesis of STX and its analogs (Stüken et al., 2011; Hackett et al., 2013; Orr et al., 2013). In both cyanobacteria and dinoflagellates, *sxtA* has four catalytic domains, with predicted activities similar (similarity scores 59–64%) to S-Adenosyl methionine (SAM)-dependent methyltransferase (*sxtA1*), GCN-5 related N-acetyltransferase (*sxtA2*), acyl carrier protein (*sxtA3*), a class II aminotransferase (*sxtA4*) (Kellmann et al., 2008). These activities are the same as the enzymes that would be required for the initial steps in the predicted biosynthetic pathway of PSTs (Kellmann et al., 2008; Shimizu, 1993). The other analogs of STX, including gonyautoxins and neosaxitoxin, are predicted to be formed in the final stages of the pathway, by modifications to the parent compound, STX (Kellmann et al., 2008).

In contrast to the bacterial homologues, the dinoflagellate transcripts had a higher GC content, were monocistronic, occurred in multiple copies and contained typical dinoflagellate spliced-leader (SL) sequences and eukaryotic polyA-tails (Stüken et al., 2011; Hackett et al., 2013; Orr et al., 2013). Genomic DNA sequences of the domains sxt*A1* and sxt*A4* from the PST-producing species *Alexandrium tamiyavanichi*, *Alexandrium ostenfeldii*, *Alexandrium tamarense* Group I, and *A. tamarense* Group V, have recently been sequenced (Stüken et al., 2011; Murray et al., 2011, 2012; Hii et al., 2012; Hackett et al., 2013; Orr et al., 2013; Suikkanen et al., 2013). Studies to date indicate that the domain *sxtA4* appears to be consistently present in dinoflagellate and cyanobacterial species that produce saxitoxin (Stüken et al., 2011; Murray et al., 2011, 2012; Hii et al., 2012; Hackett et al., 2013; Orr et al., 2013; Suikkanen et al., 2013), and absent from those that do not, unlike some other saxitoxin synthesis pathway genes such as *sxtG*, which appears to be present also in some non-toxin producing *Alexandrium* species (Orr et al., 2013).

PSTs are considered to be secondary metabolites (Shimizu, 1993). The active production of many microbial secondary metabolites is limited to certain growth phases, with production at low growth rates or the exclusive production under specific conditions (Demain et al., 1983; Zhu et al., 2002). However, this pattern appears to be less clear in dinoflagellates. The dynamics of PST production in *Alexandrium* have been investigated at different cell densities, growth stages and growth rates (Boczar et al., 1988; Anderson et al., 1990; Cembella, 1998). Previous studies have reported low level constitutive production of STX, however, results varied on which growth phase was correlated to the highest rate of toxin production, indicating that either the mid-exponential growth phase (Boczar et al., 1988; Anderson et al., 1990; Cembella, 1998), or the stationary phase (Parkhill and Cembella, 1999; Lim et al., 2005) led to higher toxin production. In addition, toxin analogues produced were found to vary with growth phases. With the identification of genes involved in STX biosynthesis, the investigation of *sxtA4* transcription using real-time reverse transcription polymerase chain reaction (RT-qPCR) in relation to the production of the various STX analogues is possible, and could potentially detect subtle changes, as it is the first metabolic regulatory level.

In this study we investigated the expression and molecular evolution of *sxtA4* in a strain of *Alexandrium catenella*, Group IV (Clade IIC). *A. catenella*, Group IV (Clade IIC) is a common bloom forming species in areas of Asia, the Mediterranean, and coastal waters of south eastern Australia (Hallegraeff et al., 1988; Miranda et al., 2012; Scholin and Anderson, 1994), where it causes annual blooms that can lead to PST uptake in shellfish (Murray et al., 2011). Few previous studies have investigated gene expression in toxic *Alexandrium* species (Fernandez et al., 2002; Harlow et al., 2007; Hosoi-Tanabe and Sako, 2005; Toulza et al., 2010; Zhuang et al., 2013), and a set of reference genes has not yet been reported for *A. catenella*. Two to three reference genes are recommended for normalization of the target gene in RT-qPCR experiments (Bustin, 2002; Bustin et al., 2005; Nolan et al., 2006). We validated the expression stability of three reference genes: cytochrome b (*cob*), as a component of the mitochondrial respiratory chain; actin, a ubiquitous cytoskeletal member; and the large subunit ribosomal RNA gene (LSU rDNA). These three genes encode metabolites belonging to different functional classes, theoretically reducing the probability of co-regulation.

Mitochondrial *cob* mRNA levels have been shown to be relatively constant over different growth conditions for the dinoflagellate *Pfiesteria piscicida*, and *cob* has been used as reference gene for gene expression studies on *P. piscicida*, *Amphidinium carterae* and *Karenia brevis* (Lin and Zhang, 2003; Lin et al., 2011, 2012; Zhang et al., 2006).

