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Algicidal Activities against *Scrippsiella trochoidea* and *Prorocentrum micans* by *Bacillus* sp. Strain N29 Isolated from Mirs Bay, China

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A bacterial strain N29 was isolated from the sediment of Mirs Bay during *Scrippsiella trochoidea* bloom. By analysis of its morphology and biochemistry, as well as homology screening using 16S rRNA gene sequences, strain N29 was identified as *Bacillus anthracis* CEB95-0033. Strain N29 showed algicidal activities against *S. trochoidea* and *Prorocentrum micans* but had no effect on *Skeletonema costatum* and *Phaeodactylum tricornutum*. Moreover, the algicidal activity correlated with bacterial concentration, algal species and incubation period. More specifically, strain N29 had little algicidal effect at low volume fraction of 0.1% and 1% for *S. trochoidea* and *P. micans*, because of the densities of *S. trochoidea* and *P. micans* increased substantially just like the controls in the incubation period. However, after incubation of strain N29 with *S. trochoidea*at volume fraction of 1% and 2%, 100% of *S. trochoidea* cells were killed within 120 h and 96 h. Further, algicidal effect of strain N29 on *P. micans* required higher bacterial concentrations. For example, at 5% volume fraction, 85% of *P. micans* cells were killed within 120 h; at 10% volume fraction, 100% of *P. micans* were killed within 72 h. It showed that strain N29 had algicidal effect on *S. trochoidea* and *P. micans* in shorter incubation time with higher bacterial concentration. In addition, algicidal effects were checked with bacterial culture filtrates and heated filtrates of strain N29 on *S. trochoidea* or *P. micans*, which showed that they lysed *P. micans* but been ineffective on *S. trochoidea*. It implied that strain N29 killed *P. micans* by releasing some heat-tolerant algicide, but killed *S. trochoidea* via direct attack or competition for nutrients.

Key words: Algicidal bacterium, *Scrippsiella trochoidea*, *Prorocentrum micans*, Mirs Bay.

Harmful algal blooms (HABs) in coastal waters have become a serious environmental problem all over the world, with a negative impact on marine ecosystems, aquaculture industries and human health[1, 2]. Therefore, techniques to reduce damage from HABs are urgently needed. Three main approaches (physical, chemical and biomanipulation methods) are currently available to address the adverse effects of HABs. Physical methods such as yellow loess and clay have side effects on the growth of bottom-dwelling organisms and do not address the fundamental issues of HABs[3, 4]. Chemical agents such as copper sulfate, potassium permanganate and bleaching powder are potentially harmful to aquatic ecosystems because they can cause secondary pollution of the environment[5]. Bio-algicidal techniques will enable bacteria, viruses, fungi and protozoans to

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inhibit HABs and with less damage to marine ecosystems[6,7].

Many bacteria isolated from coastal HABs waters have been found to have algicidal effect on species associated with HABs[4,8,9]. About 50% of such algicidal strains belong to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) groups, while about 45% are members of γ-Proteobacteria, and the remaining strains represent the Gram-positive genera *Micrococcus*, *Bacillus*, and *Planomicrobium*[7,10,11].

Algae can be killed by algicidal bacteria through direct or indirect attack[10]. A direct attack requires a physical contact which exists in the phycosphere of algae and bacterium before the algae died[5]. An indirect attack refers to a process in which the bacteria release certain algicidal compounds into the surrounding media or compete for nutrients with the algal cells[12].Algicidal compounds, including argimicin A[13], proteases[14], amino acid derivatives[15], biosurfactants[16], harmane[17], and 2,3-indolinedione/isatin[18], may be either heat labile or heat stable[7].

