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Isolation of an algicide from a marine bacterium and its effects against the toxic dinoflagellate *Alexandrium catenella* and other harmful algal bloom species

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The aim of this study was to isolate and identify bacteria demonstrating an algicidal effect against *Alexandrium catenella* and to determine the activity and range of any algicide discovered. The morphological and biochemical attributes of an algicidal bacterium, isolate YS-3, and analysis of its 16S rRNA gene sequence revealed it to be a member of the genus *Brachybacterium*. This organism, designated *Brachybacterium* sp. YS-3, showed the greatest effect against *A. catenella* cells of all bacteria isolated, and is assumed to produce secondary metabolites. When 10% solutions of culture filtrates from this strain were applied to *A. catenella* cultures, over 90% of cells were killed within 9 h. Bioassay-guided isolation of the algicide involved led to the purification and identification of an active compound. Based on physicochemical and spectroscopic data, including nuclear magnetic resonance and mass analyses, this compound was identified as 1-acetyl-β-carboline. This algicide showed significant activity against *A. catenella* and a wide range of harmful algal bloom (HAB)-forming species. Taken together, our results suggest that *Brachybacterium* sp. YS-3 and its algicide represent promising candidates for use in HAB control.

Keywords: *Alexandrium catenella*, algicidal activity, algicide, *Brachybacterium*, harmful algal bloom species, 1-acetyl-β-carboline

Introduction

In recent years, frequent large-scale harmful algal blooms (HABs) have caused mass mortality in coastal fish and bivalve cultures. Of the organisms that form HABs, dinoflagellates are among the most detrimental. Red tides of dinoflagellates are often reported in Japan, Korea, and elsewhere, where they cause severe damage to fisheries (Kim, 1997; Whyte *et al*., 2001; Glibert *et al*., 2005; Anderson *et al*., 2012). The toxic dinoflagellate *Alexandrium catenella* is one of the most frequently observed harmful species responsible for fish mortality in Korea. Moreover, blooms of toxic *Alexandrium* species are often associated with paralytic shellfish poisoning (Sala *et al*., 2005; Bravo *et al*., 2008). The relationship between *Alexandrium* spp. and bacteria has been a subject of interest for several years (Sala *et al*., 2005), however, few investigations exist concerning the effect on these dinoflagellates of algicidal bacteria (Amaro *et al*., 2005).

Many physiological and ecological studies of HABs have been conducted with the aim to reduce the resultant damage to fisheries (Kim *et al*., 2002). It has recently been revealed that many genera of marine bacteria demonstrate algicidal effects and play important roles in the eradication of HABs in natural marine environments (Yoshinaga *et al*., 1997; Park *et al*., 1998; Mayali and Azam, 2004). For example, some bacteria are known to have been involved in the elimination of algal blooms caused by *Alexandrium tamarense* (Su *et al*., 2007, 2011; Wang *et al*., 2012), *Chattonella* spp. (Imai *et al*., 2001), *Cochlodinium polykrikoides* (Jeong *et al*., 2000), *Gymnodinium breve* (Doucette *et al*., 1999), *G. mikimotoi* (Yoshinaga *et al*., 1995), *Heterocapsa circularisquama* (Imai *et al*., 1998), and *Heterosigma akashiwo* (Kim *et al*., 1998). These findings have raised the possibility of utilizing bacteria to control HABs, with algicidal bacteria being considered as potential tools to regulate the organisms responsible.

The mechanisms by which bacteria curtail HABs have not yet been well characterized. Some bacteria directly attack algal cells (Mitsutani *et al*., 1992; Imai *et al*., 1993), while several reports exist of "indirect attacks" mediated by the production of extracellular factors (Yoshinaga *et al*., 1997; Lovejoy *et al*., 1998), such as serine protease (Lee *et al*., 2002). If they are to be used in the natural environment, an understanding of the properties and functions of algicides is indispensable, yet very limited information is available regarding algicidal compounds derived from marine bacteria.

In the course of screening for algicidal bacteria, we isolated the marine bacterium *Brachybacterium* sp. YS-3 and purified its algicide, 1-acetyl-β-carboline. Here, we report the isolation, structure, and activity of this compound, which functions as an algicide against *A. catenella*, and demonstrate its algicidal properties against a wide range of other HAB-forming species. To our knowledge, this is the first report to describe the algicidal activity of 1-acetyl-β-carboline.

