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Non-toxic and toxic subclones obtained from a toxic clonal culture of *Alexandrium tamarense* (Dinophyceae): Toxicity and molecular biological feature

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ABSTRACT

Alexandrium tamarense (Lebour) Taylor strain OF935-AT6 is a rare strain of paralytic shellfish toxin (PST)-producing dinoflagellate, in which non-toxic and toxic cells are found in an approximately 1:1 ratio, isolated in Japan. The non-toxic characteristics of UAT-014-009, an axenic non-toxic subclone of OF935-AT6, have been confirmed at the attomole per cell level. Three out of nine toxic subclones of OF935-AT6 became non-toxic over a relatively short period of time (4–6 years), while the other toxic subclones retained their toxicity and the non-toxic subclones retained to be non-toxic. Two axenic subclones from OF935-AT6, UAT-014-009 (non-toxic) and Axat-2 (toxic) are indistinguishable from one another, and from popularly known *A. tamarense* by rDNA sequence analysis. The most significant difference identified by subtractive hybridization of cDNA pertains to gene fragments homologous with mitochondrial cytochrome *c* oxidase polypeptide three (cox3) and cytochrome *b* (cob). Thus, the polymorphism targeting these regions was investigated by comparison of the gene length amplified by PCR using total DNA from other subclones with a range of toxicities. No direct correlation between any allele and toxicity was observed in this study.

1. Introduction

Alexandrium is an important dinoflagellate genus that comprises many toxic species whose distribution area is spreading into various parts of the world recently. Most of toxic *Alexandrium* dinoflagellates including *A. acatenella* (Cembella et al., 1987), *A. andersoni* (Ciminiello et al., 2000), *A. catenella* (Hallegraeff et al., 1988), *A. fundyense* (Anderson et al., 1990), *A. lusitanicum* (Mascarenhas et al., 1995), *A. minutum* (Chang et al., 1997), *A. ostenfeldii* (MacKenzie et al., 2004), *A. tamarense* (Buckley et al., 1976, identified as *Gonyaulax tamarensis*, Oshima and Yasumoto, 1979, identified as *G. excavata*), and *A. tamiyavanichii* (Hashimoto et al., 2002) produce potent neurotoxins called saxitoxin analogues or paralytic shellfish toxins (PSTs), which accumulate and are metabolized in shellfish (Lin et al., 2004). The toxins cause severe symptoms in humans that ingest contaminated shellfish. In addition to *Alexandrium* species, two other dinoflagellate species, *Gymnodinium catenatum* (Oshima et al., 1987) and *Pyrodinium bahamense* var. *compressum* (Harada et al., 1982), as well as cyanobacteria (Negri et al., 1995; Carmichael et al., 1997; Lagos et al., 1999; Mahmood and Carmichael, 1986; Pomati et al., 2000) and *A. tamarense* bacteria (Kodama et al., 1988), are known producers of PSTs. It remained unsolved why such a divergent and unrelated organisms have PST synthetic genes. Lateral DNA transfer by transposable elements or viral elements had been suggested, however, evidence is still lacking to date (Plumley, 1997; Llewellyn, 2006).

The difficulty of the research on the gene sequences involved in STX synthesis in dinoflagellates is due to high variability of gene profiles among *Alexandrium* species from distinct areas even within the same species (Taroncher-Ordenburg and Anderson, 2000).

The establishment of a non-toxic subclone derived from a toxic clonal strain of *Alexandrium* species seemed to be an ideal approach to obtain excellent materials for the search of genes active in the toxin synthesis, since one non-toxic subclone and one toxic

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Abbreviations: Cox3, cytochrome *c* oxidase polypeptide three; cob, cytochrome *b*; G3PDH, glycelaldehyde-3-phosphate dehydrogenase; SAHH, S-adenosylhomocysteine-hydrolase.

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subclone derived from a same toxic clonal culture are expected to have an identical genetic profile. In 1997, one of the authors of the present study conducted a series of mutation experiments using three toxic strains of *Alexandrium* species (*A. tamarense*, *A. catenella* and *A. minutum*). Non-toxic subclones were obtained from *A. tamarense* alone (from a clonal culture strain OF935-AT6) (Omura et al., 2003). Since the ratio of non-toxic to toxic subclones isolated from strain OF935-AT6 after UV irradiation was greater than expected (78%), the non-toxic subclones were not thought to result from the effects of UV irradiation used as the mutagen. Thus, the presence of non-toxic cells in the original toxic clonal culture was suspected. Subsequently, isolation of subclones from the original culture was conducted in the absence of UV irradiation. Twenty-two of 51 isolated subclones were non-toxic, thus confirming the presence of non-toxic cells in OF935-AT6 culture. This indicates that cells within strain OF935-AT6 became heterogeneous in terms of their toxicity during a 5-year culture after the establishment, despite the fact that the strain was established from a single cell. This was not previously known to occur, even though a number of researchers have established clonal cultures of toxic *Alexandrium* species. Since toxic and non-toxic cells seem to have arisen by mitosis of a single toxic cell in a controlled environment, they are thought to arise despite having an identical genetic background.

In this study we aimed to report the non-toxic feature of a representative non-toxic subclone, to find more evidence to show that non-toxic cells are not contamination of other non-toxic species similar to *A. tamarense*, to present the major differences between the genes of the given toxic and non-toxic subclones, to investigate whether the mutant genes relates to the toxicity and to show that the non-toxic mutational event could occur in a short period of time.

