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Isolation and Characterization of a Marine Algicidal Bacterium against the Harmful Raphidophyceae *Chattonella marina*

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A bacterial strain named AB-4 showing algicidal activity against *Chattonella marina* was isolated from coastal water of Uljin, Republic of Korea. The isolated strain was identified as *Bacillus* sp. by culture morphology, biochemical reactions, and homology research based on 16S rDNA. The bacterial culture led to the lysis of algal cells, suggesting that the isolated strain produced a latent algal-lytic compound. Amongst changes in algicidal activity by different culture filtrate volumes, the 10% (100 μl/ml) concentration showed the biggest change in algicidal activity; there, estimated algicidal activity was 95%. The swimming movements of *Chattonella marina* cells were inhibited because of treatment of the bacterial culture; subsequently, *Chattonella marina* cells became swollen and rounded. With longer exposure time, algal cells were disrupted and cellular components lost their integrity and decomposed. The released algicide(s) were heat-tolerant and stable in pH variations, except pH 3, 4, and 5. Culture filtrate of *Bacillus* sp. AB-4 was toxic against harmful algae bloom (HAB) species and nontoxic against livefood organisms. *Bacillus* sp. AB-4 showed comparatively strong activity against *Akashiwo sanguinea*, *Fibriocapsa japonica*, *Heterosigma akashiwo*, and *Scrippsiella trochoidea*. These results suggest that the algicidal activity of *Bacillus* sp. AB-4 is potentially useful for controlling outbreaks of *Chattonella marina*.

***Keywords*:** *Chattonella marina*, algicidal bacteria, algicidal activity, algicide, *Bacillus*, harmful algae blooms (HABs)

Phytoplankton blooms in marine coastal waters and causes mass mortalities of fish and shellfish worldwide. In so doing, algal blooms seriously damage aquaculture industries (Nagayama *et al*., 2003) and impact environmental and human health (Jeong *et al*., 2000). Moreover, about 2,000 cases of human poisoning resulting from algal toxins are reported each year (Zingone and Enevoldsen, 2000). The raphidophycean flagellate, *Chattonella marina*, is known to be one of the most noxious red-tide organisms to cause toxic blooms in many parts of the world. Toxic and noxious algal blooms of this group have been reported in temperate and subtropical embayments in Korea, Japan, and other countries (Kahn *et al*., 1998). *Chattonella marina* has been reported to produce fat-soluble neurotoxins similar in structure to brevetoxins. Therefore, there is an urgent need to develop techniques that help predict and reduce the impact of noxious red tides (Imai *et al*., 1995).

In an effort to mitigate these problems, several control techniques have been used to manage blooms, including yellow loess (Na *et al*., 1996; Choi *et al*., 1998) and clay (Sun *et al*., 2004). These methods have been found to be effective. However, yellow loess and clay cause secondary effects on bottom-dwelling organisms (Rhoads and Young, 1970; Bricelj and Malouf, 1984). Chemical agents such as copper sulfate, hydrogen peroxide, and triosyn are effective in controlling blooms within a short period after application (Steidinger, 1983; Ryu *et al*., 1998), but their usage in aquatic ecosystems is potentially dangerous due to their side effects (Jeong *et al*., 2000). Therefore, biological control agents such as viruses (Garry *et al*., 1998), bacteria (Imai *et al*., 1995; Park *et al*., 1998), and protozoa (Sigee *et al*., 1999) are of particular interest (Kim *et al*., 2007).

Research into the relationship between bacteria and algae has resulted in the isolation of several strains of bacteria capable of inhibiting or killing harmful algal bloom (HAB) species (Lovejoy *et al*., 1998; Yoshinaga *et al*., 1998; Amaro *et al*., 2005; Su *et al*., 2007). Algicidal bacteria isolated from marine environments are classified into a common group that includes members of the genus *Cytophaga* and *Saprospira* (phylum *Bacterioidetes*), and the genera *Pseudoalteromonas* and *Alteromonas* (phylum *γ-Proteobacteria*). Based on several reviews, about 50% of the algicidal strains belong to the CFB group, while about 45% are members of *γ-Proteobacteria*; the remaining strains represent the Gram-positive genera *Micrococcus*, *Bacillus*, and *Planomicrobium* (Fukuyo *et al*., 2002; Mayali and Azam, 2004; Hare *et al*., 2005). Recently, Ahn *et al*. (2003) reported that a culture broth of *B. subtilis* completely inhibited the growth of *Microcystis aeruginosa*, a bloom-forming cyanobacterium in highly eutrophic lakes, and Mu *et al*. (2007) reported that secreted metabolites of *Bacillus fusiformis* showed algicidal activity against *M. aeruginosa*, *Chlorella*, and *Scenedesmus*.

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The Gram-positive genera were unusual algicidal bacteria (Skerratt *et al*., 2002). The Gram-positive organisms are not a major group of the water column, but are rather reported in deep-sea sediment (Li *et al*., 1999). Liu *et al*. (2008) hypothesize that algicidal bacteria–which are Gram-positive organisms–act as an important top-down control mechanism; we expect that the algicidal bacterium *Bacillus* sp. AB-4 can also function as an important controller. In this study, we report the identification, characterization, and algicidal effect of an algicidal bacterium, *Bacillus* sp. AB-4, against *Chattonella marina*.