The possession of multiple genomic copies of highly expressed genes in dinoflagellates may possibly function as a means of increasing their transcription (Bachvaroff, 2008). Investigation of *sxtA* gene copy numbers per cell performed by quantitative PCR revealed the existence of multiple copies of the *sxtA4* gene. An estimated 100–240 copies of the *sxtA4* domain were present in the genomic DNA of *A. catenella* ACSH02 (temperate Asian ribotype) Stüken et al., 2011. The mean copy number of *sxtA4* in *A. catenella* ACCC01 used in this study was reported to be 240 ± 97 copies per cell (Murray et al., 2011). We compared sequences of *sxtA4* transcripts to gDNA copies and examined transcription and evidence of preferential expression. Finally, we investigated the evolution of the genes encoding *sxtA4* in *A. catenella* in relation to similar sequences from other *Alexandrium* species and strains and *G. catenatum*.

## 2. Materials and methods

### 2.1. Culture maintenance and cell enumeration

The strain used in this study, ACCC01, a member of Group IV, Clade IIC (Murray et al., 2011), was isolated from Cowan Creek, New South Wales, NSW, in the Sydney basin (31°45′S, 121°45′E), and was obtained from the culture collection of Prof. Gustaaf Hallegraeff, University of Tasmania. *A. catenella* ACCC01 was grown in GSe medium (Blackburn et al., 1989) in a culture cabinet (Labec, Australia) at a temperature of 18 ± 1 °C and 60 μmol photon $m^{-2}$ $s^{-1}$, on a 12/12-h light/dark cycle. For growth studies, triplicate cultures were set up in 1 L volumetric culture flasks. Media was inoculated with an

**Table 1**
Details of the primers used in this study.

| Gene | Primer sequence (5′–3′) | AT (°C) | AP (bp) | References |
|---|---|---|---|---|
| ***sxtA4*** | | | | |
| sxtA4F | CTGAGCAAGGCGTTCAATTC | Forward | | |
| sxtA4R | TACAGATMGGCCCTGTGARC | Reverse | 125 | Murray et al., 2011 |
| ***cob*** | | | | |
| cob_F | TCCCATTTTTCCCTTTCWTT | Forward | | |
| cob_R | ATTTTTGTTGGGCACAGCTT | Reverse | 212 | this study |
| ***actin*** | | | | |
| act_F | ATCAAGGAGAAGCTCTGCTACATC | Forward | | |
| act_R | TCAGACTCGGCTGGAAGAGA | Reverse | 166 | Yauwenas et al. unpublished ., unpublished data) |
| **LSU rDNA** | | | | |
| catF | CCTCAGTGAGATTGTAGTGC | Forward | | |
| catR | GTGCAAAGGTAATCAAATGTCC | Reverse | 160 | Hosoi-Tanabe and Sako, 2005 |
| ***SxtA4*** | | | | |
| sxt007 | ATGCTAACATGGGAGTCATCC | Forward | | |
| sxt008 | GGGTCCAGTAGATGTTGACGATG | Reverse | 750 | Stüken et al., 2011 |

exponentially growing culture to a cell concentration of 280 cells $ml^{-1}$. The cultures were grown for 37 days. Culture growth was monitored by cell counts using a Sedgewick Rafter counting chamber (Proscitech, Australia) and an inverted light microscope (Leica Microsystems). The growth rate was calculated using the formula $\mu = \ln(N_1/N_0)/(t_1 - t_0)$ (Anderson et al., 1990), where $t$ is the time in days and $N$ is the cell number. Culture samples for RNA extraction and toxin cell quota analysis were taken on day 6, 12, 18, 27, 37 at the end of the light cycle (+11 h).

### 2.2. RNA extraction and DNase treatment

Total RNA was extracted from 40 mL of cultures harvested by centrifugation at 5000 *g* for 5 min, according to the RNA extraction protocol of Harlow et al. (2006). Briefly, cell pellets were ground with microglass pestles in 1.5 mL microcentrifuge tubes with 30 μL RLT buffer (RNeasy Kit Plant and Fungi, Qiagen) containing 1/100 volume β-mercaptoethanol on ice. Extractions were then continued following the manufacturer's instructions. Total RNA was eluted in 30 μL RNase-free water (Qiagen). Residual DNA was removed with TURBO DNase according to the TURBO DNA-free™ kit (Applied Biosystems). RNA quality and quantity was assessed using RNA 6000 Nano LabChip Kit in microcapillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies).

### 2.3. cDNA synthesis

cDNA synthesis was performed with the SuperScript™ III first-strand synthesis system for RT-PCR (Invitrogen) according to manufacturer's instructions. Briefly, reverse transcription of 100 ng total RNA was performed in 50 μl reactions with a mixture of the provided oligo(dt) primer. Reverse transcription reactions were diluted 10-fold with RNase free water and 1 μl of the dilution was used as template in successive qPCRs.