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Materials and Methods

Sampling and isolation of algicidal bacteria

Seawater samples were collected at a depth of 1 m using a Van Dorn sampler in Uljin, on the eastern coast of the Korean peninsula. To isolate bacteria with algicidal effects against *A. catenella*, samples were serially diluted and 0.1 ml aliquots of each dilution were spread onto PPES-II (Taga, 1968) agar plates, which were then incubated for 7 days at 25°C. Individual colonies of distinct morphology were streaked onto PPES-II agar plates for purification and subsequently frozen at -70°C in 20% glycerol.

In experiments testing the effects of bacteria on *A. catenella*, 24-well plates were used. Each well contained 1 ml of putative algicidal solution, to which 0.5 ml of bacterial culture, filtrate, or medium (as a control) had been added. The survival of *A. catenella* cells in each well was assessed daily using an inverted microscope (Axiovert 40 CFL; Zeiss) at a magnification of 200×. Seawater was autoclaved and filtered using a 0.1 μm pore-size membrane before being added to assay cultures as a negative control. Bacteria from wells exhibiting algicidal activity against *A. catenella* were isolated for further study. As a result, eight algicidal strains were isolated in this investigation.

Axenic cultures of *A. catenella*

A. catenella cultures (supplied by Korea Marine Microalgae Culture Center, Republic of Korea) were routinely maintained in f/2-Si medium (Guillard and Ryther, 1962). Axenic clones were obtained by repeated washing with capillary pipettes (Droop, 1967) and multiple subculturing using enriched seawater medium containing an antibiotic mixture of streptomycin, gentamicin, and cephalothin, each at 100 μg/ml. All antibiotics and the f/2-Si medium were purchased from Sigma. Each liter of f/2-Si medium consisted of GF/F-filtered seawater containing 75 mg of $NaNO_3$, 5 mg of $NaH_2PO_4 \cdot H_2O$, 4.36 mg of Na_2EDTA, 3.15 mg of $FeCl_3 \cdot 6H_2O$, 0.01 mg of $CuSO_4 \cdot 5H_2O$, 0.022 mg of $ZnSO_4 \cdot 7H_2O$, 0.01 mg of $CoCl_2 \cdot 6H_2O$, 0.18 mg of $MnCl_2 \cdot 4H_2O$, 0.006 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.1 mg of thiamine·HCl, 0.5 μg of biotin, and 0.5 μg of vitamin B_{12}. Cultures were grown in disposable sterilized tissue culture flasks (Iwaki Inc.) illuminated with 120 $\mu E/m^2/sec$ under a 12:12 h light-dark cycle at 20°C.

Identification of algicidal bacteria

Eight algicidal strains were grown at 25°C for 3 days on PPES-II agar. Assessment of morphological characteristics was carried out using Gram staining and scanning electron microscopy (S-3500N; Hitachi), while standard physiological and biochemical characteristics were examined according to the methods of MacFaddin (1980). All carbon sources for carbon-utilization tests were filter-sterilized and used at the concentrations recommended by Shirling and Gottlieb (1966). Additional biochemical tests were performed using the API 20NE system (bioMérieux).

Extraction of genomic DNA and 16S rRNA gene amplification were both carried out according to the method of Rainey *et al.* (1996). The resulting PCR product was ligated into a pGEM-T Easy Vector (Promega) and sequenced using a Termination Sequencing Ready Reaction kit and an ABI 377 genetic analyzer (both Perkin Elmer). The near-complete 16S rRNA gene sequence of isolate YS-3 (1,455 bp) was aligned using CLUSTAL W software version 1.7 (Thompson *et al.*, 1994). For phylogenetic analyses, this sequence was compared with those of other bacteria available in the DDBJ/EMBL/GenBank databases, and Kimura's two-parameter model (Kimura, 1980) was applied for the calculation of evolutionary distance. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) and bootstrap analysis with 1,000 replicates was carried out using MEGA version 5.0 (Tamura *et al.*, 2001).

Algicidal activity of isolate YS-3 culture filtrates against *A. catenella*

The algicidal effect of filtrates obtained from cultures of isolate YS-3 against *A. catenella* cells was assessed at various concentrations. After cultivation on PPES-II medium, bacterial cultures were centrifuged at 15,000 × *g* for 10 min and supernatants were filtered through 0.2-μm membranes (Millipore). Isolate YS-3 filtrates were added to *A. catenella* cultures (*ca.* 2.0×10^4 cells/ml) at concentrations of 1, 5, and 10%, while PPES-II medium was used as a control. The bioassay plates were then incubated as above and inspected every 2 h. Viable swimming cells in each well were counted with a Sedgewick-Rafter chamber under an inverted microscope.