Yoshinaga, *et al*. found that some bacterial strains lysed one kind of alga but could not effect on others[19, 20], indicating that algicidal activity might be species-specific. Bacteria with algicidal effects against toxic HABs species have been studied a lot[21-23]. However, little attention has been devoted to bacteria lysed nontoxic HABs species. Further studies are still necessary considering over 70% of HABs algal species are nontoxic, such as *Scrippsiella trochoidea* is a common and important HABs algae in the coastal waters of China, Korea, Japan, and elsewhere[24-27]. In particular, an extensive HABs of *S. trochoidea* affected > 20 km^2 waters in the South China Sea and caused economic losses about $ 100,000 in June-September, 2000[28]. *S. trochoidea* can transform into resting spores when the environment is not suitable. It was the most abundant spores in the sediment of the coastal South China Sea[25]. The formation and germination of resting spores play an important role in population succession, conservation, distribution and dispersal of HABs. There is no magic way to control HABs of *S. trochoidea* efficently. A algicidal bacterial strain N29 was isolated from Mirs Bay during *S. trochoidea* bloom. Mirs Bay, located northeast of the Sai Kung Peninsula of Hong Kong and the Pearl River Estuary, is a semi-enclosed subtropical bay with a high frequency of HABs[29]. Since the 1980s, the marine environment in Mirs Bay has been damaged by the rapid development of local marine aquaculture, tourism, and commercial shipping. As a consequence, the frequency of HABs has markedly increased[30]. In this study, we report the identification and characterization of strain N29, and its algicidal effect and mode against *S. trochoidea* and *Prorocentrum micans.*

MATERIALS AND METHODS

Isolation of marine bacteria

A bacterial strain N29 was isolated from the sediment samples of Mirs Bay (114°19'17.74"E, 22°35'43.39"N) during *S. trochoidea* bloom on July 29, 2010. Sediment samples (20 g) were diluted with autoclaved seawater (180 mL) and shaken for 20 minutes, then the diluted sediment samples were filtered through bolting-silk filters to remove large particles (e.g., zooplankton). Filtrates were serially diluted (10-fold dilutions) with autoclaved seawater and 0.1 mL aliquot of each dilution was spread onto ZoBell 2216E agar plates. After incubation at 28°C for 72 h, morphologically dominant bacteria that clearly formed colonies were randomly picked and spread onto new agar plates for separation and purification. The well isolated bacterial colonies were transferred to ZoBell 2216E agar slant culture medium, grown at 28°C for 48 h, and stored at 4°C.

Algal cultures

S. trochoidea, *P. micans*, *Skeletonema costatum* and *Phaeodactylum tricornutum* used in algicidal experiments were supplied by Institute of Hydrobiology, Jinan University, Guangzhou, Guangdong, China. Algae were incubated in f/2 medium[31, 32] under an 11 h: 13 h light-dark cycle at 23°C (except for *P. tricornutum* at 18 °C). Aliquot (1 mL) of *S. trochoidea*, *P. micans*, *S. costatum* and *P. tricornutum* cultures was added to 20 mL of f/2 medium with antibiotics to kill bacteria in the phycosphere of algae. The composition of the antibiotic cocktail used in the f/2 medium was described previously in Kim, *et al*. (2008)[8].

Algicidal ranges of bacterial strain N29

Strain N29 was inoculated in 50 mL ZoBell 2216E broth and incubated in an oscillation

incubator at 70 rpm, 28°C for 48 h. Aliquot (0.1 mL) of bacterial cultures were inoculated into the cultures of *S. trochoidea* (~ 5.0×10^4 cells.mL^{-1}), *P. micans* (~ 1.0×10^4 cells.mL^{-1}), *S. costatum* (~ 25×10^4 cells.mL^{-1}) and *P. tricornutum* (~ 65×10^4 cells.mL^{-1}) in the wells of 48-well microplates in quadruplicate. The volume fraction (v/v) of bacterial strain N29 against *S. trochoidea* was 2%, while 10% for the other algae, considering that strain N29 was isolated from *S. trochoidea* bloom. Aseptic seawater was added to *S. trochoidea* and *P. micans* cultures as controls in quadruplicate. ZoBell 2216E broth (without bacteria) was added to *S. costatum* and *P. tricornutum* cultures as controls in quadruplicate. The inoculated microplates were tightly sealed with parafilm and incubated under the same conditions as described above for the algal cultures for 120 h. The live algal cells of *S. trochoidea*, *P. micans*, *S. costatum* and *P. tricornutum* in each well were counted daily with an inverted microscope (model IX70; Olympus Corp., Tokyo, Japan). The bacterial strain N29 was considered to be algicidal when 90% or more of algal cells were killed.