2. Materials and methods

2.1. Preparation of subclones

All subclones used in this study were derived from strain OF935-AT6 that was established from a single cell isolated from natural sea water. OF935-AT6 was isolated in 1993 at Ofunato Bay, Japan, by Dr. Ogata of Kitasato University. The various subclones of OF935-AT6 examined in this experiment (Series-Axat, Series-AT-6, Series-UAT and Series-UAT-014) were generated by Dr. Omura at Tokyo University of Marine Science and Technology. Details regarding generation of the subclones are described elsewhere (Omura et al., 2003). In short, a single cell isolated under the microscope using a capillary pipette from the culture was inoculated into 1 mL of T_1 modified medium in 24- or 48-well plates. Culture conditions were identical to those of the maintenance culture. After an adequate cell density was achieved, the cells were passaged using test tubes or culture flasks.

Series-Axat was prepared from strain OF935-AT6 in 1996 to obtain axenic strain. According to the results of toxin analysis, the three subclones were all toxic, including one extremely low toxic. Series-UAT was the subclones derived from mutation experiments carried out in 1997 on strain OF935-AT6 aimed at preparing non-toxic mutants. One milliliter of culture was exposed to UV light (865 $\mu W\,cm^{-2}$) for 2 min using a UV germicidal lamp. Cells surviving after UV irradiation were isolated and inoculated as described above. Toxin analysis performed soon after establishment (at that time cell density had reached more than 1×10^4 cells/mL) revealed that 39 out of 50 subclones were non-toxic. To investigate the stability of the toxicity, toxin analysis of this series was performed four months later again, and three low toxic subclones were found to have become non-toxic and one non-toxic subclone was found to have become low-toxic. Because non-toxic mutants were obtained at a greater frequency than expected, the presence of non-toxic cells in the parent strain OF935-AT6 was suspected. Series-AT-6 was established from strain OF935-AT6 without UV irradiation in 1998. Fifty-one subclones were analyzed. Twenty-nine subclones were toxic and 22 subclones were non-toxic. Thus it confirmed the presence of non-toxic cells in the original culture. Series-UAT-014 was established from the non-toxic subclone UAT-014 in 1999 in order to provide an axenic non-toxic clone by pipette washing. All of 12 subclones were non-toxic. When we initiated this study, only two subclones (toxic Axat-2 and non-toxic UAT-014-009) were known to be axenic among all subclones from strain OF935-AT6. Thus, this pair was used for subtractive hybridization of cDNA. In 2002 the toxin and polymorphism analysis of all subclones from OF935-AT6 which were maintained for 6–3 years was performed. Totally 38 subclones (2 subclones in Series Axat, 12 subclones in Series-UAT, 12 subclones in Series AT-6 and 12 subclones in Series-UAT-014) survived until 2002 and were analyzed in this study. Series-Axat-2 was prepared from the toxic subclone Axat-2 in this study in order to investigate variations in toxicity and polymorphism in the oldest toxic subclone.

The subclone numbering system is discontinuous since subclones have been lost during the course of maintenance.

2.2. Culture conditions

All subclones were maintained and grown under a 12L:12D photocycle with light provided by cool white bulbs (100–150 μmol photons $m^{-2}\,s^{-1}$) at 15 or 17 °C in modified T_1 medium (Ogata et al., 1987a; Omura et al., 2003), without nitrilotriacetic acid and with twofold EDTA and 20 nM of selenious acid, in 50- or 250-mL tissue culture flasks. Bacterial contamination of the axenic subclones was monitored by incubation of the culture media with Marine Broth 2216 or epifluorescent microscopic observation of DAPI (4′-6-diamidino-2-phenylindole)-stained culture media.

2.3. Subclone toxicity

2.3.1. HPLC assay

Ten milliliters of culture for routine analysis, 2 L of culture for precise analysis of UAT-014-009 and 0.5 mL of 1-month-culture of Series Axat-2 were harvested during the mid-exponential growth phase. Aliquots of harvested samples were used to obtain cell counts by microscopy. Cells were collected by centrifugation ($1700 \times g$ for 10 min at ambient temperature). Cell pellets were suspended with an appropriate volume of 0.5 M acetic acid, and disrupted by sonication (three times at 100 Hz for 30 s) on ice. After centrifugation ($20{,}000 \times g$ for 5 min at 4 °C), the supernatant was subjected to ultrafiltration (Ultra-Free C3LGC, 10,000 Da cut-off, Millipore), after which the filtrate was subjected to HPLC-FL (Oshima, 1995). In case of subclones other than Axat-2 and UAT-014-009, only C-toxins were analyzed, since C1/C2 were the main toxins and the toxin profiles of subclones from OF-935-AT6 were known to be similar according to the previous results (Omura et al., 2003). Standards for PST quantitation were prepared from natural sources in our laboratory.

2.3.2. Mouse bioassay of a non-toxic subclone

A mouse bioassay was performed according to the AOAC method (AOAC, 1995). The same extracts as for HPLC analysis were used. After appropriate dilution, 1 mL of diluted extract (equivalent to 1.4×10^6 cells, or 54 MU as estimated by the HPLC analysis of Axat-2 harvested in parallel) was given to mice by intraperitoneal injection.