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A marine bacterium producing protein with algicidal activity against *Alexandrium tamarense*

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ABSTRACT

Interactions between bacteria and harmful algal bloom (HAB) species have been acknowledged as an important factor of regulating the population of these algae. In the study, two strains of algicidal bacteria, DHQ25 and DHY3, were screened out because of their probably secreting algicidal proteins against axenic *Alexandrium tamarense*. Molecular characterization classified them to the γ-proteobacteria subclass and to the genus *Vibrio* and *Pseudoalteromonas*, respectively. After centrifugation and ultrafiltration, chromatography of the cultural supernatants of DHQ25 revealed 8 peaks by HPLC. SDS-PAGE and Native PAGE determination showed that peak 7 to be a monoband peak. Both xenic and axenic culture of *A. tamarense* were susceptible to the purified protein (short for P7 below) indicated by algicidal activity assay. Observation of algicidal process demonstrated that algal cells were lysed and cellular substances were released under visual fields of microscope. P7 proved to be a challenge controller of *A. tamarense* by the above characterizations of algicidal activity assaying and algicidal process. This is the first report of a protein algicidal to the toxic dinoflagellate *A. tamarense*. The findings increase our knowledge of bacterial–algal interactions and the role of bacteria during controlling HABs.

1. Introduction

Harmful algal blooms (HABs) have been accompanied by huge economic losses through massive fish death and a threat to human from shellfish poisoning in marine and freshwater ecosystems. Thus, the management of HABs has been explored by means of predicting and mitigating their blooming in recent years (Doucette et al., 1999; Yoshinaga et al., 1998; Mayali and Azam, 2002). Up to now, different methods including chemical, physical management have been tried to control HABs in the aquatic ecosystem, but most of them are inapplicable because of the high cost and the secondary pollution they cause (Anderson, 1997). Recently, some studies have indicated biological regulation, e.g. algicidal bacteria, is a valid way to manage HABs both in sense of decreasing and removing HABs (Imai et al., 1998; Manage et al., 2001; Zheng et al., 2005; Su et al., 2007b).

In general, there are direct and indirect interactions between algicidal bacteria and algae. Direct attack means algicidal bacteria get in touch with algal cells and kill them directly, while indirect interaction is thought to be a chemically mediated aggression by algicides from bacterial secreted materials (see reviews by Azam, 1998; Salomon and Imai, 2006). What's more, what algicides should be become a hot spot due to their probable indication of algicidal mechanism. Now several different compounds have been characterized as algicides against some specific algaes in related researches. These compounds include a protease algicidal to *Skeletonema costatum* (Lee et al., 2002), peptides algicidal to *Oculina patagonica* (Imamura et al., 2000), and an antibiotic algicidal to *Heterosigma akashiwo* (Ahn et al., 2003; Wang et al., 2005). Many bacteria were isolated out but few algicides were purified out. Great difficulties exist in isolation and purification of algicidal compounds because of their apparent variation in characteristics across different species of algicidal bacteria (Skerratt et al., 2002).

Alexandrium tamarense is a notorious HABs species which is associated with the largest number of paralytic shellfish poisoning cases (Anderson et al., 1996). There are investigations mainly focusing on the bacteria associated with the growth of *A. tamarense* and their effects on the PSP production of *A. tamarense*. However, few compounds algicidal to *A. tamarense* have been reported. In this study, a protein antagonistic to *A. tamarense* was purified out from a bacterial DHQ25 isolated from the red-tide areas in East

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China See. Identification of algicidal bacteria and algicidal activity assay of the protein were undertaken in this study.

2. Materials and methods

2.1. Xenic and axenic A. tamarense

Species of harmful algae, piped from natural environment and maintained in the laboratory, harbor an attached bacterial flora in its culture (Kopp et al., 1997; Hold et al., 2001). The xenic (natural) *A. tamarense* (kindly supplied by Professor Yuzao Qi, Jinan University, China) were cultivated in f/2 medium prepared with natural seawater (Guillard, 1975) at 20 °C ± 1 °C under an illumination of 50 $\mu E\ m^{-2}\ s^{-1}$ in 12:12 (light:dark). In an earlier study, we had successfully inoculated an axenic culture of *A. tamarense* for quick screening of algicidal bacteria and a simple system to go further study of algal–bacterial interaction (Su et al., 2007a).

2.2. Algicidal bacteria

14 Strains of bacteria with algicidal activity against axenic *A. tamarense* through indirect attack, previously isolated from the red-tide areas in East China Sea (Su et al., 2007b), were cultured in 2216E medium (peptone 5 g, yeast extraction 1 g, ferric phosphorous acid 0.1 g, agar 10 g, pH 7.6–7.8, fixed capacity to 1 L using xenic seawater) at 25 °C with shaking at 120 rpm.