### 2.4. PCR primers

Three possible reference genes were chosen for analysis as they were most likely to be constitutively expressed and not co-regulated. Primers for cytochrome b, were designed with Primer 3 (Rozen and Skaletsky, 1999) based on the consensus sequence from an alignment of *Alexandrium* sequences downloaded from GenBank. All other primer details are outlined in Table 1.

### 2.5. Quantitative PCR

Primer efficiency in qPCR was determined according to Rasmussen (2001). Briefly, the standard curve was constructed from a 10-fold dilution series of a known concentration of fresh PCR product, ranging from 2 to $2 \times 10^{-5}$ ng (Hou et al., 2010). The efficiency of the reaction was calculated as $E = (10^{(-1/m)})^{-1}$, with $m$ being the slope of the equation (Rasmussen, 2001; Pfaffl, 2001). qPCR was performed in triplicate for each sample using Evagreen dye (Biorad) in a final volume of 10 μl, primers were used at a concentration of 500 nm. The reactions included 1 μl of 0.1 mg $ml^{-1}$ BSA. The following PCR protocol was used: denaturation at 95 °C for 15 s, annealing and amplification at 60 °C for 30 s, over 40 cycles with continuous fluorescence measurements. All qPCR assays were followed by dissociation curve analysis to ensure that the single PCR products matched with the standard amplicon. The melt curve analysis was performed over the temperature range 95–55 °C, including a final cooling step with continuous fluorescence measurements.

For the quantitative comparison of amplification rates of the investigated candidate reference genes and the target gene *sxtA4*, the "threshold cycle" CT was identified for each run as the cycle at which the fluorescence signal exceeded the background fluorescence of the reaction. Three technical replicates were performed and the mean values were calculated for each of the three biological replicates. For stability comparison of candidate reference genes, the Microsoft Excel add-in NormFinder was used (Andersen et al., 2004). The stability value is based on the combined estimate of intra- and intergroup expression variations of the genes studied. The gene with the least expression variability is calculated and an additional combination of the two genes is recommended that commonly reflects the lowest stability value for normalization (Andersen et al., 2004). The expression of *sxtA4* was normalized against

the reference genes recommended by NormFinder with the Pfaffl equation: Ratio $= (E_{target})^{\Delta Ct \text{ target (control-treated)}} / (E_{ref})^{\Delta Ct \text{ ref (control-treated)}}$ (Pfaffl, 2001).

The mean values of biological triplicates and standard deviations of biological replicates, as well as other statistical analyses were determined using GraphPad version 4.3 (San Diego, CA). The statistical analysis consisted of a one-way ANOVA applied to the growth rate, toxin content and production rate, and gene expression at different days. A post-hoc Tukey test was used to determine differences among samples and a $P$ value <0.05 was considered to be significant.

### 2.6. DNA extraction, cloning and sequencing of sxtA4 amplified from gDNA and cDNA

DNA was extracted from a *A. catenella* ACCC01 culture using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). PCR of the larger fragment of *sxtA4* from cDNA and genomic DNA (gDNA) was performed with the primers sxt007 and sxt008 (Table 1). Thermal cycling conditions were as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 3 min, 65 °C for 30 s, and 72 °C for 1 min, and ended with a final extension at 72 °C for 7 min. PCR products were cloned into the TOPO-TA plasmid (Invitrogen) and positive clones were screened and sequenced using the MTF/MTR primer pair. A pool of 20 cDNA and 20 gDNA *sxtA4* clones from *A. catenella* ACCC01 were sequenced. Approximately 50 ng of PCR product was used for direct sequencing. Products were sequenced using the ABI Big-Dye reaction mix (Applied Biosystems) at the Ramaciotti Centre for Gene Function Analysis, University of New South Wales (GenBank accession numbers: KF 164489–164508). A sequence alignment was constructed from the resulting *sxtA4* sequences and was analyzed at nucleotide level and the predicted amino acid level.

Dinoflagellate nucleotide sequences were aligned manually with the program Geneious v5.4 (Drummond et al., 2011) with consideration of the putative coding sequence and reading frame. For phylogenetic analyses, the sequences of gDNA clones were aligned with additional database sequences of *sxtA4* (accession numbers are given in Fig. 3) and aligned using ClustalW (Thompson et al., 1994). The program FindModel (Posada and Crandall, 1998) was implemented to evaluate optimal substitution models for the alignments, and the Generalised time-reversible (GTR)-model was chosen. Final alignments consisted of 74 sequences, 600 bp in length. Alignments were analyzed using maximum likelihood (ML) and the GTR model with parameters as implemented in the program PhyML v2.4.4 (Guindon and Gascuel, 2003). ML bootstrap analyses were performed with 100 replicates. Alignments were also analyzed using Bayesian inference (BI) using the same parameters, in the program Mr Bayes (Huelsenbeck and Ronquist, 2001). Two million generations were run, until the standard deviation of split frequencies was less than 0.01 and the potential scale reduction factors (PSRF) approached 1.00. Trees were sampled every 1000 generations, with a burning of 1500 trees. Since the genes encoding *sxtA4*, are only ~40% similar to those of cyanobacteria or any other organisms at the protein level (Stüken et al., 2011), the nucleotide alignments determined in this study were analyzed without outgroups and the trees, are presented as unrooted.

