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TOXIN COMPOSITION VARIATIONS IN ONE ISOLATE OF THE DINOFLAGELLATE *ALEXANDRIUM FUNDYENSE*

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D. M. ANDERSON, D. M. KULIS, J. J. SULLIVAN and S. HALL. Toxin composition variations in one isolate of the dinoflagellate *Alexandrium fundyense*. *Toxicon* **28**, 885–893, 1990.—A commonly accepted paradigm in the study of saxitoxin-producing dinoflagellates is that the total concentration of all toxins (toxin content) in one isolate can vary with growth conditions, but that the relative abundance of each toxin (toxin composition) does not change. We demonstrate here that dramatic changes in toxin composition do occur in one isolate of *Alexandrium fundyense*. In nitrogen- and phosphorus-limited semi-continuous cultures, toxin composition varied systematically with growth rate. When cells grew slowly under severe nutrient limitation, toxin composition was dominated by one or at most two toxin epimer pairs; as nutrient stresses eased at higher growth rates, the toxin profiles became more heterogeneous. Steady-state, sustained nitrogen limitation favored the production of toxins C 1,2 and GTX I,IV, whereas phosphorus limitation produced cells with high relative abundance of GTX II,III. STX reached its highest relative abundance when growth was most rapid. The lack of observed compositional changes in most past studies is probably not due to inherent differences in toxin biosynthetic pathways between the strains of *Alexandrium* examined, but rather to differences in the physiology of cells grown under different culturing modes (batch vs semi-continuous), methods of toxin analysis, and dominant toxins in the particular isolates examined.

INTRODUCTION

SEVERAL species within the dinoflagellate genus *Alexandrium* (formerly *Protogonyaulax;* STEIDINGER and MOESTRUP, 1990) produce an array of neurotoxins collectively called the saxitoxins (reviewed in SHIMIZU, 1987). It has long been recognized that dinoflagellate toxicity varies, both between different isolates of a species and for individual isolates under varying growth conditions (PRAKASH, 1967; PROCTOR *et al.*, 1975; SHIMIZU, 1979; ALAM *et al.*, 1979; S. HALL, Ph.D. Thesis, University of Alaska, 1982; MARANDA *et al.*, 1985; BOYER *et al.*, 1986, 1987; OGATA *et al.*, 1987). Toxin variability among isolates of a species can be due in part to differences in toxin composition (i.e. each produces different combinations of saxitoxin and its 11 derivatives; SHIMIZU, 1979; ALAM *et al.*, 1979; S.

HALL, Ph.D. Thesis, University of Alaska, 1982; CEMBELLA *et al.*, 1987). These compositional differences have important public health implications due to the significant differences in potency between the saxitoxins (GENENAH and SHIMIZU, 1981; HALL and REICHARDT, 1984). They have also proved useful as an identification tool in studies of interrelationships between dinoflagellate species and populations (S. HALL, Ph.D. Thesis, University of Alaska, 1982; CEMBELLA *et al.*, 1987) or in linking certain dinoflagellates to toxin profiles in shellfish (OSHIMA *et al.*, 1987; ANDERSON *et al.*, 1989).

Variability in the toxicity of a single isolate is generally attributed to differences in the rate of toxin production or accumulation under different growth conditions and not to any differences in toxin composition. Until recently, all studies that separately quantified saxitoxin and its derivatives concluded that toxin composition is a relatively stable or conservative property of each clone (S. HALL, Ph.D. Thesis, University of Alaska, 1982; CEMBELLA *et al.*, 1987; BOYER *et al.*, 1987; OGATA *et al.*, 1987). Some compositional differences have been observed (S. HALL, Ph.D. Thesis, University of Alaska, 1982; BOYER *et al.*, 1987; CEMBELLA *et al.*, 1987) but these were small and considered insignificant. Very different results were recently reported by BOCZAR *et al.* (1988) who described systematic changes in toxin composition of individual isolates of *A. tamarense* and *A. catenella* at various growth stages in batch culture. The changes were substantial, but were only observed in aging cultures that were two weeks or longer in plateau phase. In this paper, we report dramatic changes in toxin composition in one isolate of *A. fundyense*. Results are from semi-continuous cultures, which reflect sustained, steady-state physiological adaptations by the cells to nitrogen and phosphorus limitation.