Materials and Methods

Algal cultures

Alexandrium catenella, *Chattonella marina*, *Cochlodinium polykrikoides*, and *Heterosigma akashiwo* were supplied by the National Fisheries Research & Development Institute (NFRDI), Republic of Korea; *Akashiwo sanguinea*, *Fibriocapsa japonica*, *Gymnodinium impudicum*, *Isocrysis galbana*, *Prorocentrum micans*, *Prorocentrum minimum*, and *Scrippsiella trochoidea* were supplied by the South Sea Institute of the Korea Ocean Research & Development Institute (KORDI), Republic of Korea; and *Chlorella ellipsoidea* (C-020), *Chlorella vulgaris* (C-012), *Navicula elegans* (B-225), *Nannochloris oculata* (C-031), *Nitzschia pungens* (B-037), *Oscillatoria angustissima* (CY-003), *Pavlova gyrans* (H-012), *Phaeodactylum tricornutum* (B-255), *Skeletonema costatum* (B-687), and *Tetraselmis suecica* (P-009) were supplied by the Korea Marine Microalgae Culture Center, Busan, Republic of Korea. *A. sanguinea*, *A. catenella*, *C. polykrikoides*, *F. japonica*, *G. impudicum*, *P. micans*, *P. minimum*, and *S. trochoidea* belong to dinophyceae, and *C. marina* and *H. akashiwo* belong to raphidophyceae; all were maintained in an f/2-Si medium (Guillard and Ryther, 1962) at 20°C, pH 8, with cycles consisting of 12 h of darkness and 12 h of cool white fluorescent light (120 μmol photons $m^{-2}s^{-1}$). *N. elegans*, *N. pungens*, *P. tricornutum*, and *S. costatum* belong to bacillariophyceae, and *C. ellipsoidea*, *C. vulgaris*, *I. galbana*, *N. oculata*, *O. angustissima*, *P. gyrans*, and *T. suecica* belong to the live-food organism cultures that were maintained in an f/2-Si medium at 20°C, pH 8, with cycles consisting of 12 h of darkness and 12 h of cool white fluorescent light (30 μmol photons $m^{-2}s^{-1}$).

Isolation and screening of algicidal bacteria

Water samples were collected from coastal surface water in Uljin, Republic of Korea, from May to August 2005. Samples were serially diluted with sterile seawater and 0.1-ml aliquots of each dilution were spread onto PPES-II (Taga, 1968) agar plates, followed by incubation for seven days at 20°C. Individual colonies of distinct morphology were streaked onto PPES-II agar plates for purification and frozen at -70°C in 20% glycerol.

Isolated bacteria were precultured into 10 ml of PPES-II broth at 20°C, at 200 rpm for 24 h. *Chattonella marina* (1.0×10^4 cells/ml) at mid-exponential phase was used in experiments. In experiments testing the bacterial effects on *Chattonella marina*, we used 24-well plates; each well contained 1 ml of algal culture, to which 0.2 ml of a bacterial culture (or filtrate or medium control) had been added. The plates were monitored at a magnification of ×100 or ×400. A bacterial strain exhibiting algicidal activity against *Chattonella marina* was selected for further study.

Identification of bacteria by phylogenetic analysis

Morphological observations were carried out using Gram-staining. The results of conventional biochemical tests were examined using methods described by Smibert and Krieg (1994). The bacterial strains were identified by polymerase chain reaction (PCR) amplification of the 16S rRNA gene, BLAST analysis, and comparisons with sequences in the GenBank nucleotide database.

Two oligonucleotides, based on the report of Dunbar *et al*. (2000) were used to determine 16S rDNA of the isolate: forward; 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse; 5'-TACCTTGTTACGACTT-3'. PCR was performed using intact cells that had been treated for 5 min at 95°C, as a template. The thermal profile was 25 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and an extension for 2 min at 72°C. A final extension step consisting of 5 min at 72°C was included. The 16S rDNA amplified with PCR was purified, cloned into pGEM-T easy vector (Promega, USA), and sequenced. A comparison of nucleotide sequences was performed using the BLASTN database (http://www.ncbi.nlm.nih.gov/BLAST) at the National Center for Biotechnology Information (NCBI). Sequences were aligned using the program CLUSTAL W, and a phylogenetic tree was made by using the MEGA 2.0 program.

Algicidal effects of bacterium AB-4

Determination of mode of algicidal action

One method to test for dissolved algicides involves incubating the *Chattonella marina* and the AB-4 strain, physically separated via commercially available tissue culture inserts (Nalgene Nunc International, USA) as in Kim *et al*. (1999). This protocol was carried out with 24-well plates (Fig. 1). Tissue culture inserts (0.4-μm pore size) were added to each well, and 1 ml of algal culture (1.0×10^4 cells/ml) was inoculated into the inside of each insert. Each well was tested as follows: (A) f/2 medium added between well and insert, (B) bacterial culture added between well and insert, and (C) bacterial culture added to the inside of each insert. Equal volumes (i.e., 200 μl) of f/2-Si medium and bacterial culture (10^8 cells/ml) were added to each well. The plates were cultured in algal culture conditions, as described above.

