Science of the Total Environment 482–483 (2014) 116–124

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Novel insights into the algicidal bacterium DH77-1 killing the toxic dinoflagellate *Alexandrium tamarense*

Xiaoru Yang [a,b,*,1], Xinyi Li [b,c,1], Yanyan Zhou [b], Wei Zheng [b], Changping Yu [a], Tianling Zheng [b,**]

[a] Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China
[b] State Key Laboratory of Marine Environmental Science and Key Laboratory of Ministry of Education for Coast and Wetland Ecosystems, School of Life Sciences, Xiamen University, Xiamen 361005, China
[c] Tangdu Hospital, The Fourth Military Medical University, Xi'an 710032, China

HIGHLIGHTS

- DH77-1 is the first record of a *Joostella* being algicidal to *Alexandrium tamarense*.
- DH77-1 killed algae by indirect attack and the active substances were stable.
- Algal cells responded severely after exposure to the bacterial filtrate of DH77-1.
- The molecular weight of the algicidal substance was 125.88

ARTICLE INFO

Article history:
Received 21 October 2013
Received in revised form 20 February 2014
Accepted 26 February 2014
Available online 15 March 2014

Keywords:
Toxic *Alexandrium tamarense*
Algicidal bacteria
Algicidal substance
Algicidal mechanism
HABs control

ABSTRACT

Algicidal bacteria may play a major role in controlling harmful algal blooms (HABs) dynamics. Bacterium DH77-1 was isolated with high algicidal activity against the toxic dinoflagellate *Alexandrium tamarens*e and identified as *Joostella* sp. DH77-1. The results showed that DH77-1 exhibited algicidal activity through indirect attack, which excreted active substance into the filtrate. It had a relatively wide host range and the active substance of DH77-1 was relatively stable since temperature, pH and storage condition had no obvious effect on the algicidal activity. The algicidal compound from bacterium DH77-1 was isolated based on activity-guided bioassay and the molecular weight was determined to be 125.88 by MALDI-TOF mass spectrometer, however further identification via nuclear magnetic resonance (NMR) spectra is ongoing. The physiological responses of algal cells after exposure to the DH77-1 algicidal substances were as follows: the antioxidant system of *A. tamarense* responded positively in self-defense; total protein content decreased significantly as did the photosynthetic pigment content; superoxide dismutase, peroxidase enzyme and malondialdehyde content increased extraordinarily and algal cell nucleic acid leaked seriously ultimately inducing cell death. Furthermore, DH77-1 is the first record of a *Joostella* sp. bacterium being algicidal to the harmful dinoflagellate *A. tamarense*, and the bacterial culture and the active compounds might be potentially used as a bio-agent for controlling harmful algal blooms.

1. Introduction

Harmful algal blooms (HABs) are serious global marine disasters, causing a series of ecological, resource and environmental problems as well as significant economic losses (Yang et al., 2012; Zheng et al., 2013). To manage and mitigate the adverse impact of HABs, various management strategies have been developed. Physical techniques including the use of yellow loess (Choi et al., 1998) and chemical agents such as copper sulfate (Anderson, 1997) are effective in controlling blooms within a short period after application. However, their usage in aquatic ecosystems is considered to be potentially dangerous due to its potential secondary effects on bottom-dwelling organisms (Rhoads and Young, 1970; Bricelj and Malouf, 1984). Chemical agents can cause serious secondary pollution, and they can indiscriminately kill multiple organisms in the aquatic ecosystem, which may alter marine food webs and eventually cause a severe impact on natural fish communities (Jeong et al., 2000, 2008). Biological agents, including bacteria (Mayali and Azam, 2004; Su et al., 2007a, 2007b; Wang et al., 2010; Wang et al., 2010; Wang et al., 2012), actinomycete (Bai et al., 2011; Zheng et al., 2013; Zhang et al., 2013), viruses (Nagasaki et al., 2004; Cai et al., 2011), protozoa (Jeong et al., 2008), macrophytes (Nakai

* Correspondence to: X. Yang, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China. Tel.: +86 592 6190560; fax: +86 592 6190514.
** Correspondence to: T. Zheng, RoomC-301, School of Life Sciences, Xiamen University, Xiamen 361005, China. Tel.: +86 592 2183217; fax: +86 592 2184528.
E-mail addresses: xryang@iue.ac.cn (X. Yang), wshwzh@xmu.edu.cn (T. Zheng).
[1] X. Yang and X. Li contributed equally to this work.

http://dx.doi.org/10.1016/j.scitotenv.2014.02.125
0048-9697/

et al., 1999; Jin and Dong, 2003; Zhou et al., 2010) and microalgae (Granéli et al., 2008) are considered as potential suppressors in controlling the outbreak and control of HABs.