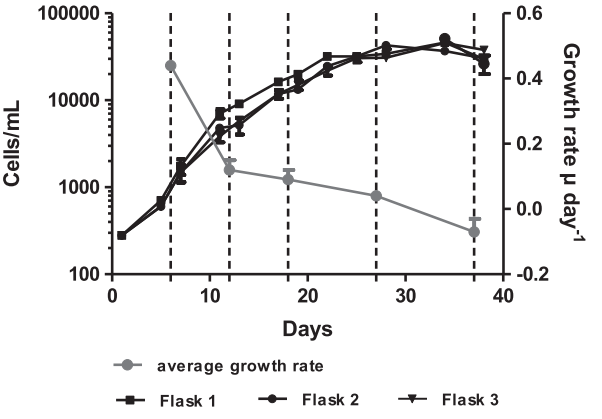



**Fig. 1.** Growth curve of triplicate cultures of *A. catenella* ACCC01 over a period of 37 days. Sampling for RNA and toxin analysis was performed on the days 6, 12, 18, 27, 37 indicated by the vertical line.

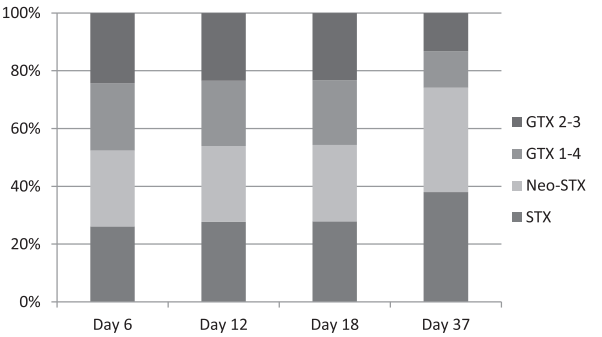



**Fig. 2.** PST analogs in Mol % (GTX-1,4, GTX-2,3, Neo-STX and STX) of total PST content at different sampling days.

### 2.7. Toxin extraction

Ten ml from each culture on sample days was harvested by centrifugation at 5000 *g* for 5 min. The toxins were extracted with 300 μl of 0.1 M HCl by hydrolysis in a boiling water bath for 5 min (Chang et al., 1997). Extracted toxins were collected from the supernatant after centrifugation.

### 2.8. Determination of STX concentration

Toxin content of the culture samples was tested using High Performance Liquid Chromatography (HPLC), according to the AOAC Official Method (2005) for paralytic shellfish poisoning toxins in shellfish (Lawrence et al., 2005); with the modification that a matrix modifier was not used, as this is only required for a shellfish matrix. HPLC was performed on a Hewlett Packard 1100 series LC with fluorescence detection (EX 340 nm, EM 395 nm). Chromatographic separation was achieved with a Zorbax SB-C18 (4.6 × 250 mm, 5 μm) reversed phase analytical column


**Fig. 3.** Results of the phylogenetic analysis of copies of *sxtA4* from different *Alexandrium* strains and *G. catenatum*, including the strains sequenced in this study (highlighted in grey). The tree depicted is based on a Maximum Likelihood (ML) analysis, using the GTR model with bootstrap (BS) values based on 100 replicates. The tree is unrooted. Bayesian analysis was also conducted, using 2,000,000 iterations. BS values and Bayesian Posterior Probabilities (PP/BS) are shown at to the left of nodes. Values greater than 1.00/90 are shown as closed circles at nodes. Support values less than 0.50/50 are not shown.

(Agilent Technologies), eluted at 2 mL $min^{-1}$. Mobile phases were 0.1 M ammonium formate (A) and 0.1 M ammonium formate in 5% acetonitrile (B), both adjusted to pH 6. The gradient consisted of a linear gradient to 5% B over 15 min, a linear gradient to 70% B over 12 min, then returning to 100% A over 6 min and held for 3 min. Analytical standards for the STX analogs were obtained from the National Research Council, Canada. Dilution curves of standards were used for quantification of the PSTs in sample material. The toxin production rate was calculated with the equation $\mu_{Tox} = \ln(T_1/T_0)/t_1 - t_0$ (16).