Relationship of algicidal activity and bacterial growth

The growth curve and algicidal activity of the AB-4 strain were inspected every 3 h, over a 36 h period. The AB-4 strain was inoculated with PPES-II (initial cell concentration; 10^3 cells/L) and incubated at optimal culture conditions (30°C, pH 7, 2% NaCl), with shaking (200 rpm); optical density (OD) was then estimated every 3 h. The activity of the culture filtrate and each bacterial growth phase (every 3 h) on the growth of *Chattonella marina* were investigated. *Chattonella marina* (1.0×10^4 cells/ml) was placed in each well of a 24-well plate, together with each culture filtrate; the plates were then cultured in algal culture conditions, as

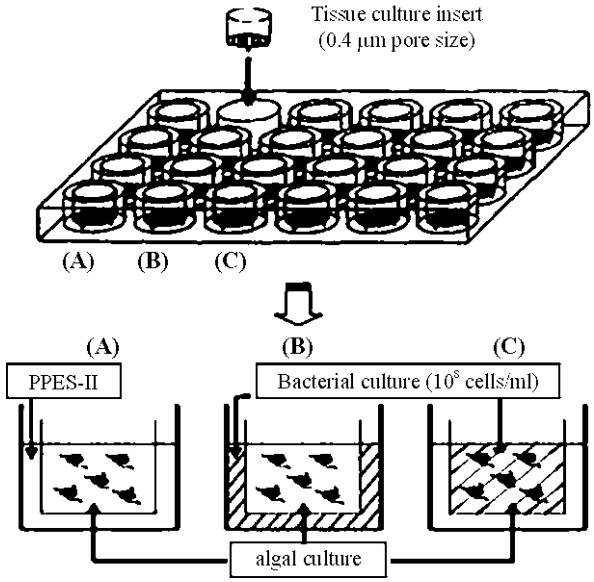

Fig. 1. Experimental procedure for determination of algicidal mode of algicidal bacteria, using commercially available tissue culture inserts (0.4 μm pore size) (Kim *et al*., 1999). One milliliter of algal culture was inoculated into each tissue culture insert of the 24-well plates. (A) f/2 media was added between the well and the tissue culture insert, (B) bacterial culture was added between the well and the tissue culture insert, and (C) bacterial culture was added to the inside of the tissue culture insert.

described above.

Impact and algicidal activity of bacterial AB-4 strain culture supernatants on *Chattonella marina* cultures

After cultivation on PPES-II, cultures (10^8 cells/ml) were centrifuged at 5,000×g for 10 min; the supernatants were then filtered through 0.2-μm Millipore membranes. The algicidal activity of the bacterial culture supernatants on *Chattonella marina* (1.0×10^4 cells/ml) was tested using 24-well plates. Each well contained 1 ml of algal culture, to which 10 μl, 50 μl, 100 μl, 500 μl, or 1,000 μl (v/v) of culture supernatants were added. The plates were monitored at a magnification of ×100, depending on the size of the organism being tested. Several transects of each well were inspected, and the condition of the algae noted. The plates were inspected every 2 h for the first 7 h, and then less frequently over the next two days.

The algicidal activity of isolate AB-4 was calculated, using the following equation (Kim *et al*., 2007): Algicidal activity (%) = (1-Tt/Ct) × 100. Tt (treatment) and Ct (control) are the cell concentrations of *Chattonella marina* with culture supernatants of AB-4 strain, and sterile PPESII broth, respectively, after inoculation time (t). A suitable volume of the culture supernatants was inoculated to the cultures of *Chattonella marina* in the treatment. In the case of two controls, equal volumes of f/2-Si medium without algae and sterile PPES-II broth were added to the *Chattonella marina* cultures in the treatment instead of bacterial culture supernatants.

Algicidal range of bacterial AB-4 strain culture supernatants

The AB-4 strain culture filtrate was prepared as described above. The algicidal range of the culture filtrate against other HAB species and livefood organisms was investigated using the following species: *A. sanguinea*, *A. catenella*, *C. marina*, *C. ellipsoidea*, *C. vulgaris*, *C. polykrikoides*, *F. japonica*, *G. impudicum*, *H. akashiwo*, *I. galbana*, *N. oculata*, *N. elegans*, *N. pungens*, *O. angustissima*, *P. gyrans*, *P. tricornutum*, *P. micans*, *P. minimum*, *S. trochoidea*, *S. costatum*, and *T. suecica*. To assess the algicidal effect on HAB species and livefood organisms, a 5% culture filtrate was added to each species at the mid-exponential growth phase (i.e., 1.0×10^4 cells/ml). The test flasks were cultivated in algal culture conditions, as described above, for 12 h. Algicidal activity was estimated using the aforementioned equation.