Algal-bacterial interactions are increasingly cited as potential regulators in the sense of both decreasing and developing algal blooms (Doucette et al., 1998). Research into the relationships between algae and bacteria has resulted in the isolation of several algicidal bacteria which belong mainly to the *Cytophaga/Flavobacterium/Bacteroidetes* (*CFB*) group and the genera *Cytophaga, Saprospira*, *Alteromonas* and *Pseudoalteromonas*, *Vibrio*, *Shewanella*, *Bacillus*, *Planomicrobium* and *Micrococcus*. These bacteria show algicidal activity through either direct or indirect attack on the target algal cells (Mayali and Azam, 2004). Most reports on algicidal bacteria have dealt with a description of the alga-lysing phenomenon, the isolation and identification of the algicidal bacteria. Only a few algicidal compounds from algicidal bacteria have been purified and identified. These algicides would be ecto-proteases (Lee et al., 2002), peptides (Imamura et al., 2000), protein (Wang et al., 2012), biosurfactants (Ahn et al., 2003; Wang et al., 2005) or antibiotic-like substances (Nakashima et al., 2006).

In this study, a strain of bacterium (DH77-1) was isolated with high algicidal activity against the toxic dinoflagellate *Alexandrium tamarense*. Algicidal mode, algicidal specificity and the basic characteristics of the bacterial supernatant of the strain against *A. tamarense* were studied. A study of the physiological responses of algal cells after exposure to the DH77-1 metabolites was also implemented in order to better understand its algicidal mechanism. In addition, the algicidal compound from DH77-1 was isolated based on activity-guided bioassay and its molecular weight was determined.

2. Materials and methods

2.1. A. tamarense culture

A non-axenic culture of *A. tamarense* ATGD98-006 was provided by the Algal Culture Collection (Institute of Hydrobiology, Jinan University, Guangzhou, China). Axenic *A. tamarense* cultures were obtained in order to investigate the interactions between this alga and bacteria (Su et al., 2007a, 2007b). The algal cultures were maintained in f/2 medium (Guillard, 1975) prepared with natural seawater (28 practical salinity units, psu) at 20 °C ± 1 °C under a 12 h light/12 h dark cycle with a light intensity of 50 μmol photons m^{-2} s^{-1}.

2.2. Algicidal activity against A. tamarense

Strain DH77-1 was isolated from surface water samples of a *Prorocentrum donghaiense* Lu bloom (~10^8 cells L^{-1}), accompanied with *A. tamarense* (~10^4 cells L^{-1}), in the East China Sea during the National Science Foundation of China 973 project MC2003-2 cruise on 19–21 May 2003. DH77-1 was inoculated in 4 mL of Zobell 2216 broth (5 g peptone, 1 g yeast extraction, 0.1 g ferric phosphorous acid, pH 7.6–7.8, in 1 L natural seawater) at 28 °C and 150 rpm for 7 days. The algicidal activity assay was carried out in the 24-well plates. A 40 μL aliquot of the bacterial supernatant, which was collected by centrifugation at 5000 ×*g* for 20 min, was inoculated in triplicate into 2 mL of logarithmic-phase (~1×10^4 cells mL^{-1}) algal cultures, and a 40 μL aliquot of 2216 liquid medium only was added to the algal cultures as a control. The growth of *A. tamarense* was monitored using fluorescein diacetate (FDA, Sigma) vital stain. FDA stain was performed according to (Widholm, 1972). FDA working stock (5 mg mL^{-1} in 100% acetone, preserved at 4 °C in the dark) was added into the samples of *A. tamarense* cultures to a final concentration of 50 μg mL^{-1} followed by incubation at room temperature for 3 min. The treated samples were kept in an ice bath and vital cells of *A. tamarense* were measured immediately by counting the green cells under an epifluorescence microscope (Olympus BX41) with blue light excitation. After co-culture for 1 d, the living algal cells were counted with FDA assay as described in previous study (Su et al., 2011). Algicidal rate, representing the algicidal activity, was calculated using the following equation:

$$\text{Algicidal rate}(\%) = (N_C - N_T)/N_C \times 100$$

where N_C refers to the initial living algal cells concentration, N_T refers to the concentration of living algal cells after incubation for 1 day.

