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Evidence for accumulation of *Synechococcus elongatus* (Cyanobacteria: Cyanophyceae) in the tissues of the oyster *Crassostrea gigas* (Mollusca: Bivalvia)

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ABSTRACT

Cyanobacteria appear to have direct relations with mollusks in several aspects. This is the first time, distinguishing Gram-negative cyanoprokaryotic *Synechococcus elongatus* as bright yellow-gold autofluorescence by Lillie's and Hiss' staining methods on paraffin-embedded tissues of *Crassostrea gigas*. Three diets: cyanoprokaryotes, cyanoprokaryotes with microalgae, and only microalgae were evaluated. Cyanoprokaryotes were intact, densely bundled, and immersed in the cytosol of the digestive gland, connective tissue, mantle, and gonad of *C. gigas*, revealing an accumulation systemic without tissue damage observed by histology. Unexpectedly, cyanoprokaryotes were slightly most accumulated with microalgae diet by each of the tissues of the *C. gigas* than with any other diets. Cyanoprokaryotes tend to be in mean slightly higher in the digestive gland than in any other tissues respectively for each diet, although these values are closely similar to connective tissue. A possible order of exposure of the oyster tissues to accumulation of cyanoprokaryotes was digestive gland, connective tissue, mantle, and gonad. Thereby, the digestive gland could be the major target tissue for the accumulation. Our observations provide a valuable insight regarding the ability of cyanoprokaryotes to penetrate, spread, and remain inside the oyster tissues, suggesting for *S. elongatus*: (1) a pre-accumulation in oyster tissues from the natural environment, (2) a phagocytosis and/or endocytosis process rather than ingestion and extracellular digestion, (3) an apparent cellular division in the cytosol of oyster tissues, (4) an apparent inter-tissue movement, and (5) a possible endosymbiosis between *C. gigas* and *S. elongatus*. Hereby, it is possible that *S. elongatus* have a well-developed host–endobiont relationship with oysters, and thereby support future work toward a description of the escape and spreading mechanisms of *S. elongatus* inside the tissues of mollusks, and put forward questions as why it is there? and are the cells active or inactive?

1. Introduction

Cyanobacteria, also known as blue-green algae, blue-green bacteria, and/or cyanoprokaryotes, are a large family of unicellular organisms (pico-planktons, prokaryotes, photosynthesis) that live in fresh, brackish, marine water (from epipelagic to bathypelagic waters: Sohrin et al., 2011), hypersaline environments, and deserts; and on a global scale, they appear to contribute significantly to ecology, primary production, and biogeochemical cycles in the ocean (Waterbury et al., 1979, 1986; Murphy and Haugen, 1985; Stockner and Antia, 1986; Senga and Horiuchi, 2004; Rosales et al., 2005; Sohrin et al., 2011).

Cyanobacteria are important organisms in the areas of agriculture (Di Piero and Pascholati, 2002; Prasanna et al., 2002), aquaculture (Gallager et al., 1994; DeMott et al., 1998; Von Elert and Wolffrom, 2001; Bec et al., 2006; Meseck et al., 2007; Basen et al., 2011), industrial processes (Radmann and Viera-Costa, 2008),

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pharmaceutical industry (Volk and Furkert, 2006; Jaiswal et al., 2008; Sánchez-Saavedra et al., 2010), and human health (Labine and Minuk, 2009), and so on.

Ecologically, there is increasing recognition that algae blooms in oceans particularly of the genus *Synechococcus* are threatening the sustainability of some marine ecosystems (Morel, 1997; Chorus and Bartram, 1999; Glibert et al., 2004; Murrell and Lores, 2004; Hudnell, 2008; Fournie et al., 2008; Havens, 2008), due that they disrupt the ecosystem structure and function of diverse marine organisms, with which are referred as ecosystem disruptive algal blooms "EDABs" rather than as harmful algae blooms "HABs" with effect toxic (Sunda et al., 2006).

Nonetheless, it has been reported that the genus *Synechococcus* is capable of producing toxigenic strains (Skulberg et al., 1993) and recently it has been suggested that *Synechococcus elongatus* might present a nonspecific neurotoxin, saxitoxin, "STX" (Maciel-Baltazar, 2006; Hernández-Enríquez et al., 2007; Hernández-Enríquez, 2007; Matus-Hernández, 2010), responsible for the human disease known as paralytic shellfish poisoning (PSP) caused by its ingestion usually through bivalve mollusks (i.e., mussels, clams, oysters, and scallops) contaminated by toxic algal blooms (Clark et al., 1999; Etheridge, 2010). Yet, there has been no consensus on whether *Synechococcus elongatu* is or not "toxic" on mollusks, so direct evidence is needed.

Accordingly, assessing the direct interactions between mollusks and individual species of harmful algae can provide insights on the responses of bivalves, as they are exposed to harmful algal blooms, and is that, some harmful algal cells have been observed into tissues of scallops (Leibovitz et al., 1984) and oysters (Wikfors and Smolowitz, 1995); therefore, it may be feasible for bivalves to identify algal cells as foreign invaders.

Thus, the current study was designed to investigate the accumulation of *S. elongatus* in tissues (i.e., digestive gland, connective tissue, mantle, and gonad) of the oyster *C. gigas*. Oyster *C. gigas* were selected as they are a common benthic invertebrate in many marine ecosystems throughout the world that represent commercial shellfish species and the coccoid cyanoprokaryotic *S. elongatus* because it is a potentially toxic or injurious species in the Southern Mexican Pacific (i.e., Oaxaca and Chiapas, see Torres-Ariño and Mora-Heredia, 2010).