## 3. Results

### 3.1. Culture growth and growth rate

The replicate cultures of *A. catenella*, strain ACCC01 were sampled over a period of 37 days. Growth rates were highest from day 4–6, with an average value of (0.44 μ $day^{-1}$) over the sampling period with values of 0.1 μ $day^{-1}$ for days 10–12, 0.17 μ $day^{-1}$ for days 16–18, 0.17 μ $day^{-1}$ for days 24–27, and −0.07 μ $day^{-1}$ for days 33–37 (Fig. 1, Table 2). Growth rates at each of these sampling days differed significantly from each other, with the exception of the days 12 and 18, between those days no significant differences were determined by one-way ANOVA and the post-hoc Tukey test ($P < 0.0001$).

### 3.2. Primer specificity

PCR primer specificity was confirmed with conventional PCR. Single bands were visualized on 3% agarose gels and were confirmed by sequencing of the amplicons. The melt curve analyses confirmed single melt temperatures for each primer pair of 77.5 °C for cytochrome B, 86 °C for actin, 80.5 °C for LSU rDNA, and 77.5 °C for *sxtA4*. Primer efficiencies were found to be: 0.90 for LSU rDNA, 0.90 for *sxtA4*, 0.90 for actin, and 0.90 for cob.

### 3.3. Reference gene expression stability

The evaluation of inter- and intragroup expression variability for the three investigated reference gene candidates cytochrome b, actin, LSU rDNA showed that the gene with the least variability in expression was cytochrome b. Its stability value was 0.041. Actin had a slightly higher stability value of 0.050 and LSU rDNA showed the highest variation between the biological replicates and at the different sampling days with an overall stability value of 0.087. The overall best stability value as reference expression is achieved when using a combination of two genes actin and cytochrome b, with a stability value of 0.036.

**Table 2**
Relative expression ratio of *sxtA4*, growth rate and toxin content of *A. catenella* ACCCO1 on the days 6, 12, 18, 37. No significant differences were determined by one-way ANOVA for the gene expression at different sampling days (*P* 0.4287). Significant differences were found for the growth rate between all sampling days (****) ($P < 0.0001$) one-way ANOVA and Tukeys posthoc test, only when comparing day 6 vs 12 no significant differences were determined. Similar for total toxin content per cell significant differences for comparison of all days (***, ****) with the exception day 6 vs 12 with no significant difference.

| Day | Growth rate μ ($day^{-1}$) | Total PST (pg $cell^{-1}$) | Relative expression ratio |
|---|---|---|---|
| 6 | 0.44 ± 0.01 | 9.48 ± 0.3 | 0.21 ± 0.78 |
| 12 | 0.10 ± 0.03 | 9.5 ± 0.3 | 0.50 ± 0.75 |
| 18 | 0.17 ± 0.03 | 11.02 ± 0.2 | 0.62 ± 0.16 |
| 37 | −0.07 ± 0.04 | 10.53 ± 0.25 | 0.35 ± 0.57 |

### 3.4. Expression normalization of sxtA4

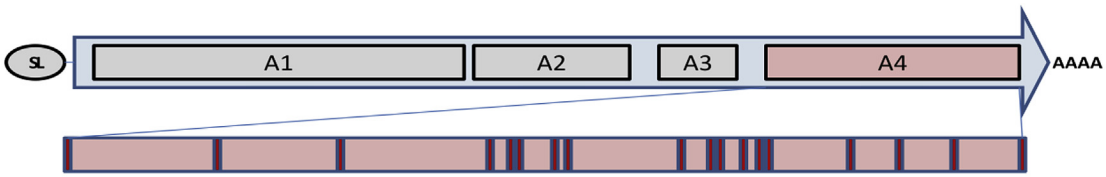
The relative expression ratio of *sxtA4* was normalized against cytochrome b and actin. The growth rate between days 26 and 27 was 0 μ $day^{-1}$. These samples were therefore treated as the control, while all other days were considered to be the treatment, in order to evaluate changes in expression related to growth rate. The domain *sxtA4* was found to be constitutively expressed throughout growth in batch culture (Table 2). There was no significant difference in expression of *sxtA4* among sampling days with significantly different growth rates, as determined using one-way ANOVA analysis ($P = 0.4287$).

### 3.5. Toxin analysis

We found that *A. catenella* strain ACCC01 produces gonyautoxins 1–4 and 2,3 (GTX-1,4, GTX-2,3), neosaxitoxin (Neo-STX) and saxitoxin (STX) (Fig. 2), as has been found previously in this strain (Murray et al., 2011). The amount of toxin produced during growth differed significantly when comparing all sampling days with each other, based on a one-way ANOVA and post-hoc Tukey test ($P < 0.0001$), with the exception of day 6 vs. day 12 (Table 2). Total toxin was found to be 9.48 ± 0.3 pg $cell^{-1}$ at lag phase, slightly increasing at exponential growth to 11.02 ± 0.2 pg $cell^{-1}$, and decreasing at stationary phase to 10.53 ± 0.25 pg $cell^{-1}$ (Table 2). The molar percent ratio of GTX-1,4 and GTX-2,3 and of Neo-STX and STX was approximately 1:2 but changed to 1:3.3 for GTX-1,4/GTX-2,3 and 1:1.4 for Neo-SXT/STX at stationary phase (Fig. 2). The molar % ratio change in the PST analogs was likely due to a conversion of analogs, as the total toxin content decreased from late exponential to stationary phase. PST production rates were calculated for the different growth stages between days 6 and 12 (0.004 ± 0.005 pg cell $day^{-1}$), days 12 and 18 (0.024 ± 0.001 pg cell $day^{-1}$) and days 18 and 37 (−0.05 ± 0.001 pg cell $day^{-1}$). The PST production rates differed significantly ($P < 0.023$) at different growth stages (Table 2).