MATERIALS AND METHODS

The *Alexandrium fundyense* strain (GtCA29) was established in January 1985 from a cyst isolated from Gulf of Maine sediments 20 miles east of Portsmouth, New Hampshire. A single cell was re-isolated from the parent culture to ensure that the culture was clonal. Prior to the experiments, all cultures were maintained at 15°C in K medium (KELLER and GUILLARD, 1985) made with filtered (0.45 μm) Vineyard Sound seawater (31 ppt salinity), under a 14:10 hr light:dark cycle (ca 250 μEinsteins $m^{-2}s^{-1}$ irradiance provided by Cool White fluorescent bulbs). All culture glassware was autoclaved partially filled with distilled, deionized water which was decanted after sterilization and the sterile medium added aseptically. The cultures were not axenic.

Semi-continuous cultures

Initial attempts to grow strain GtCA29 in true continuous cultures failed due to the organism's sensitivity to stirring. Consequently, a semi-continuous mode was used whereby medium was pumped into the unstirred growth chamber continuously at the desired dilution rate, but cells and medium were removed manually once each day following brief stirring. Details of the culturing equipment and protocol are described in ANDERSON *et al.* (in press). Briefly, culture was initiated with 5 ml of exponentially growing, axenic *A. fundyense* cells inoculated into 1-l, water-jacketed reactor vessels. Each vessel contained 1-l of modified K medium (KELLER and GUILLARD, 1985), prepared with 0.2 μM filtered Sargasso seawater reduced in salinity to 30 ppt with distilled water. Seawater was autoclaved in teflon bottles and nutrients were later added aseptically (BRAND *et al.*, 1981). Modification of the K medium consisted of a decrease in the NaH_2PO_4 and $NaNO_3$ concentration to 0.91 μM and 44.2 μM for the P-limited and N-limited series, respectively, with NH_4^+ omitted in the latter as well. The cultures were continuously bubbled with sterile laboratory air and were maintained at 15°C with continuous illumination of approximately 100 μEinsteins $m^{-2}s^{-1}$ provided by Cool White bulbs.

Before the cultures reached maximum cell density in batch growth in the reactor vessels, a continuous supply of fresh medium was pumped from a 4–l polypropylene reservoir stored at room temperature. All components of the continuous culture assembly were autoclaved and aseptically connected prior to inoculation. At the same time each day, the cultures were mixed for a few sec with a magnetic stir bar and the sample withdrawn equivalent to the volume of fresh medium pumped in during the previous 24 hr. Daily triplicate cell counts were performed on the withdrawn sample. The culture was considered to be at steady state and was harvested when cell density remained constant for a minimum of two weeks.

Toxin analyses

Medium and cells were withdrawn from the growth chamber and centrifuged for 3 min at 1700 × *g* at 23°C. The supernatant was aspirated and discarded, and 1.0 ml of 0.05 M acetic acid added to the pellet. The samples were frozen and thawed three times prior to filtration (0.45 μm) into 1.5 ml teflon capped borosilicate autoanalyzer vials. This procedure had been found to be equivalent to sonication in efficiency of toxin extraction. Toxin was analyzed by HPLC using the method of SULLIVAN and WEKELL (1988). The C toxins were quantified in a second HPLC run using the methods of SULLIVAN (in press).

Abbreviations used throughout this text are: STX = saxitoxin; NEO = neosaxitoxin; GTX I,IV = gonyautoxins I and IV; GTX II,III = gonyautoxins II and III; C 1,2 = toxins C1 and C2; B1 = toxin B1. Several of these designations represent the pooled concentrations of two toxins due to problems with epimerization. Toxicities (in STX equivalents/cell) were calculated from the molar composition data using individual potencies (GENENAH and SHIMIZU, 1981; HALL and REICHARDT, 1984).

RESULTS

The focus of this paper is on changes in dinoflagellate toxin composition in semi-continuous cultures. Details of the cell physiology and rates of toxin production in these same experiments are presented elsewhere (ANDERSON *et al.*, in press).

