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Novel algicidal evidence of a bacterium *Bacillus* sp. LP-10 killing *Phaeocystis globosa*, a harmful algal bloom causing species

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HIGHLIGHTS

- An algicidal bacterium strain LP-10 was isolated and identified as *Bacillus*.
- Strain LP-10 could lyse more than 5 HAB causing species.
- The mode of action of LP-10 on *P. globosa* was determined as indirect attack.
- The active compound was heat-stable, low molecular weight and mid-polarity.

GRAPHICAL ABSTRACT

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ABSTRACT

While searching for effective bio-agents to control harmful algal blooms (HABs), the bacterial strain LP-10, which has strong algicidal activity against *Phaeocystis globosa* (Prymnesiophyceae), was isolated from surface seawater samples taken from the East China Sea. 16S rDNA sequence analysis and morphological characteristics revealed the strain LP-10 belonged to the genus *Bacillus*. The lytic effect of *Bacillus* sp. LP-10 against *P. globosa* was both concentration- and time-dependent. Algicidal activities of different growth stages of the bacterial culture varied significantly. The lytic effect of different parts of the bacterial cultures indicated that the algal cells were lysed by algicidal active compounds in the cell-free filtrate. Analysis of the properties of the active compounds showed that they had a molecular weight of less than 1000 Da and that the active compounds were stable between −80 and 121 °C. The algicidal range assay indicated that five other algal species were also suppressed by strain LP-10, including: *Alexandrium catenella*, *A. tamarense*, *A. minutum*, *Prorocentrum micans* and *Asterionella japonica*. Our results suggested that the algicidal bacterium *Bacillus* sp. LP-10 could be a potential bio-agent to control the blooms of harmful algal species.

1. Introduction

Harmful algal blooms (HABs), often referred to as red tides, have serious negative consequences on ecosystems, aquaculture, recreational activities and human health (Hallegraeff, 1993; Landsberg, 2002; Sellner et al., 2003). Shading effects and depletion of dissolved oxygen, as well as production of toxins by HABs, obstruct other phytoplankton in seawater (Van Dolah, 2000; Landsberg, 2002; Matthijs et al., 2012). Frequent blooms of harmful algal species have resulted in significant economic losses worldwide (Hoagland et al., 2002).

Many chemical and physical methods have been proposed to mitigate or control HABs (Anderson, 1997; Kim, 2006) and chemical reagents have strong inhibitory effects on the growth of

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harmful algae. However, chemical methods to control HABs *in situ* have received much criticism because of the disadvantages of high costs, frequent reappearance and secondary pollution. Physical methods, such as clay flocculation (Sengco and Anderson, 2004), ultrasonic irradiation (Lee et al., 2001) and mechanical salvage, also create problems, with difficulties in large scale applications and detrimental effects on other phytoplankton while not eradicating the problem (Shi et al., 2013).

Biological methods have gradually gained attention by virtue of their advantage of being environmentally friendly. Plants (Yan et al., 2012), protozoa (Tillmann, 2004), microalgae (Yamasaki et al., 2010) and microorganism (Luo et al., 2013; Mayali and Azam, 2004; Zheng et al., 2013), have been reported to exert inhibitory effects on the growth of HABs. Among these, microorganisms (Zhang et al., 2013) were considered to provide better HAB control, especially the algicidal bacteria (Jia et al., 2010; Nakano et al., 2003; Su et al., 2011; Wang et al., 2010). A great deal of research has focused on the interactions between algicidal bacteria and algae, suggesting that algae-lysing bacteria play an important role in regulating the growth of algal cells and have the potential to mitigate or eradicate the frequent outbreaks of HABs (Cole, 1982; Grossart and Simon, 2007).

Many bacteria with strong algicidal activity against HAB species have been isolated and investigated (Kodani et al., 2002; Ma et al., 2011; Su et al., 2007). Lovejoy et al. (1998) obtained a yellow-pigmented *Pseudoalteromonas* strain Y which can lyse harmful algal species in the genera *Chattonella*, *Gymnodinium* and *Heterosigma*. However, research on the harmful alga *Phaeocystis globosa* Scherffel (Prymnesiophyceae), a widely distributed bloom-forming microalga (Chen et al., 1999), is still not well documented. In our study, an algicidal bacterial strain LP-10 with high efficacy was isolated from the East China Sea. The characteristics of the lytic effect, the algae-lysing range and the mode of action of this strain were investigated to determine the potential of strain LP-10 as a bio-agent for mitigating or controlling HABs.