Algicidal effects of bacterial strain N29 against *S. trochoidea* and *P. micans*

The cultures of the bacterial strain N29 and algae were prepared as described above. Strain N29 cultures were added in algae cultures at three volume fractions (v/v) to check the algicidal effects in the wells of 48-well microplates in quadruplicate, which for *S. trochoidea* were 2%, 1% and 0.1%, but for *P. micans* were 10%, 5% and 1%, considering that strain N29 was isolated from *S. trochoidea* bloom. The initial density of *S. trochoidea* and *P. micans* cultures was ~ 5.0×10^4 cells.mL^{-1} and ~ 1.0×10^4 cells.mL^{-1}, respectively. Aseptic seawater was added as controls in quadruplicate. The inoculated microplates were tightly sealed with parafilm and incubated for 120 h under the same conditions as the algal cultures described above. The live algal cells in each well were counted with a blood corpuscle counting plate using optical microscopy (model LB30s; Leica Microsystems GmbH, Wetzlar, Germany) every 24 h for 120 h. The algicidal effect of strain N29 was calculated as follows: Algicidal effect (%) = $(1-N_t/N_0)\times100\%$, where N_t and N_0 are the numbers of living algal cells at time t and the beginning of the treatment. Meanwhile, the live bacterial cells of strain N29 in each well were counted by the spread plate count method.

Algicidal modes of bacterial strain N29 against *S. trochoidea* and *P. micans*

The algicidal activities of three types of bacterial cultures were examined against *S. trochoidea* and *P. micans* in order to finding out whether the algicidal effects resulted from bacteria or its extracellular polymeric substance excreted by strain N29. Three types were as follows: (A) bacterial cultures without treatment; (B) culture filtrates: aliquots of bacterial cultures were centrifuged at 12,000×g (relative centrifugal force) for 20 minutes at 4°C and filtered through 0.22 µm pores to remove bacterial cells; (C) heated filtrates: aliquot of the culture filtrates was heated for 5 minutes at 105 °C. Then, aliquot (0.1 mL) of the culture filtrates and heated filtrates was spread on ZoBell 2216E agar plates in duplicate, and incubated at 28°C for 96 h to test whether the bacteria had been completely removed. Aliquot of bacterial cultures, culture filtrates and heated filtrates was inoculated at volume fraction of 2% and 10% into cultures of *S. trochoidea* (~ 5.0×10^4 cells.mL^{-1}) and *P. micans*(~ 1.0×10^4 cells.mL^{-1}) in the wells of 48-well microplates in triplicate, respectively. Aseptic seawater was added as controls in triplicate. The inoculated microplates were tightly sealed with Parafilm and incubated under the same conditions as the algal cultures as described above. The live algal cells in each well were counted with a blood corpuscle counting plate using optical microscopy after incubation for 48 h.

Identification of bacterial stain N29

To identify the bacterium, its morphology was evaluated using transmission electron microscopy (model 7650; Hitachi, Tokyo, Japan), conventional biochemical tests were performed using methods described by Dong and Cai (2001)[33], and the bacterial 16S rRNA gene was amplified by the polymerase chain reaction (PCR) method. Bacterial genomic DNA was extracted and used as a template. Subsequently, 16S rRNA was PCR-amplified with the primers 27F (5'-AGAGTTTGAT CATGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')[34]. The thermal profile was comprised of 35 cycles of denaturation for 35 seconds at 95°C, annealing for 35 seconds at 55°C, and an extension for 90 seconds at 72°C followed by a final extension step of 8 minutes at

72°C. The PCR-amplified 16S rRNA was purified and sequenced. A comparison of nucleotide sequences was performed 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 Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0[35].