Semi-continuous cultures limited by either nitrogen or phosphorus were established at a broad range of dilution (= growth) rates. Under nitrogen limitation, the total toxin content of the cells was relatively constant between 10–20 fmole/cell at low and intermediate growth rates, but increased rapidly to 50–80 fmole/cell at the higher growth rates (i.e. when nutrients were more abundant but still limiting; Fig. 1A). Across the entire range of growth rates examined (between 0.035 and 0.37/day) only toxin B1 remained relatively constant as a percentage of total toxins (Fig. 1B). As growth rate increased, C 1,2 and GTX I,IV decreased from ca 50 to 25 and from 30 to 14 mole % respectively (Fig. 1B,C). Conversely, NEO, GTX II,III, and STX increased from 10 to 22, from 6 to 18, and from 0 to 20 mole %, respectively (Fig. 1B,C).

Under phosphorus limitation in semi-continuous culture, the toxin content of the cells decreased with increasing growth rate (Fig. 2A). As this occurred, the relative molar abundances of toxins B1, NEO, GTX I,IV and C 1,2 all remained relatively constant, whereas STX increased from 10–30 mole % and GTX II,III decreased from 55–35 mole % (Fig. 2B,C).

Linear regressions of the mole % of each toxin vs growth rate were calculated for each semi-continuous culture series. Under nitrogen limitation, the slopes of the curves for toxins GTX I,IV, GTX II,III NEO, STX and C 1,2 were all significantly different from zero ($P < 0.01$); only B1 showed no relationship. Under phosphorus limitation, only GTX II,III and STX had non-zero slopes ($P < 0.01$).

DISCUSSION

Contrary to consistent reports in the literature that toxin composition of individual isolates of the saxitoxin-producing dinoflagellates remains essentially constant under a wide variety of growth stages and environmental conditions (S. HALL, Ph.D. Thesis, University of Alaska, 1982; CEMBELLA *et al.*, 1987; BOYER *et al.*, 1987; OGATA *et al.*, 1987), our data on *Alexandrium fundyense* and that of BOCZAR *et al.* (1988) on *A. tamarense* and *A. catenella* show conclusively that major compositional changes can occur. These changes are in the relative abundance (mole %) of the different toxins; the specific suite of toxins produced does not vary. Toxin composition is thus a variable characteristic of the dinoflagellate that reflects adaptations to nutritional and environmental conditions. It is not a fixed genetic trait or "fingerprint".