2. Materials and methods

2.1. Algal cultures

Cultures of *P. globosa* PG03 and other algal species used in this study were supplied by the State Key Laboratory of Marine Environmental Science, Xiamen University, China. *Alexandrium tamarense* ATGD98-006 was obtained from the Algal Culture Collection, Institute of Hydrobiology, Jinan University (Guangzhou, China). f/2 medium (without silica) was used to prepare algal cultures and the light conditions were set to a 12 h/12 h light–dark cycle under cool-white fluorescent light with an intensity of 50 μmol photons m^{-2} s^{-1} at 20 ± 1 °C. The f/2-Si medium contained 75 mg $NaNO_3$, 5 mg $NaH_2PO_4{\cdot}H_2O$, 4.36 mg Na_2EDTA, 3.15 mg $FeCl_3{\cdot}6H_2O$, 0.01 mg $CuSO_4{\cdot}5H_2O$, 0.022 mg $ZnSO_4{\cdot}7H_2O$, 0.01 mg $CoCl_2{\cdot}6H_2O$, 0.18 mg $MnCl_2{\cdot}4H_2O$, 0.006 mg $Na_2MoO_4{\cdot}2H_2O$, 0.1 mg thiamine·HCl, 0.5 μg biotin, and 0.5 μg vitamin B_{12} per liter of filtered seawater.

2.2. Isolation and screening of algae-lysing bacteria

Water samples collected from the surface seawater in the East China Sea (120°29′24″E-126°E, 25°52′30″N −30°N) were stored at −20 °C until they were used. The samples were serially diluted with sterilized seawater and 100 μL aliquots of each dilution were spread on Zobell 22l6E agar plates, followed by incubation for 3 days at 28 °C. Individual colonies were randomly picked out to streak on fresh 22l6E agar plates. Repeated streaking was performed until the obtained colonies were confirmed to be pure cultures. The purified cultures were frozen in 30% (v/v) glycerol at −70 °C for further analysis.

To screen the algae-lysing bacteria, all the strains obtained were inoculated into 250 mL flasks with 100 mL 2216E media, followed by incubation for 3 days at 28 °C. 0.5 mL aliquots of each bacterial culture were inoculated into 20 mL axenic logarithmic-phase cultures of *P. globosa*, and 0.5 mL blank 2216E broth was added as the control. The growth of the algal cultures was monitored and those strains exhibiting obvious algicidal activity (>75%) were analyzed further. The algicidal activity was calculated as:

$$\text{Algicidal activity}(\%) = (1 - \text{Nc}/\text{Nt}) \times 100\%$$

where Nc and Nt are the chlorophyll *a* content of *P. globosa* treated with bacterial cultures of the tested strains, and the blank 2216E broth control.

Chlorophyll *a* content was measured using the methods described by Holm-Hansen and Riemann (1978) and Marr et al. (1995) with slight modifications. 20 mL axenic logarithmic-phase cultures of *P. globosa* were harvested and the pellets were re-suspended and mixed with 5 mL 90% ethanol overnight to extract the chlorophyll *a*. After complete extraction, the residuals were removed by centrifugation, and the optical density of the ethanol extract was measured at wavelengths 645 nm and 664 nm using a VARY-50 spectrophotometer. The chlorophyll *a* concentration was calculated using the formula:

$$\text{Chlorophyll } a \ (\text{mg L}^{-1}) = 12.7A_{664} - 2.69A_{645}$$

where A_{645} and A_{664} were the optical densities of the ethanol extract at wavelengths of 645 nm and 664 nm.

2.3. Identification of algae-lysing bacterial strain LP-10

Morphological observations were carried out both with the naked eye and using a transmission electron microscope (JEM2100, Japan). Conventional biochemical tests were made using methods described by Kim et al. (2009), and the extraction of genomic DNA from strain LP-10 cultures was performed as described by Su et al. (2007), followed by PCR amplification of 16S rRNA gene with genomic DNA as a template. The two primers used for PCR amplification were 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The thermal profiles involved denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and another 10 min of extension time as the last step. The amplification products were subsequently purified using a GeneClean Turbo Kit (Qbiogene) after loading in the agarose gel. Purified products were then ligated with the pMD-19T vector and the ligated products were transformed into *Escherichia coli* DH5α competent cells. Positive clones which grew on the plates were picked out and sent to be sequenced by the Shanghai Invitrogen Biotechnology Co., Ltd. Comparison of the obtained sequences with deposited nucleotide sequences was carried out using the Basic Local Alignment Search Tool (BLAST) database (http://www.ncbi.nlm.nih.gov/BLAST) at the National Center for Biotechnology Information (NCBI). A neighbor-joining phylogenetic tree was constructed using MEGA 5.0.

2.4. Characterization of algicidal effect of bacterial strain LP-10 against *P. globosa*

2.4.1. The effect of bacterial cultures concentration on algicidal activity

Bacterial cultures were inoculated into 20 mL algal cultures at a volume fraction of 1, 3, 5, 7 and 10%, and Zobell 2216E broth was inoculated into algal cultures as the control. All operations were performed in triplicate. The cultures were incubated for 72 h under