2.3. Screening bacteria for their probably producing algicidal proteins against axenic A. tamarense

Cultures (in stationary phase) of 14 algicidal isolates were centrifuged for 5 min at 8000 rpm to collect the cultural supernatants. A portion of the cultural supernatants was ultrafiltrated at 5000 × *g* for 50 min by centrifugal filter devices (Ultracel-3 kD) to cultivate the concentrated supernatants. All operation was undertaken at 4 °C. The remainder of the cultural supernatants was incubated in enclosed Eppendorf tubes in a water bath at 120 °C for 20 min.

50 mL axenic cultures of *A. tamarense* (in stationary phase) was added with an aliquot (0.5 mL) of the supernatants (with ultrafiltration treatment above) or the supernatants (with heat treatment above) to observe the growth of algal cells by microscopy. 50 mL axenic algal cultures added with 0.5 mL autoclaved 2216E broth acted as a control. The supernatants (with heat treatment) or the supernatants (with ultrafiltration treatment) were considered to be algicidal to axenic *A. tamarense* when *A. tamarense* cells were lysed.

2.4. Identification of bacteria

Bacterial cells from cultures of the above screened bacteria were collected by centrifugation (5000 × *g* for 10 min) and subjected to sequential digestion of lysozyme (2.5 $mg\ mL^{-1}$, 37 °C for 1 h) and proteinase K (200 $mg\ mL^{-1}$ in 1% SDS, 55 °C for 1 h), followed by incubation in 1% CTAB and 0.7 M NaCl at 65 °C for 15 min. After extraction with phenol/chloroform, DNA was recovered by ethanol precipitation and was dissolved in ddH_2O. The 16S rRNA gene was amplified by PCR in a 50 mL reaction system using primers 27F and 1492R (DeLong, 1992) under the following conditions: 1 U Ex Taq buffer, 0.2 mM of each dNTP, 0.2 mM of each primer and 1 U Ex Taq polymerase (TAKARA). An initial denaturation period of 5 min was followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and the final extension (72 °C) time was 10 min. PCR products were checked using 1% agarose gel electrophoresis. The amplified 16S rDNA was purified from agarose gel with a GeneClean Turbo Kit (Qbiogene) and ligated with pMD18-T vector (TAKARA). The ligation products were transformed into *Escherichia coli* DH5a competent cells and the clone with a 1.5 kb insert was sent to be sequenced (Invitrogen Biotechnology Co. Ltd.). The sequence was aligned and a phylogenetic tree was constructed with known algicidal bacterial sequences obtained from GenBank.

2.5. Isolation and purification of algicidal protein

All the experiments during various stages of protein purification were carried out at 4 °C, unless otherwise indicated. Culture of DHQ25 (screened out above for probably producing algicidal protein) was centrifugated to collect the cultural supernatants. Then, the cultural supernatants were ultrafiltrated by centrifugal filter devices (Ultracel-3 kD) to gather the concentrated supernatants. Subsequently, the concentrated supernatants were chromatographed on a C18 capillary column (Varian Polaris), which had been equilibrated with 20 $mmol\ L^{-1}$ Tris–HCl (pH 8.0) buffer. Chromatography was carried out on a HPLC chromatograph (VarianProstar 210) under a flow rate of 0.2 $mL\ min^{-1}$ at room temperature. The 7th fraction, showing out obvious algicidal activity, represented the algicidal protein. Then it was pooled and freeze-dried to be powder for storing and the following characterization.

2.6. SDS-PAGE gel electrophoresis and Native PAGE

Laemmli and Favre (1973) method was used. The protein samples were denatured using Laemmli's buffer and electrophoresed on 15 – 4% SDS-polyacrylamide slab gel system under reducing and non-reducing conditions.

For Native PAGE the purified fraction was treated with Laemmli's sample buffer lacking SDS and β-mercaptoethanol. Purified protein was loaded without heat treatment and electrophoresed on 10 – 2.5% polyacrylamide slab gel system at 4 °C. Protein bands were visualized by 0.1% Coomassie Brilliant Blue staining.

2.7. Protein content measurement

The measurement was conducted according to the method of Lowry et al. (1951). Bovine serum albumin was used as the standard.

2.8. Characterization of algicidal activity of purified protein and its algicidal process

Assay of algicidal activity against axenic and xenic culture of *A. tamarense* was done to determinate whether the purified protein to be a challenge algicide. Aliquots (4 mL) of axenic or xenic culture of *A. tamarense* (in stationary phase) were transferred to a 12-well plate for 2 days and then each well in the plate was added with 40 μL purified protein (dissolved in 20 $mmol\ L^{-1}$ Tris–HCl (pH 8.0)). The final concentrations of purified protein in wells were controlled to be 2 μM, 5 μM, 8 μM and 12 μM, respectively. Each concentration was repeated in triplicate.

The algicidal rate was calculated according to the following formula:

$$\text{Algicidal rate}\ (\%) = \frac{N_c - N_E}{N_c} \times 100$$

N_c represents the number of cells in the control group, and N_E represents the number of cells in the experimental group.