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Research Paper

Evidence for the presence of cell-surface-bound and intracellular bactericidal toxins in the dinoflagellate *Heterocapsa circularisquama*

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ABSTRACT

Heterocapsa circularisquama, a harmful dinoflagellate, has multiple haemolytic toxins that are considered to be involved in the toxic mechanism against shellfish and certain species of zooplankton. To evaluate the further nature of the toxins of *H. circularisquama*, we investigated its effects on several species of bacteria. By colony formation assay, we found that *H. circularisquama* had antibacterial activity toward the marine bacterium *Vibrio alginolyticus* in a cell density-dependent manner. When the inoculated bacterial cells were co-cultured with *H. circularisquama* under dinoflagellate cell culture conditions, the bacterial growth was significantly suppressed, whereas the number of live bacterial cells increased when cultured in the medium alone. Since the cell-free culture supernatant and the ruptured dinoflagellate cell suspension showed no toxic effects on *V. alginolyticus*, it is speculated that direct cell-to-cell contact mediated by the live dinoflagellate cells may be the major toxic mechanism. The decrease in bactericidal activity of theca-removed dinoflagellate cells may further support this speculation. *H. circularisquama* also showed bactericidal activities towards *Escherichia coli* and *Staphylococcus aureus*. In the dinoflagellate/bacteria co-culture system, the number of live bacterial cells declined with increasing incubation time. Light-dependent antibacterial activity of the ruptured dinoflagellate cells against *S. aureus* was observed, whereas no such activity was detected against *E. coli*. These results suggest that intracellular photosensitising bactericidal toxins, which were previously found to be porphyrin derivatives, may have specificity towards gram-positive bacteria. Based on these results together with previous studies, it is obvious that *H. circularisquama* possesses antibacterial activity, which may be mediated through toxins located on its cell surface. It is likely that such toxins play a role in the defence mechanism against predators and infectious bacteria. Although the exact biological significance of intracellular photosensitising toxins is still unclear, such toxins may have potential to be developed as novel photo-controllable antibiotics.

1. Introduction

Heterocapsa circularisquama, a dinoflagellate that causes harmful algal blooms (HABs), has resulted in the mass mortality of bivalves in the coastal area of western Japan since 1988 (Horiguchi, 1995; Matsuyama et al., 1996). This dinoflagellate can be identified on the basis of its characteristic morphological features and swimming patterns. The incidences of *H. circularisquama*-caused HABs have been increasing since early 1990. *H. circularisquama* shows potent lethal effects on various bivalves, such as pearl oyster (*Pinctada fucata*), short-necked clam (*Ruditapes philippinarum*), and oyster (*Crassostrea gigas*), whereas harmful effects on wild and cultured finfish and other marine vertebrates as well as public health hazards in general have not been reported so far (Matsuyama et al., 1992; Yamamoto and Tanaka, 1990). The toxic effect of *H. circularisquama* on pearl oysters was confirmed under laboratory conditions, where exposure to a high cell density ($> 10^3$ cells mL^{-1}) of the dinoflagellate caused the oysters to immediately contract their mantles and close their valves, which led to paralysis and eventually death (Nagai et al., 1996). These abnormal symptoms of bivalves have also been pointed out in field observations

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(Matsuyama et al., 1996). Recent laboratory studies have shown that *H. circularisquama* impaired early stage of development of bivalves (Basti et al., 2013).

H. circularisquama was shown to have a cell density-dependent lethal effect on the tintinnid ciliate *Favella taraikaensis* (a microzooplankton) (Kamiyama, 1997; Kamiyama and Arima, 1997). Similar to their findings, we found that *H. circularisquama* also had a cell density-dependent lethal effect on another microzooplankton, the rotifer *Brachionus plicatilis* (Kim et al., 2000). Furthermore, it has been reported that *H. circularisquama* showed cell contact-dependent lethal effects on other phytoplankton species (Yamasaki et al., 2011). In the recent extensive pathological studies of the Mediterranean mussel, *Mytilus galloprovincialis* exposed to *H. circularisquama*, Basti et al. reported that the most affected organ was the gill, followed by the labial palps and mantle, the stomach and intestines, and the hepatopancreas. Based on the results, they concluded that the cell-contact dependent toxicity of *H. circularisquama* does not seem to be the only mechanism of toxicity (Basti et al., 2015). Hence, it seems that the toxic mechanisms of *H. circularisquama* on bivalves and certain zooplankton species are still controversial and not yet fully understood. It has been speculated that unstable toxic substances may be the responsible agents (Matsuyama et al., 1997). However, because of the extremely unstable nature of the supposed toxins of *H. circularisquama*, the isolation and characterization of such toxins from the organism has not yet been successful. We have previously found that the *H. circularisquama* cell suspension causes potent haemolysis of rabbit erythrocytes. The cell-free culture supernatant prepared from the live cell suspension of *H. circularisquama* also showed the haemolytic activity but at a much lower level, suggesting that part of the haemolytic toxin might be discharged from the cells into the culture medium (Oda et al., 2001; Sato et al., 2002). Furthermore, a comparative study of the haemolytic activities of several strains of *H. circularisquama* isolated from different localities in Japan revealed that the activity was well correlated with their toxicity towards shellfish (Kim et al., 2002).

Recently, Matsuyama (2012) has proposed that live *H. circularisquama* cells must come into direct contact with bivalves in order for their lethal effects to be executed, and that certain toxins located on the cell surface might play an important role in this regard. Based on these previous findings, it seems likely that the haemolytic substance on the cell surface of *H. circularisquama* is a toxin responsible for the shellfish-killing activity.