Statistical analysis

All statistical analyses were performed using Statistica 6.0 software (Statsoft, Tulsa, OK, USA). The data are presented as the mean ± standard error (SE). Significant differences in algicidal activity between the treatments and the control were analyzed using *t*-tests, where $p < 0.05$ was considered statistically significant.

RESULTS

Algicidal ranges of strain N29

Strain N29 showed strong algicidal activity against *S. trochoidea* and *P. micans*, but could not kill *S. costatum* or *P. tricornutum*. The algicidal activity caused the morphological changes of *S. trochoidea* and *P. micans*. Normal cells of *S. trochoidea* (Fig. 1 A) exposed to strain N29 became irregular and the cellular components lost their integrity and decomposed. Finally, the cellular membranes were disrupted and cellular components were released (Fig. 1 B). Normal cells of *P. micans* (Fig. 1 C) exposed to strain N29 inflated to ellipsoid and their cellular components were aggregated. Finally, the cellular membranes were disrupted and cellular components were released (Fig. 1 D).

Algicidal effects of strain N29 against *S. trochoidea* and *P. micans*

The algicidal activity of strain N29 against *S. trochoidea* and *P. micans* correlated with bacterial concentration, algae species and incubation period. More specifically, after incubation with high volume fractions at 2% and 10% for *S. trochoidea* and *P. micans*, respectively, with bacterial density of 39.4×10^6 CFU.mL^{-1} and 177.3×10^6 CFU.mL^{-1}, strain N29 showed significantly algicidal effect on both algae compared to the controls ($p < 0.05$) for 90% of *S. trochoidea* and *P. micans* were killed within 72 h and 24 h, respectively, and all of them were killed within 96 h and 72 h (Fig. 2 A & D). At middle volume fractions of 1% and 5% with bacterial density of 19.7×10^6 CFU.mL^{-1} and 92.9×10^6 CFU.mL^{-1} for *S. trochoidea* and *P. micans*, algicidal activity remained effectively with a rise of both algal populations in the middle incubation period, then 90% and 100% of *S. trochoidea* were killed within 96 h and 120 h, respectively; while 85% of *P. micans* were dead after 120 h (Fig. 2 B & E). At low volume fraction of 0.1% and 1% with bacterial density of 2.0×10^6 CFU.mL^{-1} and 19.3×10^6 CFU.mL^{-1} for *S. trochoidea* and *P. micans*, strain N29 showed little algicidal effect on both algae for algal densities increased substantially over their initial values and had no significant difference compared to the controls ($p>0.05$) (Fig. 2 C & F).

The density range of strain N29 in the phycosphere of *S. trochoidea* cultures was different with that in *P. micans* cultures at the effective algicidal volume fractions. More specifically, at 2% and 1% volume fractions for *S.*

Table 1. Characteristics of strain N29

Characteristic	N29
Colony characteristic	
Color	Orange
Surface	Smooth
Elevation	Convex
Hardness	Moderate
Transparency	Opaque
Gram staining	+
Motility	+
Spore shape	Oval
Spore swollen	-
Facultative anaerobe	+
Nitrate reduction	-
Oxidase test	-
Lactic acid	-
Sulfate reduction	-
Catalase	+
Methyl red	+
Glucose fermentation oxidation reaction	+
Growth condition	
Temperature range (°C)	4-45
Optimal temperature (°C)	30-37
NaCl concentration (‰ w/v)	0-70
Optimal salinity (‰ w/v)	5
pH range	5-8.5
Optimal pH	7

Symbols: +, positive; -, negative.