Algicidal activity was estimated using the flowing equation: Algicidal activity (%) = (1−Tt/Ct) × 100, where T (treatment) and C (control) represent the number of viable *A. catenella* cells in the presence or absence of bacterial culture filtrates, respectively, and t signifies the exposure time. The treatment consisted of the addition of a suitable volume of each bacterial supernatant to *A. catenella* cultures. The two controls comprised equal volumes of algae-free f/2-Si medium or sterile PPES-II medium in place of culture filtrates.

Isolation of algicide from isolate YS-3 and elucidation of its structure

In order to obtain the algicide of interest, isolate YS-3 was grown in a 5-L flask containing 2 L of PPES-II medium at 25°C on a rotary shaker at 150 rpm for 3 days. The culture broth was then centrifuged at 15,000 × *g* for 20 min at 4°C, and filtered using a 0.2 μm pore-size membrane to obtain cell-free culture filtrates. Finally, the solution was partitioned with ethyl acetate. The ethyl acetate layer was concentrated using a rotary evaporator, and the residual aqueous suspension was subjected to ODS open column chromatography (YMC-GEL, 10 × 20 cm) with aqueous MeOH followed by CH_2Cl_2. The active fractions at 60 and 80% MeOH were combined and final purification was achieved by reversed-phase HPLC (COSMOSIL 5C18-MS, 10 × 250 mm; 30–80% MeOH in 60 min; flow rate, 2.0 ml/min; UV detection at 254 nm).

The structure of the isolated algicide was identified using physicochemical and spectroscopic data, including nuclear magnetic resonance (NMR) and mass analysis. ^1H-, ^{13}C-, and 2D-NMR spectra were measured on a JNM-A600 NMR spectrometer (JEOL Ltd.) in CD_3OD-d_4 at 300 K. ^1H- and

^{13}C-NMR spectra were measured at 500 and 125 MHz, respectively. The resonances of residual CD_3OD-d_4 at δ_H 3.30 and δ_C 49.0 were used as internal references for ^{1}H- and ^{13}C-NMR spectra, respectively. Heteronuclear multiple-bond correlation (HMBC) spectra were acquired with a delay time of 60 ms. Liquid chromatography-mass spectrometry (LC-MS) data, including high-resolution mass measurements, were acquired on a JMS-SX 102A mass spectrometer (JEOL). ^{1}H- and ^{13}C-NMR spectroscopic assignment analyses were performed using correlation (COSY) and HMBC spectroscopy.

Algicidal range of algicide against HAB-forming species

The range of HAB-forming organisms affected by the algicide purified from isolate YS-3 was investigated using the following algal species: *Chattonella marina*, *C. polykrikoides*, *A. catenella*, and *Heterosigma akashiwo*, which were provided by the National Fisheries Research & Development Institute, Republic of Korea, and *Akashiwo sanguinea* (*G. sanguineum*), *G. impudicum*, *Fibriocapsa japonica*, *Prorocentrum micans*, *P. minimum*, and *Scrippsiella trochoidea*, provided by the South Sea Institute of the Korea Ocean Research & Development Institute, Republic of Korea.

All algal cultures were grown in f/2-Si medium and illuminated with 120 μE/m^2/sec under a 12:12 h light-dark cycle at 20°C. Cultures of HAB-forming species were prepared in the same manner as the *A. catenella* assay. The algicide (10 μg/ml) was added to each culture during the mid-exponential growth phase, and after a 6 h incubation, viable swimming cells were counted as above.

Results

Isolation of bacteria demonstrating algicidal effects against *A. catenella*

To isolate bacterial strains exhibiting algicidal activity, we identified wells in which all *A. catenella* cells were dead. In total, 213 colonies showing distinct color and morphology were selected. Each strain was cultured and inoculated once again into *A. catenella* cultures to confirm its algicidal activities. As a result, eight algicidal strains were isolated in this study, of which, YS-3 exhibited the strongest effect against *A. catenella* cells.