2.3. Characterization and identification of strain DH77-1

The colony appearance of DH77-1 was described after incubation on the 2216 agar plate for about 2–3 days at 28 °C. The bacterium was dyed with 2% phosphotungstic acid and examined using a transmission electron microscope (JEM2100). Bacterial cells from DH77-1 cultures grown in 2216 liquid cultures were collected by centrifugation (5000 ×*g* for 20 min). Extraction of genomic DNA from DH77-1 and PCR amplification for the 16S rRNA gene were performed as described by Su (Su et al., 2007a, 2007b). The PCR products were purified from agarose gel with a GeneClean Turbo Kit (Qbiogene) and ligated with a pMD 19-T vector; the ligation products were transformed into *Escherichia coli* DH5α competent cells, followed by sequencing, which was performed by Shanghai Invitrogen Biotechnology Co., Ltd. The sequence for DH77-1 was compared with other 16S rRNA genes obtained from GenBank using the BLAST program. Alignments and similarity comparison were calculated by the Clustal *X*2 software (Thompson et al., 1997), and a phylogenetic tree was constructed using MEGA 5.0 with the neighbor-joining method. Bootstrap values were determined according to Felsenstein's method (Felsenstein, 1985).

2.4. Characterization of the algicidal substances

2.4.1. Algicidal mode

The pure DH77-1 isolate grown in 3 mL 2216 broth were inoculated as a 1% inoculum into 100 mL tryptone broth and grown to the stationary phase (28 °C at 150 rpm for 22–24 h, based on the results of the growth curve). Bacterial cells were collected using centrifugation (8000 rpm, 10 min), washed three times using sterile f/2 medium and re-suspended in sterile f/2 medium. The supernatants were filtrated through 0.22 μm Millipore membrane filters. To determine the algicidal mode of the DH77-1 bacterium, 40 μL (2%) bacterial cultures, filtrates or cell suspensions were inoculated into 2 mL exponential phase axenic *A. tamarense* cultures (~1×10^4 cells mL^{-1}). Cultures with the addition of 40 μL 2216 broth but no bacteria served as a control, and a no addition control was also involved in the experiments. All treatments and controls were performed in triplicate. Algicidal mode was illustrated by the algicidal activity, calculated after co-cultured for 24 h, and microscopically observed.

2.4.2. Host range of DH77-1

The algicidal range of DH77-1 was tested on 22 different algal species, some of which are typical HABs species: Cyanobacteria, *Dunaliella salina* (provided by Professor Gao Yahui, Xiamen University, Xiamen, China); *Chlorella autotrophica*, *Platymonas helgolandica*, *Prasinophyceae*, *Chlorella*, *Prorocentrum donghaiense*, *Dicrateria inornata*, *Isochrysis galbana*, *Heterosigma akashiwo*, *Chattonella marina*, *Phaeodactylum tricornutum*, *Chaetoceros compressus*, *Thalassiosira pseudonana*, *Amphiprora alata*, *Thalassiosira weissflogii*, *Asterionella japonica* (provided by College of Ocean and Earth Sciences, Xiamen University, Xiamen, China); *Alexandrium minutum* TW01, *Alexandrium catenella* DH01, *Scrippsiella trochoidea* XM01 (provided by Vice Professor Zhou Lihong, Jimei University, Xiamen, China); *Phaeocystis globosa* (provided by the Algal Culture Collection, Institute of Hydrobiology, Jinan University, Guangzhou, China); and *Nannochloropsis* (provided by the Center of Marine Biotechnology, University of Maryland, Biotechnology Institute, Baltimore, USA).