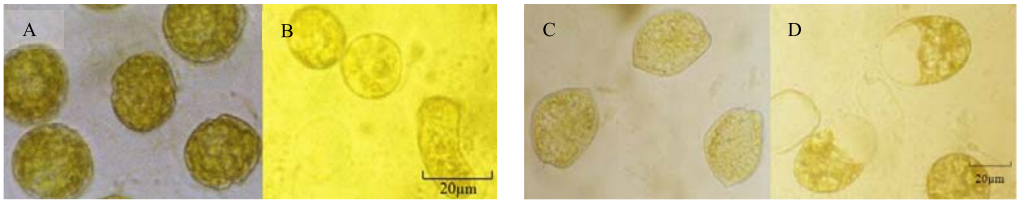

Fig. 1. Morphological changes of *Scrippsiella trochoidea* (A & B) and *Prorocentrum micans* (C & D) induced by algicidal bacterial strain N29 by inverted microscope:A & C normal algal cells; B & D algal cells exposed to strain N29

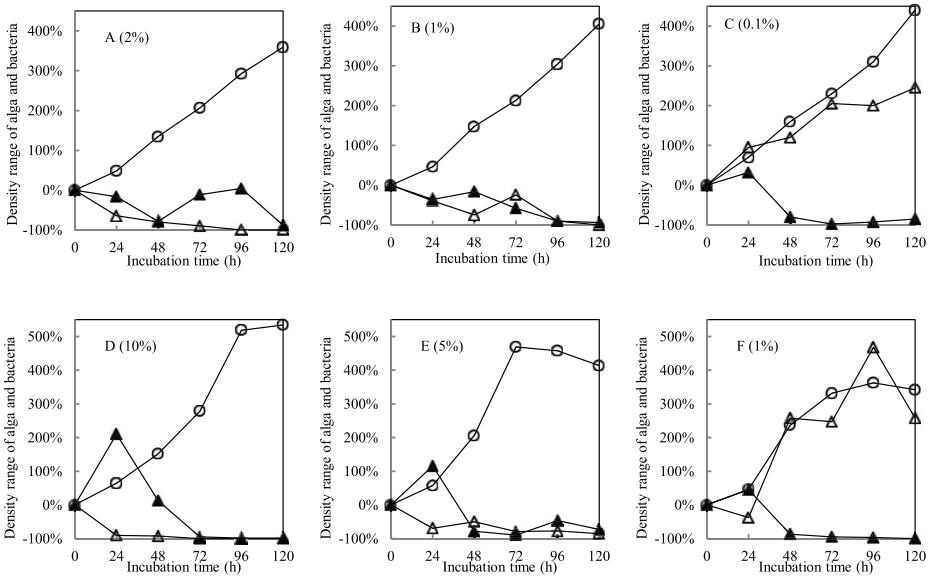

Fig. 2. Algicidal activities of bacterial strain N29 cultures against *Scrippsiella trochoidea* (A, B, &C) and *Prorocentrum micans* (D, E, &F) at different volume fraction of (A) 2%, (B) 1%, (C) 0.1%, (D) 10%, (E) 5% and (F) 1%.○, algal density of control. Δ, algal density of experimental group; ▲, bacterial density of experimental group. Aseptic water was added as control.

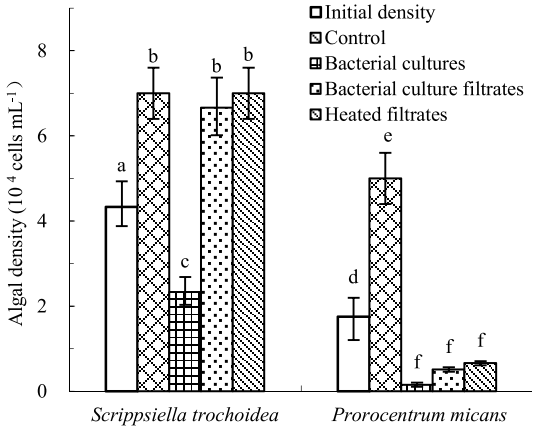

Fig. 3. Algicidal activities of strain N29 bacterial cultures, bacterial culture filtrates and heated filtrates against *Scrippsiella trochoidea* (2% v/v) and *Prorocentrum micans* (10% v/v) after 48 h. Data are expressed as the mean ± SE from triplicate assays. Data of each group with the same letter did not differ significantly from each other ($P > 0.05$).

trochoidea groups, bacterial density of strain N29 decreased at first, then rose near the initial density, and then decreased at last. However, at 10% and 5% volume fraction for *P. micans* groups, bacterial density of strain N29 rose dramatically to triple and double of the initial density at 24 h; then reduced (Fig. 2 A, B, D & E).