Identification of isolate YS-3

The morphological and biochemical characteristics of isolate YS-3 are summarized in Table 1. This algicidal bacterium was gram-positive, cocci-shaped, and showed yellow pigmentation on PPES-II agar. However, cells varied in shape from coccoid forms (single or in agglomerates) in the stationary phase, to short rods during the exponential phase. Cells were non-motile and did not form endospores. The optimal conditions for growth were 25°C, at pH 7.0, in the presence of 2.0% (w/v) NaCl. Isolate YS-3 did not grow at temperatures below 5°C or above 40°C, pH values below 5 or above 10, and NaCl concentrations above 7.0%. Catalase and urease were produced, while oxidase and indole were not. Gelatin and esculin were hydrolyzed but starch was not. Nitrate was reduced to nitrite. The test for arginine dihydrolase was positive. Glucose, D-xylose, and D-galactose were utilized, while D-fructose, L-rhamnose, and inositol were not. The near-complete 16S rRNA gene sequence (1,455 bp) of isolate YS-3 was aligned with those of other bacteria available from the DDBJ/EMBL/GenBank databases. This isolate shared the highest degree of similarity with *Brachybacterium paraconglomeratum* LMG 19861^T (accession number AJ415377, 99% similarity; Fig. 1). Morphological and biochemical characteristics in addition to phylogenetic analysis supported the identification of isolate YS-3 as a member of the genus *Brachybacterium*. Thus, we designated this isolate *Brachybacterium* sp. YS-3, and investigated it in more detail.

Algicidal activity of *Brachybacterium* sp. YS-3 culture filtrates against *A. catenella*

The algicidal effect of isolate YS-3 filtrates against *A. catenella* cultures (*ca.* 2.0 × 10^4 cells/ml) was measured at concentrations of 1, 5, and 10% (Fig. 2). At concentrations greater than 1%, significant differences between the control

Table 1. Morphological and biochemical characteristics of *Brachybacterium* sp. YS-3

Characteristics	Isolate YS-3
Gram stain	+
Cell morphology	Coccus
Colony color	Yellow
Catalase	+
Oxidase	–
Urease	+
Indole	–
MR	–
VP	–
Citrate	+
KIA	K/K
O/F	+/–
L-Lysine decarboxylase	+
L-Ornithine decarboxylase	+
L-Arginine dehydrolase	+
Utilization of :	
Glucose	+
D-Xylose	+
D-Fructose	–
L-Rhamnose	–
D-Galactose	+
Inositol	–
D-Mannose	+
Mannitol	–
Sucrose	–
Lactose	–
L-Arabinose	+
Hydrolysis of :	
Esculin	+
Casein	+
Starch	–
Gelatin	+
Reduction of nitrates	+

+, positive result or growth; –, negative result or no growth

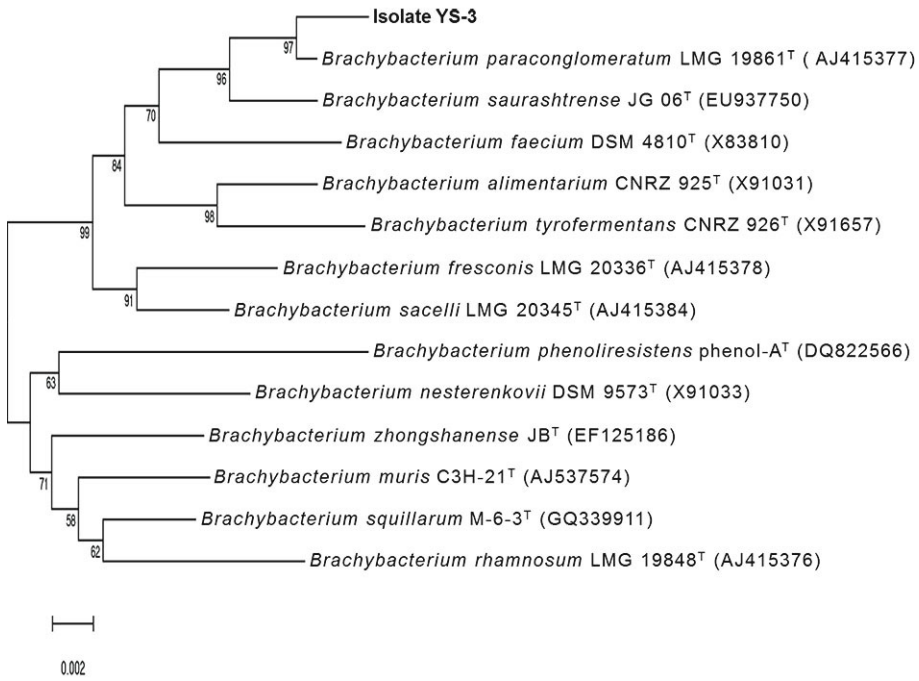

Fig. 1. Phylogenetic tree based on comparison of 16S rRNA gene sequences, indicating the taxonomic position of isolate YS-3. The phylogenetic tree was generated using the neighbor-joining method. Bootstrap values, expressed as percentages of 1,000 replicates, are given at branching points. The bar represents 0.002 substitutions per nucleotide position.