### 3.6. Phylogenetic analysis

*A. catenella* ACCC01 was found to possess several non-identical genomic copies of *sxtA4*. Twenty clones were sequenced, and 19 of the clones clustered on a highly supported branch (0.92/92) (Fig. 3) with *sxtA4* sequences from three other *A. catenella* (Group IV) strains (ACSH02, ACTRA02, CCMP1493), and two *A. fundyense* (Group I) strains (CCMP1719, CCMP1979). Only clone 13 from ACCC01 formed a separate clade with copies from *Alexandrium minutum* and *G. catenatum*, however this clade had minimal support (Fig. 3). Sequences from five different strains of




| 1 | 90 | 170 | 235 | 268 | 275 | 307 | 385 | 404 | 409 | 430 | 437 439 | 493 | 521 523 | 558 | 600 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| gDNA_c101 TAC | GAA | GAA | -TA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| gDNA_c102 TAC | GAA | GAA | -TA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| gDNA_c103 TAC | GAA | GAA | -TA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ATT | TTC | AAG | TAA |
| gDNA_c104 TAC | GAA | GAA | -TA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CA- |
| gDNA_c105 TAC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | TTT | TTG | AAG | TAA |
| gDNA_c106 TAC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGT | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| gDNA_c107 TAC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| gDNA_c108 TAC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CA- |
| gDNA_c109 TAC | GAA | GAA | GCA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| gDNA_c110 TAC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| gDNA_c111 TAC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| gDNA_c112 TAC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | ACC | TGT | AAT | GTT | ACT | TCC | AAG | CAA |
| gDNA_c113 GAC | GAG | GGA | GTA | CGC | GAC | GGC | AAC | GGC | TGC | AAG | ATC | ACT | CTC | AAG | CAG |
| gDNA_c114 TAC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACC | TTC | AAG | CAA |
| gDNA_c115 TAC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| gDNA_c116 TAC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| gDNA_c117 TAC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| gDNA_c118 TGC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| gDNA_c119 TGC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| gDNA_c120 TGC | GAA | GAA | GTA | CGC | AAC | GGC | AAT | AGC | TGT | AAT | GTT | ACT | TTC | AAG | CAA |
| cDNA_c101 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c102 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c103 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c104 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c105 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAC |
| cDNA_c106 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAC |
| cDNA_c107 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c108 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c109 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c110 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c111 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c112 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c113 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c114 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c115 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c116 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACG | CTG | AGG | CAG |
| cDNA_c117 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c118 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c119 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |
| cDNA_c120 GAC | GAG | GGA | GTG | CGA | GAC | GGT | AAC | GGC | TGC | AAG | ATC | ACC | CTG | AGG | CAG |

**Fig. 4.** *SxtA* gene model and 15 of the *sxtA4* sequence positions at which cDNA clones differ from the gDNA clones nucleotide acid sequences of *A. catenella* ACCC01 derived from mRNA and gDNA.

*A. minutum* (CCMP1888, CS-324, CCMP113, ALSP01, ALSP02) formed two separate fully supported clades (1.00/99). Similarly, all sequences from four different strains (ATNWB01, ATEB01, ATBB01, ATCJ33) of *Alexandrium tamarense*, Group V genotype, formed a separate fully supported clade (1.00/99).

### 3.7. Sequence comparison of sxtA4 cDNA and gDNA

The comparison of the amplified fragment of *sxtA4* cDNA and gDNA sequences (600 bp) revealed the presence of several non-identical copies of the *sxtA4* gene in both mRNA and gDNA in *A. catenella* ACCC01 (Fig. 4). Clones amplified from gDNA and cDNA all contained SNPs at multiple positions in the sequence. At least two distinctly different gDNA copies of *sxtA4* were identified (clone 13 with a GC content of 63% and clones 1–12 and 14–20 with a GC content of ~65%). cDNA sequences contained SNPs, but did not differ more from each other, sequences did not contain major deletions and insertions of gene fragments. The comparison of cDNA and gDNA (Fig. 4, Table 3) shows that the cDNA sequences consistently differed at several