Algicidal modes of strain N29 against *P. micans* and *S. trochoidea*

No bacterial colonies were found in the agar plates of strain N29 bacterial culture filtrates and heated filtrates by the spread plate method. The density of *S. trochoidea* exposed to strain N29 bacterial culture filtrates or heated filtrates was

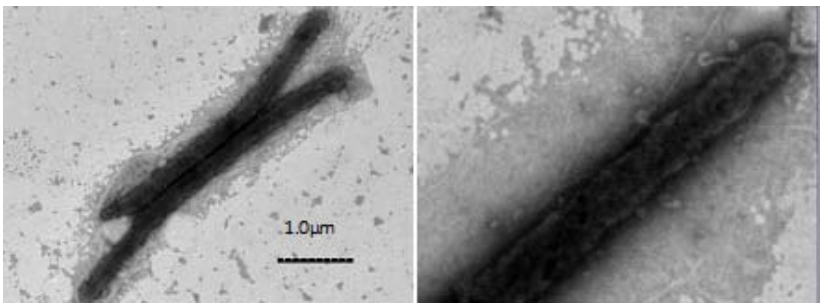

Fig. 4. Transmission electron micrographs of bacterial strain N29

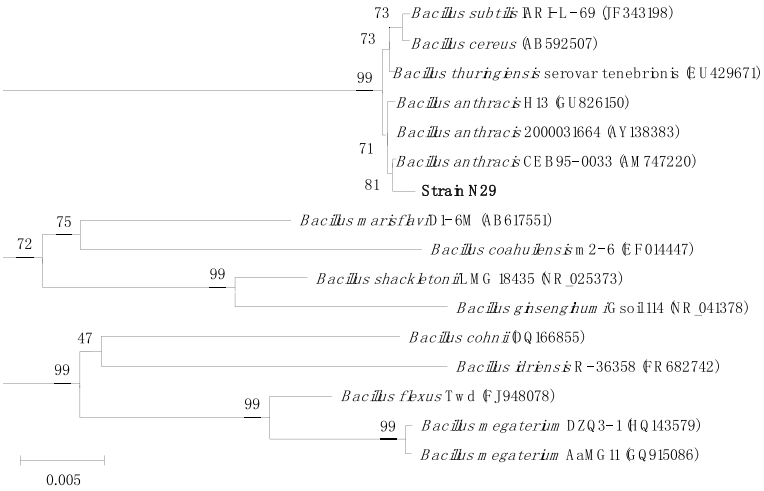

Fig. 5. Phylogenetic trees based on 16S rRNA gene sequences. The numbers at the nodes are levels of bootstrap support (%), based on neighbor-joining analyses of 1000 resampled datasets. Strain N29 and its closely related members in the genus *Bacillus*. Bar, 0.005 nucleotide substitutions per position

same as the control ($P > 0.05$), but not like it exposed to bacterial strain N29 cultures which decreased significantly in comparison to the control ($P < 0.05$) (Fig. 3). It showed that the bacterial culture filtrates and heated filtrates of strain N29 were ineffective to lyse *S. trochoidea* unlike bacterial cultures.

In contrast to *S. trochoidea*, the density of *P. micans* decreased significantly whether exposed to bacterial cultures, or culture filtrates and heated filtrates in comparison to the control ($P < 0.05$) and had no significant difference between the three treatments ($P > 0.05$) (Fig. 3). It showed culture filtrates and heated filtrates lysed *P. micans* as well as bacterial cultures. So it indicated that strain N29 killed *P. micans* by releasing some heat-tolerant algicide, but killed *S. trochoidea* via direct attack or competition for nutrients.