and culture filtrates were observed after incubation for 3 h. Although almost all *A. catenella* cells remained motile, their speed had decreased within 3 h at a concentration of 1%. Using 5% filtrates, over 50% of cells had died after 6 h, while in the presence of 10% filtrates, over 90% of cells were killed within 9 h. These results suggest that culture filtrates of *Brachybacterium* sp. YS-3 inhibit the growth of *A. catenella* in a concentration- and time-dependent manner.

The algicidal process induced by the 5% filtrate was observed under light microscopy (Fig. 3). When the algicide

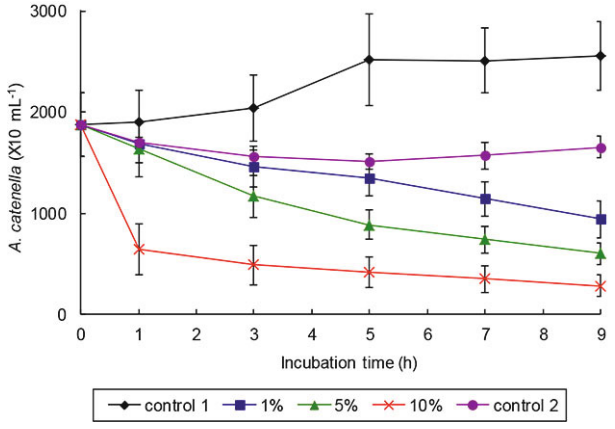

Fig. 2. Algicidal activity of *Brachybacterium* sp. YS-3 culture filtrate against *Alexandrium catenella* at various concentrations. *Brachybacterium* sp. YS-3 was cultured at 25°C for 3 days in PPES-II medium. Each culture filtrate was added to *A. catenella* cultures (*ca.* 2.0×10^4 cells/ml) during the mid-exponential growth phase. Control 1 consisted of algal cultures to which f/2-Si broth had been added. Control 2 comprised algal cultures to which PPES-II medium had been added. Data are expressed as means ± standard deviations of ten-time assays. (◆) control 1; (■) 1%; (▲) 5%; (×) 10%; (●) control 2.

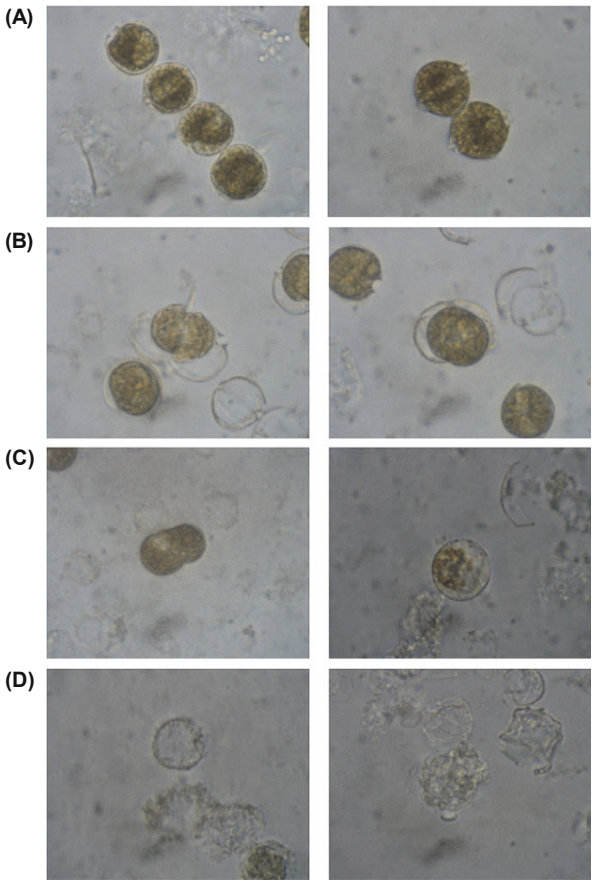

Fig. 3. Micrographs of the lysing process induced by 5% *Brachybacterium* sp. YS-3 culture filtrates on *Alexandrium catenella* cells. (A) living cells; (B) cell walls becoming detached (ecdysis) after 1 h; (C) release of cellular components after 3 h; (D) broken thecae after 6 h. Once *A. catenella* cells had burst, they could not be restored to living cells until 2 days.