positions from the gDNA sequences. All 20 cDNA *sxtA4* sequences were most similar to the *sxtA4* gDNA sequence of the clone 13. These polymorphic sites differed from the other gDNA clones (Table 3) at 13 positions, 6 times at the first codon base, twice at the second codon base and 6 times at the third codon base, leading to ten amino acid replacements. Furthermore, several positions have been detected at which the cDNA clones differ from all sequenced gDNA clones (Table 3). Five of those changes were at the third codon position and one occurred within the codon at the second base, two of these nucleotide changes would lead to an amino acid exchange (Table 3). Most of the observed nucleotide substitutions were due to transitions. Further comparison of cDNA and gDNA sequences of *sxtA4* of the toxic strain *A. fundyense* CCMP sequences derived from an EST study and high throughput sequencing (Stüken et al., 2011) were also conducted and revealed variable sites at eight of the same positions in one or more sequences (Supplementary material B).

## 4. Discussion

This is the first study to examine factors impacting the regulation and molecular evolution of a gene involved in STX biosynthesis in a dinoflagellate. An understanding of factors such as the growth stages, in regulating the production of STXs and the expression of genes involved in STX biosynthesis is crucial for the prediction and possibly mitigation of harmful blooms of STX producing dinoflagellates.

We detected significant differences in the quantity of STXs produced throughout growth stages (Table 2), and also changes in the proportion of the STX congeners produced (Fig 2). Previously, it has been reported that STX production in *Alexandrium* can be largely independent of environmental conditions such as nutrient or light regimes, but change due to culture growth phases (Parkhill and Cembella, 1999).

The relative expression of the target gene *sxtA4,* normalized against the reference genes, *cob* and actin, did not change significantly during the different growth stages of *A. catenella*. This suggests that *sxtA4* may be translationally or post-translationally regulated. Transcription level regulation may play a minor role in the expression of many dinoflagellate genes, compared to regulation in other organisms. Microarray studies of dinoflagellates have suggested that only about 10–27% of genes might be regulated transcriptionally (Lidie et al., 2005; Erdner and Anderson, 2006; Lin et al., 2010; Lin, 2011). Several physiological processes may be regulated at the translational level in dinoflagellates, including bioluminescence (Mittag et al., 1998; Morse et al., 1989), carbon fixation (Fagan et al., 1999), photosynthesis (Le et al., 2001), and the cell cycle (Brunelle and Van Dolah, 2011; Van Dolah et al., 2007).

Other studies of dinoflagellate gene expression have indicated that these organisms use both transcriptional and post-transcriptional regulation in roughly equal measure, with the iron superoxide dismutase of *Lingulodinium polyedrum* exhibiting both modes, depending on the stimulus (Okamoto and Colepicolo, 2001). Transcriptional regulation has been shown for the peridinin–chlorophyll *a* binding protein in *Heterocapsa pygmaea* (Triplett et al., 1993), and for cyclin B (*Afcyc*), S-adenosyl-homocysteine-hydrolase-like protein, methionine-aminopeptidase-like protein, and histone like protein in *A. fundyense* (Taroncher-Oldenburg and Anderson, 2000; Zhuang et al., 2013).

A possible mechanism to explain a prevalence of post-transcriptional gene regulation in dinoflagellates is trans-splicing, as in the kinetoplastid *Trypanosomas* sp., in which trans-splicing is present and most genes are regulated post-translationally (Morey et al., 2011). Trans-splicing of dinoflagellate mRNAs is a process by which a specific 22 base oligonucleotide, a spliced leader, is added to the 5′ end of a heterogenous group of RNA (Lidie and Van Dolah, 2007; Zhang et al., 2007). The trans-splicing system in dinoflagellates may provide a level of regulation in addition to transcription (Bachvaroff, 2008) by creating a pool of fully mature translationally active mRNA (Lidie and Van Dolah, 2007; Zhang et al., 2007; Bachvaroff, 2008).

Dinoflagellates possess a number of remarkable genetic characteristics that distinguish them from other eukaryotes. One such striking feature is the large amount of gDNA that they contain, 3–250 pg cell$^{-1}$ (Rizzo, 1991; Lin, 2011). Up to 100–378 genomic copies of *sxtA4* per cell have been reported for three investigated *A. catenella* strains previously (Stüken et al., 2011; Murray et al., 2011.). The expressed *sxtA4* gene copies have been reported to differ due to single nucleotide polymorphisms (SNPs), in *A. fundyense*, of which two thirds were silent (Stüken et al., 2011). Stüken et al. defined SNPs as a base pair change that occurred in at least two of the reads within the EST library (Stüken et al., 2011). In this study we found multiple copies of *sxtA4* all containing SNPs (Fig. 4). Additionally, one

