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Effects of salinity variation on growth and yessotoxin composition in the marine dinoflagellate *Lingulodinium polyedra* from a Skagerrak fjord system (western Sweden)

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ABSTRACT

The marine dinoflagellate *Lingulodinium polyedra* is a toxigenic species capable of forming high magnitude and occasionally harmful algal blooms (HABs), particularly in temperate coastal waters throughout the world. Three cultured isolates of *L. polyedra* from a fjord system on the Skagerrak coast of Sweden were analyzed for their growth characteristics and to determine the effects of a strong salinity gradient on toxin cell quotas and composition. The cell quota of yessotoxin (YTX) analogs, as determined by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS), ranged widely among strains. For two strains, the total toxin content remained constant over time in culture, but for the third strain, the YTX cell quota significantly decreased (by 32%) during stationary growth phase. The toxin profiles of the three strains differed markedly and none produced YTX. The analog 41a-homo-YTX (*m/z* 1155), its putative methylated derivative 9-Me-41a-homo-YTX (*m/z* 1169) and an unspecified keto-YTX (*m/z* 1047) were detected in strain LP29-10H, whereas strain LP30-7B contained nor-YTX (*m/z* 1101), and two unspecified YTX analogs at *m/z* 1159 and *m/z* 1061. The toxin profile of strain LP30-8D comprised two unspecified YTX analogs at *m/z* 1061 and *m/z* 991 and carboxy-YTX (*m/z* 1173). Strain LP30-7B cultured at multiple salinities (10, 16, 22, 28 and 34) did not tolerate the lowest salinity (10), but there was a statistically significant decrease (by 21%) in toxin cell quota between growth at the highest versus lower permissible salinities. The toxin profile for strain LP30-7B remained constant over time for a given salinity. At lower salinities, however, the proportion of the unspecified YTX analog (*m/z* 1061) was significantly higher, especially with respect to nor-YTX (*m/z* 1101). This study shows high intra-specific variability in yessotoxin composition among strains from the same geographical region and inconsistency in toxin cell quota under different environmental regimes and growth stages in culture. This variation has important implications for the kinetics of YTX production and food web transfer in natural bloom populations from diverse geographical regions.

1. Introduction

Massive algal proliferation events can pose a threat to ecosystem function, marine fauna and human health, especially in cases of toxigenic harmful algal blooms (HABs) (Hallegraeff, 2003). Such toxins can accumulate in suspension feeding bivalve mollusks, for example, and cause acute human illnesses upon consumption (van Dolah, 2000).

Among these algal toxins, yessotoxins (YTXs) were first isolated from the digestive gland of the Japanese scallop *Patinopecten yessoensis* from Mutsu Bay, Japan (Murata et al., 1987). Yessotoxins are lethal to mice when administered intraperitoneally (Ogino et al., 1997; Tubaro et al., 2003, 2010), and the behavioral symptoms are similar to those induced by the diarrheic shellfish poisoning (DSP) toxins. On this basis, YTXs were initially included within the DSP toxin complex, but when it later became evident that YTX analogs, in contrast to DSP toxins, do not induce diarrhea in oral dosages (Ogino et al., 1997), they were removed from the group. To date no case of YTX intoxication in humans has been reported, but studies on mice and rats as well as on human cell lineages showed several cytotoxic effects caused by YTX. For example, cytotoxic effects are expressed in cardiomyocytes (Bianchi et al., 2004; Dell'Ovo et al., 2008), myoblasts (Korsnes and Espenes, 2011), the thymus (Franchini et al., 2004) and the immune system (La Rosa de et al., 2001). The exact mode of action of YTX remains unknown, but interference with Ca^{2+} ion channels regulating cell homeostasis is strongly

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indicated. As a precaution, regulatory limits of exposure for human consumption are still in effect.

Yessotoxins are planar ladder-frame polyethers bearing two sulfate groups. Around 100 YTX analogs have been reported from marine dinoflagellates or as metabolites within suspension feeding mollusks. The known producers of YTX are four closely related planktonic dinoflagellate species, *Protoceratium reticulatum* (Claparède & Lachmann) Bütschli (Satake et al., 1997), *Lingulodinium polyedra* (F.Stein) J.D. Dodge (Paz et al., 2004), *Gonyaulax spinifera* (Claparède & Lachmann) Diesing (Rhodes et al., 2006) and *G. taylorii* Carbonell-Moore (Álvarez et al., 2016). Cell quotas of YTX analogs vary dramatically depending on species, strain and environmental conditions. The greatest diversity of YTX analogs has been discovered in *P. reticulatum*; 40 distinct putative YTX structures were determined by NMR and LC-MS/MS (Miles et al., 2005a, b; Miles et al., 2006). Whereas most strains of *P. reticulatum* have been reported to be toxigenic, the toxicity of *G. spinifera* and *L. polyedra* has been documented in fewer cases and non-toxic populations are frequently prevalent.

The majority of studies on production of YTX have been performed on *P. reticulatum*, often focusing on YTX cell quotas during stationary phase and on comparison of the toxin profile among strains from the same or different locations (Mitrovic et al., 2005; Paz et al., 2007, 2013; Sala-Pérez et al., 2016). In some cases, the toxin content per cell was compared between different growth phases or as a function of salinity, temperature, and nutrient availability (Guerrini et al., 2007; Röder et al., 2012). The gonyaulacoid dinoflagellate *P. reticulatum* is often considered as a model species for studies on ecophysiology and dynamics of YTX production. This is based upon the assumption that since *P. reticulatum*, *L. polyedra, G. spinifera* and *G. taylorii* are close phylogenetic relatives within the order Gonyaulacales, they probably share elements of the same biosynthetic gene cluster for YTX production. Less experimental evidence is available for *G. spinifera, G. taylorii* and *L. polyedra*, but the characteristic mechanisms of toxin production for *P. reticulatum* could be similar to these other species.