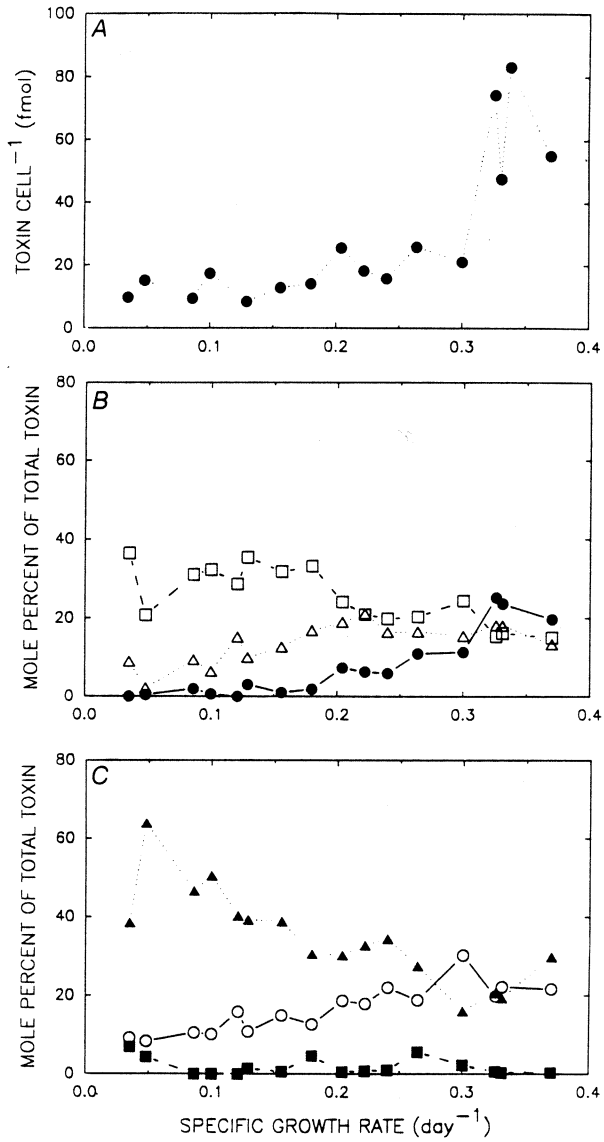

FIG. 1. TOXIN CONTENT AND COMPOSITION OF *Alexandrium fundyense* AS A FUNCTION OF SPECIFIC GROWTH RATE IN NITROGEN-LIMITED SEMI-CONTINUOUS CULTURE.
(A) Toxin content (all saxitoxins combined); (B and C) relative abundance (mole % of STX (●), GTX II,III (△), GTX I,IV (□), C 1,2 (▲), NEO (○) and B1 (■).

BOCZAR *et al.* (1988) demonstrated toxin composition variability in two different isolates of *Alexandrium*, but those changes were only evident in old batch cultures that had been in plateau phase for several weeks. Since those cells had also decreased in toxicity nearly 10-fold from peak values, the results might be considered to represent an unusual physiological condition reflecting differential catabolism of the various toxins, with little relevance to actively growing cells. This is not the case, however, as our data demonstrate

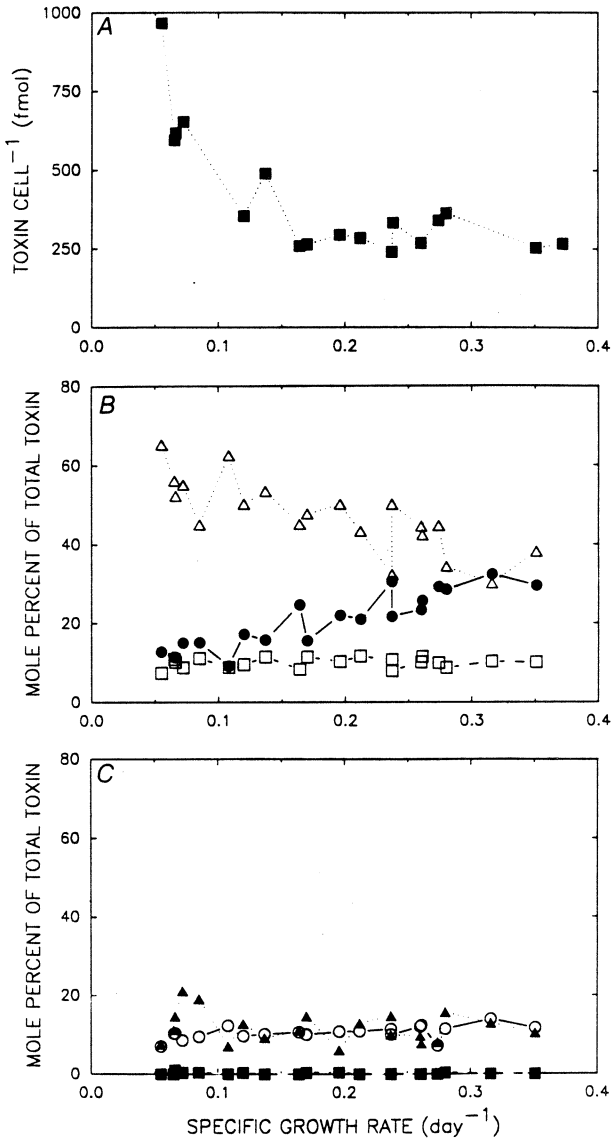

FIG. 2. TOXIN CONTENT AND COMPOSITION OF *Alexandrium fundyense* AS A FUNCTION OF SPECIFIC GROWTH RATE IN PHOSPHORUS-LIMITED SEMI-CONTINUOUS CULTURE.
(A) Toxin content (all saxitoxins combined); (B and C) relative abundance (mole %) of STX (●), GTX II,III (△), GTX I,IV (□), C 1,2 (▲), NEO (○) and B1 (■).

that toxin composition changes can be sustained in dividing cells that are adapted to different degrees of nutrient deficiency in approximate steady-state conditions. In these semi-continuous cultures, increases or decreases in certain of the major toxins were observed as linear functions of growth rate. Since each data point represents a separate culture maintained at a specific dilution rate for a minimum of two weeks and since the culturing and HPLC analyses occurred in random order over a two year interval, the

results for each dilution rate are independent and the observed trends real. Further confirmation of the statistical validity of these trends comes from linear regressions of mole % abundance vs growth rate in which five of the toxins under nitrate limitation and two under phosphorus limitation had slopes that were significantly different from zero.