Stability of algicidal activity

To investigate the effect of heat treatment on algicidal activity, filtrates from the bacterial cultures grown in PPES-II broth at 30°C were incubated in a water bath at 4, 10, 20, 30, 40, 50, 70, and 100°C for 30 min. In addition, to investigate the effect of pH, the filtrates were suspended in 0.1 M citrate phosphate buffer with a pH range of 3 to 7 and

Table 1. Biochemical characteristics of *Bacillus* sp. AB-4

Characteristics	AB-4 strain
KIA (Kligler's iron agar) test	K/K
O/F (Oxidation/Fermentation) test	+/–
Production of:	
Indole	–
MR (Methyl red)	–
VP (Voges-Proskauer)	–
Citrate	+
Catalase	+
Oxidase	+
L-lysine decarboxylase	+
L-ornithine decarboxylase	+
L-arginine dehydrolas	+
Utilization of:	
Glucose	+
D-Xylose	–
D-Fructose	+
L-Rhamnose	+
D-Galactose	+
Inositol	–
D-Mannose	+
Mannitol	+
Sucrose	+
Lactose	–
L-Arabinose	–
Adonitol	+
Maltose	+
Salicin	+
Sorbitol	+

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

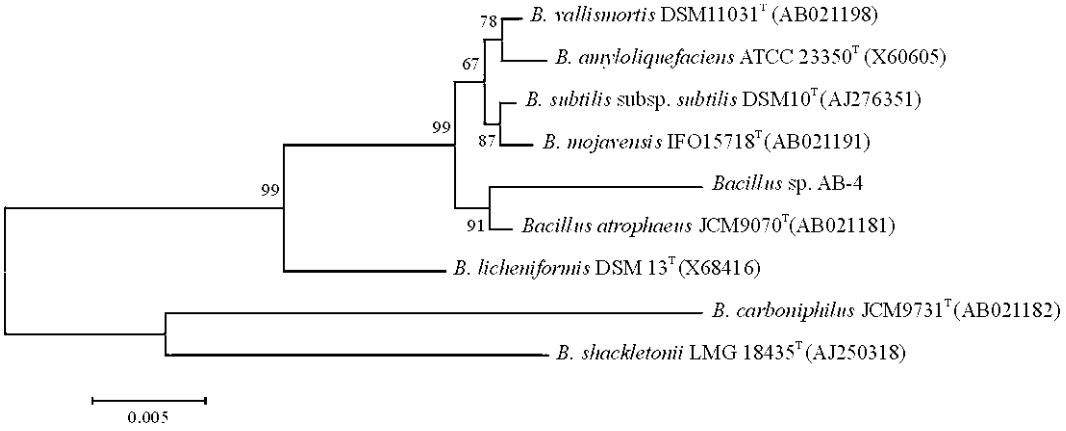

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of the isolated AB-4 strain and closely related members in the genus *Bacillus*. Numbers at nodes are levels of bootstrap support (%), based on neighbor-joining analyses of 1,000 resampled datasets. Bar, 0.005 nucleotide substitution per position.

0.1 M Tris-HCl buffer with a pH range of 8 to 10; in both cases, they were kept in the buffer for 30 min. The treated filtrates (10%, v/v) were subsequently inoculated into *Chattonella marina* cultures (1.0×10^4 cells/ml), to test for algicidal activity. PPES-II broth subjected to the same treatments was added to *Chattonella marina* cultures as a control.

Results

Screening of algicidal bacteria

A total of 350 bacterial strains were isolated; six isolates showed algicidal activity against *Chattonella marina*. The algicidal activities of strains AB-1, 2, 3, 4, 5, and 6 were 90%, 87%, 84%, 97%, 75%, and 78%, respectively. Among these six isolates, AB-4 exhibited the strongest algicidal activity against *Chattonella marina*.

Characterization and identification of AB-4 strain

To identify the AB-4 strain, morphological, biochemical, and genetic analyses were performed. The algicidal bacterium AB-4 was Gram-positive, rod-shaped, and non-pigmented in a PPES-II agar plate. The optimal conditions for growth of the AB-4 strain were 30°C, pH 7.0, and salinity 20‰. The AB-4 strain did not grow at temperatures <10°C or >50°C, or at a pH<4 or >10 (data not shown). The results of the biochemical tests are listed in Table 1.