In addition to the cell-surface haemolytic activity of *H. circularisquama*, our previous studies suggested the presence of an intracellular haemolytic agent that can be extracted into ethanol. Interestingly, the haemolytic activity of such agent was absolutely light-dependent (Oda et al., 2001; Sato et al., 2002). Purification and characterisation studies suggested that one of these agents (named H2-a) has structural similarity to porphyrin derivatives, which are well-known photosensitising haemolytic agents (Miyazaki et al., 2005). Further studies on H2-a showed that this porphyrin derivative induced necrotic cell death on human tumor cells (Kim et al., 2008).

With regard to the relationship between phytoplankton and bacteria, it has been reported that the growth of *Vibrio alginolyticus* was promoted in a mixed culture with the diatom *Chaetoceros muelleri* (Gomez-Gil et al., 2002). The authors explained that the growth-promoting effect of *C. muelleri* on *V. alginolyticus* might be derived from organic nutrients produced by the diatom (Gomez-Gil et al., 2002). On the other hand, Kogure et al. (1979) reported that the marine diatom *Skeletonema costatum* significantly inhibited the growth of *Vibrio* sp., and Naviner et al. (1999) also reported that organic solvent-extractable compounds from *S. costatum* inhibited *Vibrio* sp. growth. Furthermore, we have found that the raphidophycean flagellate *Chattonella marina*, which is a well-known fish-killing HAB species, shows bactericidal activity against the marine bacterium *V. alginolyticus* (Oda et al., 1992). As an opposite relationship between bacterium and phytoplankton, it has been reported that *Cytophaga* sp. exhibited algicidal activity against *H. circularisquama* (Nagasaki et al., 2000). These findings prompted us to examine whether or not *H. circularisquama* can be toxic to bacteria. In this study, therefore, we investigated the effects of *H. circularisquama* on *V. alginolyticus*, *Staphylococcus aureus*, and *Escherichia coli* in dinoflagellate/bacteria co-culture systems under various experimental conditions.

2. Materials and methods

2.1. Plankton culture

H. circularisquama was kindly provided by Dr. Y. Matsuyama (Seikai National Fisheries Institute, Japan) in 2000. Since then the strain has been maintained in our laboratory under the conditions described below. This strain was originally isolated by Dr. Matsuyama from Ago Bay, Japan in 1994 (Matsuyama, 1999). The plankton culture was maintained at 26 °C in 100 mL flasks containing 60 mL of a modified seawater medium (SWM-3) at a salinity of 25 (Yamasaki et al., 2007), under a 12:12 h photoperiod which was maintained by using a cool-white fluorescent lamp (200 ± 5 μmol m^{-2} s^{-1}). The modified SWM-3 contained a Tris-HCl buffer system and was autoclaved for 15 min at 121 °C before use. The plankton cell numbers of the cultures were counted microscopically using a haemocytometer (Erma Inc., Tokyo, Japan). The plankton culture used throughout the experiment was in its late exponential growth phase unless otherwise specified, and was handled with sterilised instruments. A cell-free culture supernatant of *H. circularisquama* was prepared from a cell suspension in the late exponential growth phase (1–3 × 10^5 cells mL^{-1}) by centrifugation at 5000 × *g* for 10 min at 4 °C. A ruptured cell suspension was prepared by ultrasonic treatment of the cell suspension (1–3 × 10^5 cells mL^{-1}) in a bath-type ultrasonic apparatus for 5 min at 20 °C. A theca-removed cell suspension was prepared by repetitive ultrasonic treatment of the cell suspension for 20 s and hand-shaking agitation in a 1.5 mL centrifuge tube. Subsequently, microscopic observation confirmed that the cells were ruptured or the theca had been removed depending on the duration of ultrasonic treatment.

2.2. Bacterial cultivation

V. alginolyticus (NBRC15630), *E. coli* (NBRC13898), and *S. aureus* (NBRC12732) were obtained from NITE Biological Resource Center (Tsukuba, Japan). Zorbell marine agar medium (for *V. alginolyticus*) or nutrient agar medium (for *S. aureus* and *E. coli*) were used to maintain the strains. *V. alginolyticus* was sub-cultured overnight at 34 °C in plankton medium containing 2% glucose before use. *S. aureus* and *E. coli* were cultured overnight at 34 °C in nutrient broth, and then the cells were harvested and washed with phosphate-buffered saline (PBS) by centrifugation at 15,000 × *g* for 10 min at 4 °C. The final cell pellets were diluted to appropriate concentrations with plankton culture medium and immediately used for the experiments.

2.3. Measurement of antibacterial activity

Experiments for the antibacterial effects of the cell-free culture supernatant, theca-removed cell suspension, and ruptured cell suspension of *H. circularisquama* were conducted immediately after their preparation, as described in Section 2.1. Each bacterial cell suspension in plankton culture medium was inoculated into the respective *H. circularisquama* test suspensions in 0.5–1.5 mL of total assay mixture. After incubation for the indicated periods of time under the plankton culture conditions at 26 °C in the light (200 ± 5 μmol m^{-2} s^{-1}) or in the dark, 10–50 μL aliquots of the reaction mixtures were withdrawn for the enumeration of viable bacteria. Each reaction mixture was suitably diluted with the plankton culture medium, and was inoculated in triplicates into Muellar-Hinton agar medium (for *V. alginolyticus*) or nutrient agar medium (for *S. aureus* and *E. coli*). After 24 h incubation at