In both semi-continuous culture series, STX increased as growth rate increased. This is consistent with the batch culture results of BOCZAR *et al.* (1988) who showed highest relative abundance of STX in mid-exponential growth cells of *A. tamarense,* decreasing thereafter as the cells entered plateau phase. In our nitrogen-limited cultures, GTX I,IV and C 1,2 relative abundances were highest at the lowest growth rates, whereas only one toxin epimer pair (GTX II,III) was dominant at low growth rates with phosphorus limitation. Thus at low growth rates under severe nutrient limitation, toxin composition was dominated by one or at most two toxins in our *A. fundyense* strain. As nutrient stresses eased at higher dilution (= growth) rates, the toxin profiles became more heterogeneous. If each culturing series (i.e. nitrogen- and phosphorus-limited) had been extended to the maximum growth rate for this organism (approximately 0.5/day in batch culture), we would expect the trends in toxin composition for the two nutrients to converge to equivalency since neither nutrient would have been limiting. Our highest growth rates were 20% lower than the maximum achieved in batch culture, so nutrient limitation was still occurring in those cultures and strict equivalence would not be expected. However, it is clear from Figs 1 and 2 that in each nutrient series, the various toxins did trend towards a consistent "nutrient-replete" toxin composition profile for this strain of *A. fundyense:* 20–30% STX; 10–15% GTX I,IV; 10–20% NEO; 10–20% C1,2; 15–30% GTX II,III, and virtually no B1. This is a markedly different composition from those observed at the lowest growth rates in Figs 1 and 2.

Several investigators looked for toxin composition variations in *Alexandrium* (= *Protogonyaulax*) species, but found none or concluded that observed differences were insignificant (S. HALL, Ph.D. Thesis, University of Alaska, 1982; CEMBELLA *et al.*, 1987; BOYER *et al.*, 1987; OGATA *et al.*, 1987). Now our data and that of BOCZAR *et al.* (1988) show that significant changes are possible. We believe that the discrepancy between these newer data and negative findings of others is not due to inherent differences in toxin biosynthetic pathways between the strains of *Alexandrium* examined, but instead reflects differences in experimental protocols, methods of toxin analysis, and dominant toxins in the particular isolates examined.

HALL (Ph.D. Thesis, University of Alaska, 1982) tested a variety of *Alexandrium* isolates for toxin composition. Some differences were occasionally seen, but were considered small relative to the inaccuracies of the mouse bioassay—the only available analytical methodology at the time. Studies by BOYER *et al.* (1986, 1987), CEMBELLA *et al.* (1987), and OGATA *et al.* (1987) used the more accurate HPLC method for toxin quantification so their results should be comparable to ours. However, OGATA *et al.* (1987) did not analyze samples at different growth stages, used nutrient-replete cultures, and only compared toxin profiles at one point in time (late exponential phase) between treatments.

BOYER *et al.* (1986) used HPLC to look for toxin composition differences in 2 isolates of *Alexandrium* grown in nutrient-replete batch culture. A gradual increase in GTX I,IV was balanced by a decrease in GTX II,III (from 45–20 mole %) as the culture of one isolate aged. This difference between stationary phase and exponential phase cells is the same pattern observed by BOCZAR *et al.* (1988). In a subsequent study using a different *Alexandrium* isolate, BOYER *et al.* (1987) tested for toxin composition differences in nutrient-replete, low PO_4^{3-} and low NO_3^- medium, but saw only a minor increase in GTX

I,IV with culture age for the P-limited cuture. The lack of observed changes in other treatments or in other toxins may reflect the toxin composition of the strains analyzed. Their isolate had B2 as the major toxin (ca 50 mole %), with C 1,2 (20 mole %) and GTX I,IV (8 mole %) as minor components. Since our isolate had no detectable B2, and that of BOCZAR *et al.* (1988) had at most 2 mole %, we do not know how that specific toxin would have varied. Variability in the less abundant C toxins would have been difficult to detect relative to the precision of the analytical method. CEMBELLA *et al.* (1987) used HPLC to analyze several isolates at multiple points during nutrient-replete batch culture growth, but once again, most clones contained predominantly B2 and C toxins. Interestingly, the only isolates in which those authors did notice compositional changes were two that contained neither B nor C toxins. In one, a decrease in GTX II,III was observed in stationary phase, but the low relative abundance of those toxins (2.6 mole %) made this difference seem insignificant. These authors also noted that toxin B1 was detected in a senescent culture (3.8 mole %) although it was typically absent at other growth stages.