To genetically characterize the AB-4 strain, a PCR was

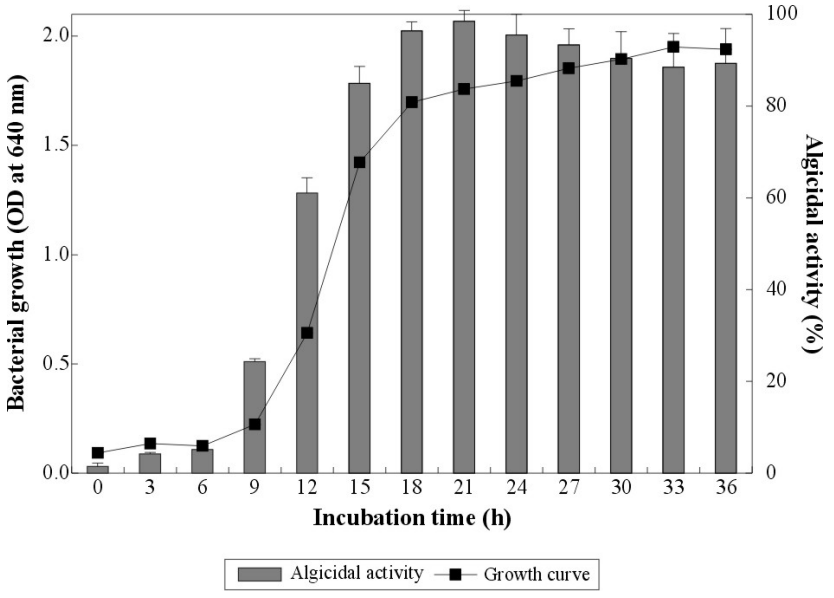

Fig. 3. Growth curve of *Bacillus* sp. AB-4 at optimal culture conditions (i.e., 30°C, pH 7, 2% NaCl, 200 rpm) and algicidal activity by the culture filtrate of each growth phase of *Bacillus* sp. AB-4 against *Chattonella marina*. (–■–), bacterial growth curve (OD at 640 nm). Bar graph, algicidal activity (control=algal cultures with PPES-II broth added).

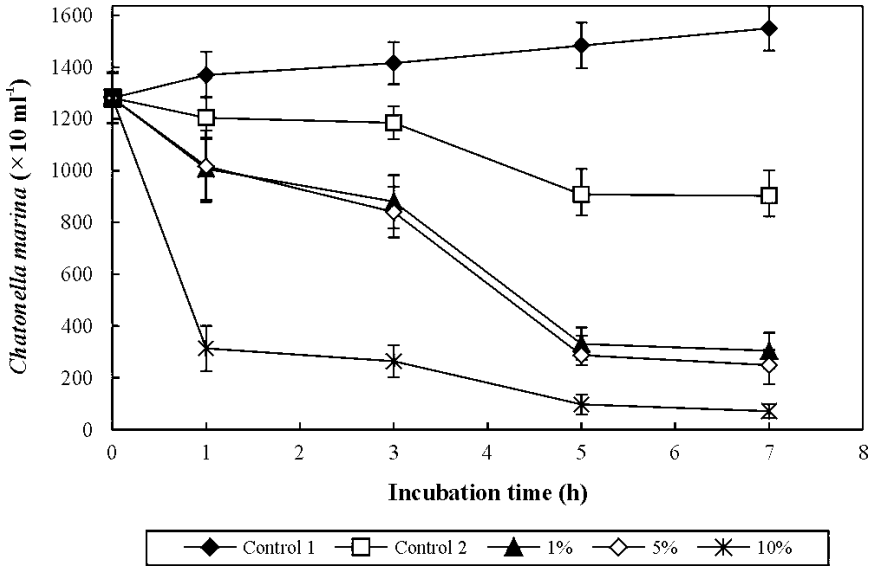

Fig. 4. Algicidal activity of the culture filtrate of *Bacillus* sp. AB-4 against *Chattonella marina*, at various concentrations [(◆) control 1; (□) control 2; (▲) 1% (10 µl); (◇) 5% (50 µl); (×) 10% (100 µl)]. Control 1=algal cultures with f/2-Si broth added. Control 2=algal cultures with PPES-II broth added. Data are expressed as Mean±SD, from ten-times assays.

carried out to amplify 16S rDNA, as described above. The 16S rDNA sequences (1,484 bases) of the AB-4 strain were aligned through comparison with available sequences from the GenBank database. The sequences of the AB-4 strain shared the greatest identity with those of *Bacillus atrophaeus* JCM 9070^T (AB021181) (99.3% identity; data not shown). A phylogenetic tree based on bacterial 16S rDNA sequences showed close relationships between AB-4 and the genus *Bacillus* (Fig. 2). The isolated strain was identified as *Bacillus* sp. via culture morphology, biochemical reactions, and homology research based on 16S rDNA.

Algicidal activity of *Bacillus* sp. AB-4 against *Chattonella marina*

Relationship of algicidal activity and bacterial growth

The growth curve and algicidal activity of the AB-4 strain were inspected every 3 h, over a 36 h period (Fig. 3). It seemed that the algicidal activity of the AB-4 strain was bacterial growth-dependent, since the strongest algicidal activity occurred in early stationary-phase cultures, in treatments involving the addition of a bacterial culture filtrate.

Determination of mode of algicidal action

Our results using commercially available tissue culture inserts revealed that *Bacillus* sp. AB-4 killed *Chattonella marina* through algicide release, as an indirect attacker. Each (A), (B), and (C) well showed negative, positive, and positive, respectively, where a negative result means there was no algicidal activity and a positive result means there were signs of algicidal activity.