The toxin profiles of three strains of *L. polyedra* isolated from within a fjord system subject to strong salinity gradients on the west coast of Sweden were compared with respect to their compositional stability over a culture cycle. The yessotoxin composition of one cultured strain grown at different salinities was analyzed to determine the effect of decreased salinity on growth, total toxin cell quota and toxin profile.

2. Materials and methods

2.1. Species identification and culture of isolates

Multiple clones (> 40) of *L. polyedra* were isolated from plankton samples within fjords along the west coast of Sweden (Skagerrak) during the HABcyst oceanographic cruise HE431 aboard *R.V. Heincke* in late summer 2014. Two vertical net tows with a 20 µm mesh Nitex plankton net were conducted through the upper 10–30 m of the water column of each station, depending upon the water depth. The net tow concentrates were examined under a stereomicroscope (M5A, Wild, Heerbrugg, Switzerland) for cell isolation of candidate species. The three clonal isolates in the present study originated from 10 m vertical net tows conducted on September 09, 2014 from St 29 (58° 5.01′ N 11° 47.02′ E; maximum water depth: 11.7 m) or St 30 (58° 14.88′ N 11° 50.44′ E: 16.7 m). During this sampling at both stations, the water column was homogeneous with respect to temperature; in the upper 10 m temperature was 17–18 °C. The water column was stratified by salinity with a shallow halocline at 5 m and range of salinity from 22 to 24 within the upper 10 m sampled by net tow.

Single cells of *L. polyedra*, designated as isolate numbers LP29-10H, LP30-8D and LP30-7B, were isolated on board by micropipette by transfer into individual wells of 96-well tissue culture plates (TPP, Trasadingen, Switzerland), each containing 250 µL of 1/10 strength K-medium (Keller et al., 1987) prepared from 0.1 µm sterile-filtered natural Antarctic seawater diluted with 0.45 µm filtered seawater from the sampling location. For the stock K-medium, the micro- and macronutrients, as well as vitamins and trace metals, were added after autoclaving; as a final step the medium was 0.1 µm sterile-filtered. The plates were incubated on board at 15 °C under dim light (40 µmol photons $m^{-2} s^{-1}$) in a controlled environment growth chamber (Model MIR 252, Sanyo Biomedical, Wood Dale, USA).

Upon return to the laboratory the plates were transferred into a walk-in growth chamber at 15 °C and a light intensity of 50 µmol photons $m^{-2} s^{-1}$ on a 16:8 h light:dark cycle. After several weeks of growth, isolates were examined for viability and contamination, and unialgal survivors were then transferred to 24-well tissue culture plates, each well containing 2 mL of K-medium diluted 1:1 with filter-sterilized and autoclaved Antarctic seawater. The cultures were scaled up to 250 mL in plastic tissue culture flasks on K-medium prepared with autoclaved North Sea seawater. Identification of the experimental cultures as *L. polyedra* was confirmed by probing the 28S rDNA gene (A. Mera and A. Cembella, unpublished data).

Stock cultures were kept in a walk-in growth chamber at 15 °C and at a light intensity of 110 µmol photons $m^{-2} s^{-1}$ on a 16:8 h light:dark cycle. All cultures were grown in K-medium based on autoclaved North Sea seawater with salinity around 34.

2.2. Acclimated growth and toxin comparison experiment

For this experiment three different clonal isolates, LP29-10H, LP30-8D and LP30-7B, were grown in triplicate in 2 L borosilicate glass Erlenmeyer flasks. A stock culture of 1 L volume was acclimated for four weeks to the experimental conditions for optimal growth (15 °C, 110 µmol photons $m^{-2} s^{-1}$). After acclimation, experimental cultures were initiated in fresh K-medium in triplicate 2 L Erlenmeyer flasks, each with a starting cell density of around 500 cells mL^{-1}. Every two to three days the cell density was determined by microscopic counting and toxin samples were taken. The cultures were left to grow until they reached stationary phase, indicated by at least three consecutive cell density determinations without further increase.

2.3. Salinity-dependent growth and toxin production experiment

Strain LP30-7B was selected for the salinity-dependent growth experiment. Within six weeks the culture was stepwise acclimated to lower salinities, generating 1 L of stock culture for each salinity. Triplicate cultures were grown for 27 days in K-medium at five different salinities ranging from 10 to 34; salinity was adjusted by adding deionized water prior to autoclaving. At higher salinities (22, 28 and 34), triplicate 2 L cultures with a starting cell density of around 500 cells mL^{-1} were initiated. At the two lowest salinities (10 and 16), the cell densities in the stock cultures remained so low that only one 2 L culture could be maintained. Every two to three days the cell density was determined, and toxin samples taken.

2.4. Cell densities

Cell densities were determined by optical microscopic counts in cylindrical polymethacrylate sedimentation chambers. The chambers had a fixed volume of 2 mL, but the volume of culture added by pipette varied depending upon the cell density. After thoroughly mixing the culture, one to two droplets of Lugol´s iodine solution were added to an appropriate volume of culture to count at least 400 cells per chamber. In cases of dense cell suspensions, the sample was diluted with K-medium. The samples were then allowed to sediment in the chambers for 10–15 min prior to counting with an inverted microscope (Axiovert 40 C and Axiovert 200, Carl Zeiss AG, Feldbach, Switzerland).