took effect, the swimming movements of *A. catenella* were inhibited and cell walls detached from the main bodies of the cells (Fig. 3B). After a longer exposure, cell structure was disrupted and eventually, chloroplasts and cytoplasms lost their integrity and disintegrated (Fig. 3C), resulting in the appearance of abundant broken thecae (Fig. 3D).

Isolation of algicide from isolate YS-3 and elucidation of its structure

In order to purify the algicide present in cultures of *Brachybacterium* sp. YS-3, the organic solvent EtOAc was used for extraction. EtOAc extract (17 g) was then further separated to obtain a compound active against *A. catenella*. The last purification step was achieved by reversed-phase HPLC, as described above. Finally, we obtained 0.8 mg of a pure compound, which was then subjected to structural analysis. The LC-MS spectrum of this algicide indicated a molecular weight of 210.07 (data not shown). In the mass spectrum, a CO peak at *m/z* 182 and an $[(M-(COCH_2)]^+$ base peak at *m/z* 168 established the presence of a β-carboline moiety (data not shown). The molecular formula of the algicide was established as $C_{13}H_{10}N_2O$ by LC-MS, ^1H-, and ^{13}C-NMR spectroscopic analyses. COSY and HMBC analyses revealed the structure to be that of 1-acetyl-β-carboline. This identification was confirmed by comparison with data from the literature regarding chemical shifts (Proksa *et al*., 1990; Lee *et al*., 2013).

Algicidal range of 1-acetyl-β-carboline

Further algicidal effects of 1-acetyl-β-carboline against HAB-forming species were investigated (Fig. 4). To each algal culture, 10 μg/ml of 1-acetyl-β-carboline was added during the mid-exponential growth phase. After incubation for 6 h, this compound demonstrated a wide spectrum of activity, affecting all the dinoflagellates and raphidophytes tested. Algicidal activity against HAB-forming species was as follows: *A. catenella* (97%), *P. micans* (88%), *P. minimum* (48%), *C. polykrikoides* (7.3%), *A. sanguinea* (68%), *G. impudicum* (72%), *F. japonica* (36.7%), *H. akashiwo* (12.2%), *S. trochoidea* (76%), and *C. marina* (65%).

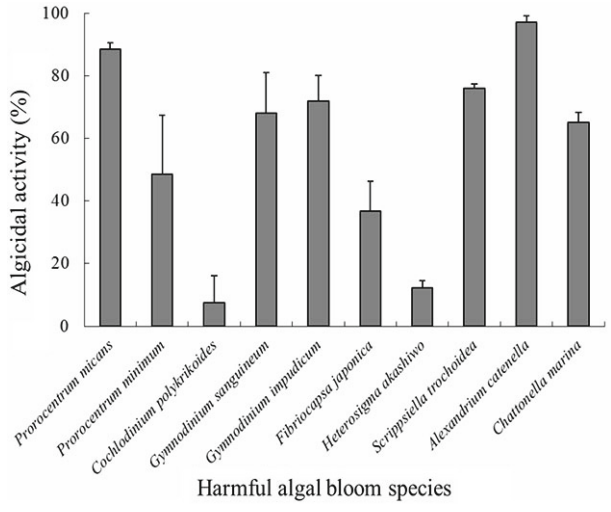

Fig. 4. The range of harmful algal bloom-forming species affected by algicide derived from *Brachybacterium* sp. YS-3. To each algal culture, 10 μg/ml of 1-acetyl-β-carboline was added during the mid-exponential growth phase. After incubation for 6 h, algicidal activity was evaluated. The control consisted of algal cultures to which methanol had been added. Data are expressed as means ± standard deviations from triplicate assays.