Impact of bacterial culture supernatant on *Chattonella marina* cultures

The experiment testing for different concentrations (1, 5, 10, 50, and 100%) of culture supernatant additions showed various levels of algicidal activity on the *Chattonella marina* culture (Fig. 4). *Chattonella marina* was killed by high-concentration rather than low-concentration additions. A concentration of <5% showed little algicidal activity against *Chattonella marina*; concentrations of 10, 50, and 100% exhibited algicidal activities of 95.4%, 99.9%, and 100%, respectively, after a 7 h incubation period. Algicidal effects were observed in the control 2 wells, which involved 50 and 100% PPES-II broth; consequently, at a high concentration (i.e., >50%), results were not significant. Therefore, results

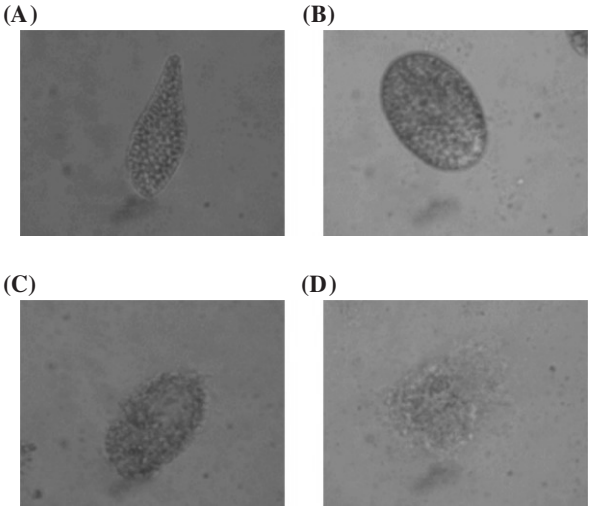

Fig. 5. Light microscopic observation of *Chattonella marina*: (A) normal cell; (B) cell walls becoming swollen after 1 h; (C) cell membrane rounded or disrupted after 7 h; (D) release of cellular components and destruction of cell contents after 48 h.

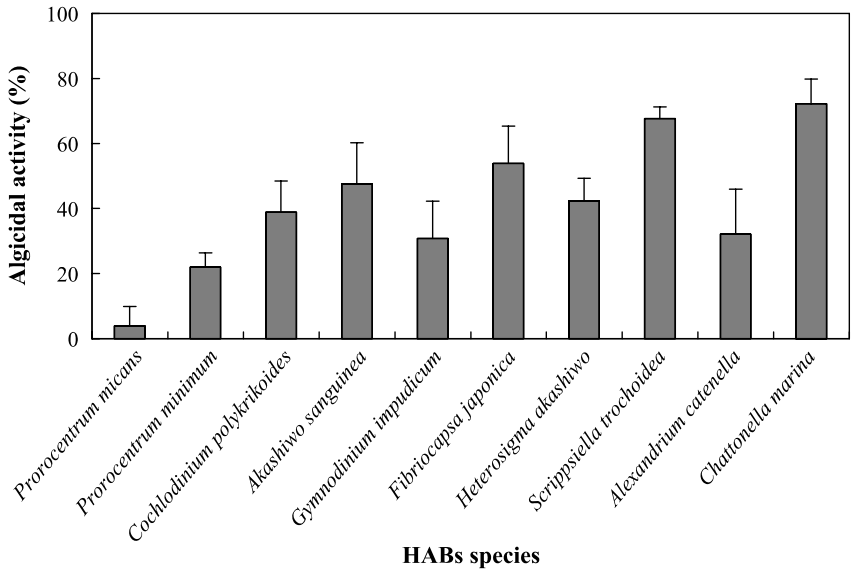

Fig. 6. The algicidal range of *Bacillus* sp. AB-4 against other HAB species. The 5% culture filtrate was added to each algal culture at the mid-exponential growth phase. Algicidal activity was evaluated after 12 h. Control=each algal culture with sterile PPES-II broth added. Data are expressed as Mean±SD, from triplicate assays.

with >50% concentration are not shown, and in control 2, *Chattonella marina* cells were added to PPES-II broth in a 10% concentration (Fig. 4). Amongst changes in algicidal activity by different culture filtrate volume, the 10% (100 μl added) concentration showed the biggest change in algicidal activity after 1 h. Although all of the cells of *Chattonella marina* remained motile, their speed of motility had decreased markedly within 3 h, with a low concentration of culture supernatant (i.e., 5% added).

Table 2. Algicidal effect of *Bacillus* sp. AB-4 on the growth of Bacillariophyceae and livefood organisms (data are expressed as Mean±SD, from triplicate assays)

Species	Algicidal activity (%)
Bacillariophyceae	
Navicula elegans	28.6±12.4
Nitzschia pungens	21.6±14.4
Skeletonema costatum	4.1±23.7
Paeodactylum tricornutum	–
Chlorophyceae	
Chlorella ellipsoidea	–
Chlorella vulgaris	–
Nannochloris oculata	–
Cyanphyceae	
Oscillatoria angustissima	–
Haptophyceae	
Isocrysis galbana	20.0±4.0
Pavlova gyrans	–
Prasinophyceae	
Tetraselmis suecica	–

– , negative result (i.e., no algicidal activity)

The algicidal effect of *Bacillus* sp. AB-4 against *Chattonella marina* cells during the algicidal process was observed using light microscopy (Fig. 5). When the algicidal bacterium took effect, the swimming movements of the *Chattonella marina* cells became inhibited and the cell walls swollen, both within 1 h (Fig. 5B). With longer exposure time (i.e., 7 h), *Chattonella marina* cells became rounded or disrupted (Fig. 5C). Subsequently, cellular components also lost their integrity and decomposed, resulting in the appearance of abundant broken cellular components after 48 h (Fig. 5D).