Identification of strain N29

Strain N29 is a Gram-positive and rod-shaped bacterium (Table 1 & Fig. 4). The biochemical tests show the optimal conditions for growth of strain N29 are 30~37°C, pH 7.0 and

salinity 5‰ (w/v). Strain N29 cannot grow at temperatures <4°C or >45°C, or at a pH below 5 or above 8.5 (Table 1). Based on bacterial 16S rRNA gene sequence analysis, strain N29 belongs to the *Bacillus* sp. of the *Bacillaceae* family (Fig. 5) and the closest specie is *Bacillus anthracis* CEB95-0033 (100% homology, GenBank accession number: AM747220).

DISCUSSION

Previous studies have reported that about 50% of algicidal bacterial strains belong to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) groups, about 45% of strains are members of γ-Proteobacteria, and the remaining strains represent Gram-positive genera *Micrococcus*, *Bacillus*, and *Planomicrobium*[7,10,11]. In this study, strain N29 belongs to genera *Bacillus*. Several members belong to genera *Bacillus* have been also reported having algicidal activities against *Microcystis aeruginosa*, *Chlorella* sp. and *Scenedesmus* sp.[36], *Cochlodinium polykrikoides*, *Akashiwo sanguinea*, *Fibriocapsa japonica*, *Heterosigma akashiwo* and *S. trochoidea*[8, 32]. Moreover, Gram-positive organisms do not comprise a major group in water column, but they are rather found in the deep-sea sediment[37]. It was hypothesized that algicidal bacteria which are Gram-positive may act as an important top-down control mechanism[38]. We expect that strain N29 isolated from the sediment can also function as an important controller of HABs.

It was reported that most members of genus *Bacillus* showed strong algicidal activity against algae, mediated by the release of heat-tolerant algicidal compounds[32, 36, 39]. In this study, bacterial strain N29 heated filtrates could lyse *P. micans* (Fig. 5 B). However bacterial strain N29 filtrates whether be heated or not could not lyse *S. trochoidea* (Fig. 5 A). It suggested that strain N29 lysed *P. micans* by indirect attack through heat-stable algicidal at 105°C, but lysed *S. trochoidea* via direct attack or competition for nutrients. To our knowledge, this is the first report that a member of genus *Bacillus* exhibits strong algicidal activity against two kinds of algae using different algicidal modes.

Algicidal effects of bacteria are influenced by bacterial density and algicidal substance cincentration in direct attack and indirect mode, respectively[7,21,40]. In agreement, strain N29 proliferated to peak over 1-2 times of its initial density within 24h, to accumulate its algicidal substance concentration and play its indirect algicidal effect to *P. micans* cultures (Fig. 4 D&E). However strain N29 barely topped near the initial density in lysing *S. trochoidea* directly.

In this study, *P. micans* cells exposed to strain N29 first became swollen, followed by aggregation of their cellular components and finally decomposition (Fig. 1D). Kim, et al. (2009) observed a similar process when *Chattonella marina* cells were lysed by *Bacillus* sp. through indirect attack[32]. However, *S. trochoidea*cells exposed to strain N29 became irregular instead of swollen, and their cellular components lost their integrity and decomposed (Fig. 1B). This difference in morphological phenomena is probably related to the different algicidal modes of strain N29, which will be interesting to investigate further.

CONCLUSIONS

These experiments provide evidence that bacterial strain N29 in its phycosphere could play a key role in the population growth of *S. trochoidea* and *P. micans*, and have implications for future bio-control of HABs. However, further studies are needed before this algicidal bacterium can be practically applied to the regulation of HABs. These include studies on the identification of algicidal compounds released by bacteria, the mechanisms of death of algal cells, and the impact of algicidal bacteria on other marine organisms.

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