Discussion

A series of reports concerning interactions between algicidal bacteria and HAB-forming species (Lovejoy *et al*., 1998; Jeong *et al*., 2000; Lee *et al*., 2002; Wang *et al*., 2005, 2012; Su *et al*., 2007, 2011) supports the hypothesis that bacteria can regulate the development of algal blooms (Fukami *et al*., 1991). In this study, bacteria demonstrating algicidal activity against *A. catenella* were isolated from coastal water in Uljin, Republic of Korea. Of the bacteria tested, isolate YS-3 exhibited the strongest effect against *A. catenella* cells. This bacterium was tentatively identified as a *Brachybacterium* sp., and designated *Brachybacterium* sp. YS-3. However, members of the genus *Brachybacterium* are not commonly considered algicidal bacteria.

At present, algicidal bacteria are classified into four groups, including members of the genera *Cytophaga* (reclassified *Cellulophaga*) and *Saprospira* of the phylum Bacteroidetes, and *Pseudoalteromonas* and *Alteromonas* of the Proteobacteria (Mayali and Azam, 2004). Other researchers (Hold *et al*., 2001; Su *et al*., 2007; Yang *et al*., 2014) have observed that the phylogenetic diversity of bacteria associated with *Alexandrium* spp. is limited to α-Proteobacteria, γ-Proteobacteria, and the Cytophaga-Flavobacter-Bacteroides (CFB) group. For instance, three bacterial strains belonging to the groups *Cytophaga* (*Cytophaga* sp. AMA-01), γ-Proteobacteria (*Pseudoalteromonas* sp. AMA-02), and α-Proteobacteria (*Ruegeria atlantica* AMA-03) have been found to be potentially lytic to *A. catenella* cells in high-nutrient media (Amaro *et al*., 2005).

Algicidal bacteria kill algae using two main mechanisms: direct contact or the release of algicide as an indirect attack (Mayali and Azam, 2004). For example, *Saprospira* prey upon bacteria and algae by attaching to their target (Lewin, 1997), while *Alteromonas* and *Pseudoalteromonas* destroy algal cells by releasing dissolved substances (Mayali and Azam, 2004). In addition, many *Pseudoalteromonas* species produce extracellular bioactive molecules (Holmström and Kjelleberg, 1999) and release algicidal solutes. Some putative algicides remain active following autoclaving and are thus unlikely to be enzymes (Skerratt *et al*., 2002). However, their chemical structures remain uncharacterized. In this study, we isolated a bacterium, *Brachybacterium* sp. YS-3, that showed significant algicidal activity against *A. catenella*. This activity was also observed using culture filtrates, indicating that this bacterium is capable of releasing algicidal compounds into the culture broth. Thus, *Brachybacterium* sp. YS-3 is believed to act on algae in an indirect manner.

The isolation, purification, and characterization of algicides can prove challenging due to their wide variety of characteristics across different species of bacteria (Skerratt *et al*., 2002). Therefore, only a small number of bacterial algicides have

been purified and identified. Algicides active against HAB-forming species include antibiotic-like substances (Nakashima *et al*., 2006), biosurfactants (Ahn *et al*., 2003; Wang *et al*., 2005), peptides (Jeong *et al*., 2003), proteases (Lee *et al*., 2002), and other proteins (Mitsutani *et al*., 2001; Wang *et al*., 2012).

In this study, we isolated an algicide derived from *Brachybacterium* sp. YS-3 active against *A. catenella*. This compound, 1-acetyl-β-carboline, was lethal to not only naked dinoflagellates and raphidophytes, but also thecate dinoflagellates. β-Carboline alkaloids are found in many plant and animal cells, and norharmane, the simplest β-carboline, is used as an efficient photosensitizer in photodynamic therapy. During an investigation into agents capable of controlling cyanobacterial blooms, Kodani *et al*. (2002) isolated harmane (1-methyl-β-carboline) from the algicidal bacterium *Pseudomonas* sp. K44-1. This compound was first isolated from filtrates of *Streptomyces kasugaensis* cultures (Proksa *et al*., 1990) and recently, Lee *et al*. (2013) reported that 1-acetyl-β-carboline derived from *Pseudomonas* sp. UJ-6 demonstrates anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity. However, the current work is the first to report that 1-acetyl-β-carboline exerts algicidal effects against HAB-forming species. The algicidal activity of 1-acetyl-β-carboline was effective against a wide spectrum of dinoflagellates and raphidophytes, including all of those tested. This broad algicidal range affecting various HAB-forming species may prove to be a beneficial attribute. In conclusion, our results suggest that algicidal bacteria and the algicide they produce may be promising candidates for use in HAB control. Further studies are needed to elucidate their exact mechanism of action.

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