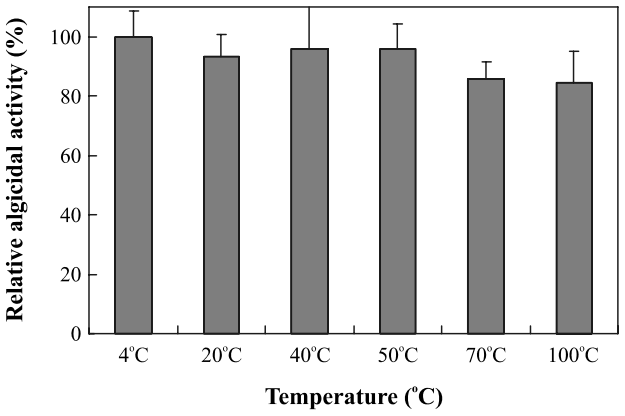

Fig. 7. Thermal stability of culture filtrates of *Bacillus* sp. AB-4. For thermal stability, the extract was incubated at an indicated temperature (4, 20, 40, 50, 70, or 100°C) for 30 min. The relative algicidal activity was estimated for relative data, when the algicidal activity at 4°C was defined as 100%. Data are expressed as Mean±SD, from triplicate assays. $P>0.05$. Heat treatment and algicidal activity are not significantly different (one-way ANOVA on SPSS).

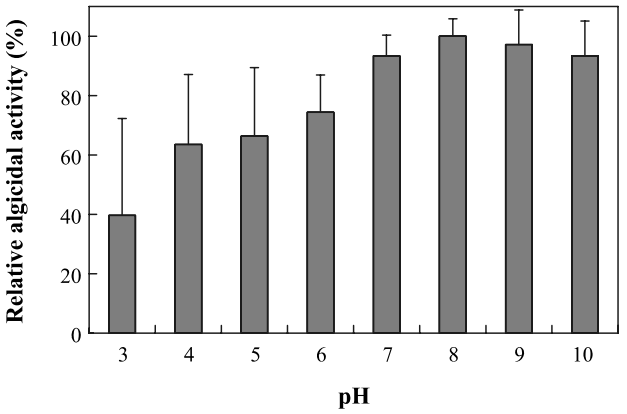

Fig. 8. pH stability of culture filtrates of *Bacillus* sp. AB-4. For pH stability, the extract was suspended in 0.1 M citrate phosphate buffer with a pH range of 3 to 7 and 0.1 M Tris-HCl buffer with a pH range of 8 to 10; it was then kept in each buffer for 30 min. The relative algicidal activity was estimated for relative data, when the algicidal activity at pH 8.0 was defined as 100%. $P<0.01$ (one-way ANOVA on SPSS).

Algicidal range of *Bacillus* sp. AB-4 culture supernatants

The algicidal range of the culture filtrate against other HAB species and livefood organisms is presented in Fig. 6 and Table 2. The algicidal activities against dinophyceae and raphidophyceae were as follows: *A. catenella* (32.2%), *P. micans* (3.8%), *P. minimum* (22.0%), *C. polykrikoides* (38.9%), *A. sanguinea* (47.6%), *G. impudicum* (30.8%), *F. japonica* (53.8%), *H. akashiwo* (42.4%), *S. trochoidea* (67.7%), and *C. marina* (72.3%). *Bacillus* sp. AB-4 showed particularly strong activity against *A. sanguinea*, *F. japonica*, *H. akashiwo*, and *S. trochoidea*. However, strong inhibitory effects were not found *vis-à-vis* the growth of bacillariophyceae or livefood organisms. *N. pungens*- and *S. costatum*-causing HABs and *N. elegans* showed weak algicidal activity (21.6, 4.1, and 28.6%, respectively). No species of tested livefood organism showed algicidal activity, except *I. galbana*.

Stability of algicidal activity

The stability of algicidal activity following heat treatments was tested. All filtrates after incubation at 4, 10, 20, 30, 40, 50, 70, and 100°C for 30 min exhibited algicidal activity, indicating that the algicidal compounds were heat-tolerant (Fig. 7). A loss of algicidal activity occurred when the pH of the filtrates was adjusted to 3, 4, or 5, while algicidal activity continued in filtrates when pH was adjusted to 10 (Fig. 8).

Discussion

In coastal seawater where red tides occur frequently, marine bacteria play an important role in the interactions of phytoplankton, in terms of decreasing or developing algae blooms (Yoshinaga *et al*., 1995). Species of harmful algae harbor an attached bacterial flora, and various free-living bacteria also coexist with algal cultures maintained in the laboratory (Kopp *et al*., 1997; Hold *et al*., 2001). Marine bacteria are considered key biological controllers in the dramatic termination of phytoplankton blooms, as observed in coastal seawaters (Van Rijssel *et al*., 2000; Schoemann *et al*., 2005). Lewitus *et al*. (2003) reported that algicidal bacteria and other microbes that are toxic to or prey upon raphidophytes (e.g., viruses, microzooplankton, and mixotrophic algae) serve as more important factors in phytoplankton bloom termination than nutrient depletion.