We can conclude that one difficulty in observing toxin composition changes in batch cultures could be due to the predominance of toxins that may not vary as much as those in the isolate that we examined. More importantly, we feel the differences between our results and those of other workers reflect fundamental differences in the physiology of cells grown in batch vs semi-continuous cultures. In fact, had the isolates of CEMBELLA *et al.* (1987) and BOYER *et al.* (1987) been grown in semi-continuous culture, we would expect compositional differences to be evident, if only in the minor toxin components. This is because nutrient uptake, cell division, and general vegetative metabolism would be sustained during a steady-state maintained for at least several weeks, providing more time for interconversions, leakage, or differential synthesis to occur relative to low-nutrient batch cultures, which change from nutrient-sufficient conditions to complete nutrient depletion in a day or less. The resulting differential inhibition of various metabolic pathways might make it difficult for cells to change the toxin composition that they possessed at the onset of the nutrient exhaustion. The implication is that the true extent of toxin composition variation will only be evident when cells are given time to adjust their physiology to an unchanging environment ("balanced growth"), as in a semi-continuous culture or a very old batch culture.

Our results offer new insights into the complexities of toxin biosynthesis. Phosphorus limitation did not stop toxin synthesis completely, but instead resulted in the accumulation of predominantly GTX II,III. Likewise, even severe nitrogen limitation was still associated with toxin production, with the major products being toxins C 1,2 and GTX I,IV. STX accumulation was maximal when growth conditions were optimal. These observations are tantalizing clues to the longstanding mystery of the function of toxins in dinoflagellate metabolism, but biochemical explanations will be possible only with more detailed information than that presently available on saxitoxin biosynthetic pathways (SHIMIZU *et al.*, 1984) or on the physiological changes that occur in cells when nutrients or other environmental parameters limit growth (ANDERSON *et al.*, in press).

The final aspect of our study that bears discussion relates to the use of toxin composition profiles in inter- and intraspecific comparisons of *Alexandrium* populations (S. HALL, Ph.D. Thesis, University of Alaska, 1982; CEMBELLA *et al.*, 1987) or in identifying the dinoflagellates responsible for particular toxin profiles in shellfish (OSHIMA *et al.*, 1987; ANDERSON *et al.*, 1989). In the former instance, CEMBELLA *et al.* (1987) performed cluster analysis on *Alexandrium* isolates from different regions, using the mole % of each toxin as the basis for comparison. As described earlier, those authors

were careful to test some of their isolates for toxin composition changes and found none. As long as future studies of this type harvest cells during carefully-defined and consistent stages of batch culture growth, comparisons between isolates should be valid. Nevertheless, the most accurate (but most coarse) comparison of geographically-dispersed isolates would be based on the presence or absence of each toxin, not on its relative concentration. Even then, Fig. 1 demonstrates that there can be growth conditions where one toxin (in our case STX) can be essentially undetectable and other conditions in which it is a prominent or even dominant component.

The same caution must be exercised when comparing toxin profiles in shellfish with those from cultured dinoflagellates. In several cases, this has been successful in establishing linkages (OSHIMA *et al.*, 1987; ANDERSON *et al.*, 1989), but it is now clear that the environmental variability of natural waters might result in dinoflagellate (and thus shellfish) toxin profiles which differ significantly from those of the same species grown in nutrient-replete batch cultures.

The potential for toxin composition variation within individual isolates of *Alexandrium* adds a new level of complexity that must henceforth be incorporated into the design and interpretation of experiments and field programs. However, these data also provide new insight that is important with respect to studies of the synthesis of the saxitoxins, their movement through the food chain, and the geographic distribution of the causative organisms.

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