2. Materials and methods

2.1. Obtention, maintenance, and morphometric measurements of oysters

C. gigas (Thunberg, 1793) adults ($n = 204$ oysters) used for this study were purchased from Acuícola San Quintín S.A. de C.V., located at the great coastal lagoon of the San Quintín Bay, Baja California, Mexico. All oysters were packed at $15 \pm 2\,^{\circ}C$ in a plastic storage box and transported mainly by air (around 6 h) to the Universidad del Mar "UMAR" at Puerto Angel, Oaxaca, Mexico, for experimental treatment. On arrival to the laboratory, organisms were washed and scrubbed to eliminate epibionts; but due to natural spawning of the oyster at about the second day, they were packed in sacks and carried by boat to La Boquilla Bay, Puerto Angel, Oaxaca (15°40′51″ N, 96°27′56″ W), located 20 min from the University, where they were maintained for three months (June–August 2008) at 20 m depth, salinity of 34 ± 1; and temperature $28 \pm 2\,^{\circ}C$ before starting experimental treatment. Once back in the laboratory, the organisms were washed and scrubbed to eliminate epibionts, and total length ($TL \pm 0.1$ mm), total width ($TWI \pm 0.1$ mm), and total height ($TH \pm 0.1$ mm) were measured for each oyster shell with a Vernier caliper, and total weight including the shell ($TW \pm 0.1$ g) was recorded (Galtsoff, 1964).

2.2. Obtention and maintenance of cyanoprokaryotes and microalgae

The cyanoprokaryotes *S. elongatus* (Nägeli) Nägeli 1849-strain SYNE-3, were primarily isolated from several water samples collected from diverse environments around the Oaxaca region during 2002–2004 (Torres-Ariño and Mora-Heredia, 2008, 2010); and maintained until the present at the Biotechnological Microalgae Laboratory "BML" of the UMAR, at 33 ± 2 of Salinity, and $23 \pm 1\,^{\circ}C$ in ASN-III culture medium (Rippka et al., 1979) and in Q-Foska Foliar®, a foliar fertilizer used routinely for mass production (Anzueto-Sánchez, 2005; Hernández-Enríquez, 2007; Torres-Ariño and Mora-Heredia, 2010). Cyanoprokaryotic cells were harvested in their late-exponential growth phase (i.e., around 30 days in culture) when possible toxic effects were suggested at the experimental level, i.e., around 80 cell millions per milliliter (Anzueto-Sánchez, 2005; Hernández-Enríquez et al., 2007; Hernández-Enríquez, 2007; Matus-Hernández, 2010) and later concentrated through continuous centrifugation, followed by frozen storage (Rosales et al., 2005; Torres-Ariño and Mora-Heredia, 2010). Microalgae *Tetraselmis suecica* Butcher 1959 and *Isochrysis galbana* Parke 1949 variety *tahitiana* used for this study are routinely used in the BML and were maintained at 33 ± 2 of Salinity, and $23 \pm 1\,^{\circ}C$ in f/2 culture medium (Guillard and Ryther, 1962) and in Q-Foska Foliar® during mass production (Anzueto-Sánchez, 2005; Hernández-Enríquez, 2007; Torres-Ariño and Mora-Heredia, 2010).

2.3. Experimental accumulation of the cyanoprokaryotic in the oyster

To determine the accumulation of the cyanoprokaryotic *Synechococus elongatus* on the tissues of the oysters *C. gigas*, three general patterns of diets (i.e., cyanoprokaryotes, cyanoprokaryotes with microalgae, and only microalgae) was evaluated per triplicate in cylindrical-tanks. Nine oyster groups ($n = 20$ oysters per group, three groups per each diet pattern) were maintained by 30 days in 19-L white cylindrical-tanks (0.29 m diameter × 0.35 m height) arranged in two levels ($n = 10$ oysters per level) separated each by 0.10 m from the bottom (Fig. 1). All oyster groups had similar morphometric measurements ($P > 0.05$) at the start of the experiment ($TL = 100 \pm 3.5$ SD mm; $TWI = 56 \pm 2.5$ SD mm; $TH = 29 \pm 1.1$ SD mm; $TW = 86.7 \pm 4$ SD g; $n = 20$ oysters per group). During the experiment, sea water in the cylindrical-tanks was maintained at $23 \pm 1\,^{\circ}C$, practical salinity at 31 ± 2; dissolved oxygen higher than $6\ mg\ L^{-1}$; and pH above 8. Whole water in each tank was renewed each third day with water that was filtered biologically and aged for 72 h in white tanks.

2.4. Diets preparation and oysters feeding

Diets were elaborated with daily food rations (DFR) at 4% and 6%, following the percentages recommended by Helm et al. (2006). Three diets with DFR at 4% during the first 15 days were provided, becoming three diets with DFR at 6% on day 16 and onward. General pattern of diets was cyanoprokaryotes, cyanoprokaryotes with microalgae, and only microalgae (as control). Diets with DFR at 4% was: (A) *S. elongatus* at 4%, (B) mix of *S. elongatus* at 2%, *T. suecica* at 1%, and *I. galbana* at 1%, and (C) *T. suecica* at 2% and *I. galbana* at 2% (as control). Diets with DFR at 6% was: (D) *S. elongatus* at 6%, (E) mix of *S. elongatus* at 4% and *T. suecica* at 2%, and (F) *T. suecica* at 6% (as control). Diets changes were: A became D; B became E; and C became F (Fig. 1). Oysters were fed twice a day (900 h and 1800 h) during 30 experimental days.

To estimate the daily food rations (DFR) per oysters group, was used a no-experimental group of haphazardly selected oysters ($n = 20$ oysters) similar to oysters used in this experimental study,