**Table 3**
Loci of the *sxtA4* sequences derived from cDNA and gDNA that differed throughout the clone pools. Part I of the table contains potential mRNA editing position these nucleotide differences were present between all cDNA clones and all gDNA clones. Part II of the table contains nucleotide differences present in all but one gDNA clone (clone 13). Amino acids encoded by the codons and amino acid exchanges are listed. The last column indicates if changes have been found in the *sxtA4* sequences of *A. fundyense* CCMP1790 see Supplementary Data.

| Position in alignment | gDNA | cDNA | Amino acid | *Alexandrium fundyense* |
|---|---|---|---|---|
| **I** | | | | |
| 235 | GUA | GUG | Val | no |
| 268 | CGC | CGA | Arg | yes |
| 274 | CCC | CCA | Pro | yes |
| 307 | GGC | GGU | Gly | no |
| 523 | UUC | CUG | Phe → Leu | no |
| 558 | AAG | AGG | Lys → Arg | no |
| **II** | | | | |
| 1 | UAC | GAC | Tyr → Asp | yes |
| 90 | GAA | GAG | Glu | yes |
| 170 | GAA | GGA | Glu → Gly | yes |
| 274 | AAC | GAC | Asn → Asp | no |
| 313 | AUC | GUC | Ile → Val | yes |
| 385 | AAU | AAC | Asn | no |
| 404 | AGC | GGC | Ser → Gly | yes |
| 409 | UGU | UGC | Cys | no |
| 430 | AAU | AAG | Asn → Lys | no |
| 437 | GUU | AUC | Val → Ile | no |
| 439 | GUU | AUC | Val → Ile | yes |
| 493 | ACU | ACC | Thr | no |
| 521 | UUC | CUG | Phe → Leu | no |

gDNA copy which differed substantially from other gDNA copies amplified from *A. catenella*, was identified (Fig. 3). One group of sequences (19 clones) formed a well-supported clade (Fig. 3, 0.92/92) together with sequences from other *A. tamarense* species complex taxa *A. catenella* and *A. fundyense* Group I. The other type of *sxtA4* gDNA copy identified (clone 13), appeared to be not closely related to this clade.

The species *Alexandrium minutum* and *A. tamarense* Group V appear to have distinctive *sxtA4* copies; and there is little evidence of widespread horizontal transfer amongst all species of *Alexandrium* (Fig. 3). However, within closely related species, for example those of the *A. tamarense* species complex, *A. catenella* Group IV and *A. fundyense* Group I, there may be some genetic exchange, as many clones from these two species formed a single clade with reasonable support (Fig. 3). The presence of multiple, slightly different genomic copies of *sxtA4* may provide adaptive plasticity. Multiple copy numbers and copy number polymorphism (CNP) within genomes have been described for humans, mice, *Drosophila* and other eukaryotes (Freeman et al., 2006; Cutler et al., 2007; Dopman and Hartl, 2007). CNP is also common in the alveolate *Plasmodium falciparum*, a sister group to the dinoflagellates, and the manipulation of copy numbers has been shown to alter the response to drugs (Sidhu et al., 2006) suggesting CNP can allow for adaptive evolution (Nair et al., 2008).

We found indications of a preferential transcription of a particular *sxtA4* gene copy in this study, and identified consistent differences between all clones of the sequences of *sxtA4* from genomic DNA and that of cDNA, at 6 nucleotide positions (Fig. 4, Table 3). It has been previously suggested that gDNA copies might be preferentially transcribed in *Alexandrium* species, possibly according to GC content (Hsiao et al., 2010; Stüken et al., 2011). While it is highly likely that not all genomic copies were identified in our screening subset, we have no reason to suppose this was an unrepresentative sample.

Additionally, mRNA editing may play a role in the maturation of *sxtA4* transcripts. RNA editing is a process by which sequences at the nucleotide (nt) sites may be changed during or after transcription (Benne et al., 1986; Gray, 2003). Recently, editing was detected for mitochondrial genes *cob* and *cox1* in about 20 species of dinoflagellates (Nash et al. 2008; Lin et al. 2002; Zhang and Lin, 2008). RNA editing of transcripts of chloroplast origin, genes encoded in the dinoflagellate minicircles, was demonstrated in *Ceratium horridum* (Zauner et al., 2004). Editing events have been found to re-establish proper reading frames and evolutionary conserved functions, or may potentially create protein diversity (Ochsenreiter and Hajduk, 2006; Ochsenreiter et al., 2008; Knoop, 2011). Although the critical function of mRNA editing in dinoflagellates is not yet clear, the clustering of the edited sites in previous studies suggests that targets for editing were selected (Lin et al., 2002; Zauner et al., 2004). The potential role of both the preferential transcription of certain genomic copies of *sxtA4*, and RNA editing, in further maturing transcripts, need to addressed in order to fully tease out the regulatory processes for STX biosynthesis in this important harmful algal bloom-forming organism.

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## Conflict of interest

The authors declare that there are no conflicts of interest.

## Transparency document

Transparency document related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2014.09.015.

## Appendix I. Supplementary data

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