Based on several reviews, about 50% of algicidal strains belong to the CFB group, while about 45% are members of *γ-Proteobacteria*; the remaining strains represent the Gram-positive genera *Micrococcus*, *Bacillus*, and *Planomicrobium* (Fukuyo *et al*., 2002; Mayali and Azam, 2004; Hare *et al*., 2005). Other researchers have observed that the phylogenetic diversity of bacteria associated with *Chattonella* spp. was limited to *Cellulophage*, *Zobellia*, *Planomicrobium*, *Alteromonas*, *Cytophaga*, *Bacillus*, and *Pseudoalteromonas* (Imai *et al*., 1995; Lovejoy *et al*., 1998; Skerratt *et al*., 2002; Jeong *et al*., 2003; Liu *et al*., 2008). A strain closely related to *Bacillus* sp. was unusual, because most algicidal bacteria belong to either the CFB group or the genus *Pseudoalteromonas* (Imai *et al*., 1993, 1995, 2001; Skerratt *et al*., 2002). Recently, Ahn *et al*. (2003) reported that a culture broth of *B. subtilis* completely inhibited the growth of *Microcystis aeruginosa*, a bloom-forming cyanobacterium found in highly eutrophic lakes; additionally, Mu *et al*. (2007) reported that the secreted metabolites of *Bacillus fusiformis* showed algicidal activity against *M. aeruginosa*, *Chlorella*, and *Scenedesmus*. The Gram-positive genera comprised unusual algicidal bacteria (Skerratt *et al*., 2002); moreover, the Gram-positive organisms do not comprise a major group of the water column, but are rather found in deep-sea sediment (Li *et al*., 1999). Liu *et al*. (2008) hypothesize that algicidal bacteria–which are Gram-positive organisms–may act as an important top-down control mechanism; we expect that the algicidal bacterium, *Bacillus* sp. AB-4, can also function as an important controller.

The testing of algicidal activity against *Chattonella marina* was performed with PPES-II, because *Bacillus* sp. AB-4 cannot grow in an f/2-Si medium. Nakashima *et al*. (2006) and Mu *et al*. (2007) reported that algicidal activity increased as bacterial cell density increased. It seemed that the algicidal activity of *Bacillus* sp. AB-4 was also bacterial concentration-dependent, since the strongest algicidal activity occurred in stationary-phase cultures in treatments involving the addition of a bacterial culture filtrate. When the strain entered the logarithmic and stationary phases, its algicidal activity increased; algicidal activity then decreased a little in the stationary phase. From this result, it was deduced that the algicides produced might be secondary metabolites.

Algicidal bacteria kill their prey via one of two main mechanisms: direct or indirect (Mayali and Azam, 2004). *Saprospira* prey upon bacteria as well as algae by attaching to their prey (Sangkhobol and Skerman, 1981; Sakata, 1990; Lewin, 1997). It was noted that *Bacterioidetes* are often particle-associated (Kirchman, 2002); in contrast, algicidal bacteria–such as *Alteromonas* and *Pseudoalteromonas*–are killed by releasing dissolved substances (Mayali and Azam, 2004). Many specimens of *Pseudoalteromonas* produce extracellular

bioactive molecules (Holmström and Kjelleberg, 1999) and release dissolved algicide (Lee *et al*., 2002; Amaro *et al*., 2005). Each well, physically separated by a 0.4-μm pore-size filter insert that allows algicides to diffuse across, was tested to determine mode of algicidal action. Our results, using tissue culture inserts, revealed that *Bacillus* sp. AB-4 killed *Chattonella marina* through algicide release as an indirect attacker. Therefore, we used culture filtrate in experiments, to test algicidal activity; the supernatant showed algicidal activity. Algicidal strains were characterized according to their algicidal mechanism, with approximately 70% showing an indirect mode and the remaining (~30%) requiring direct contact with the target prey. Alternatively, algicidal bacteria exhibiting an indirect killing mechanism release dissolved algicide(s) effective in the absence of physical contact with the target host. Algicidal activity via released algicides clearly provides a competitive benefit to this segment of the microbial community (Doucette, 1995; Mayali and Azam, 2004; Roth *et al*., 2008).

The isolation, purification, and characterization of algicidal compounds are difficult, due to their various characteristics across different species of algicidal bacteria (Doucette and Powell, 1998; Skerratt *et al*., 2002). The compounds–e.g., proteases (Lee *et al*., 2002), peptides (Imamura *et al*., 2000), biosurfactants (Ahn *et al*., 2003; Wang *et al*., 2005), antibiotic-like substances (Dakhama *et al*., 1993), argimicin A (Imamura *et al*., 2000), bacillamide (Jeong *et al*., 2003), or Prodigiocin-like pigments (Jeong *et al*., 2005; Nakashima *et al.*, 2006)–had been reported as algicidal compounds. We had expected *Bacillus* sp. AB-4 to break down *Chattonella marina* cells using algicidal compounds similar to the aforementioned compounds, which are excreted by bacteria, and that they would not require contact with algal target cells. Heat-tolerant algicidal activity suggests that thermally stable algicide(s) produced by *Bacillus* sp. AB-4 could not be enzymatic, since the filtrate, after treatment at 100°C for 30 min, exhibited similar algicidal ability. Algicidal compounds could be either heat-labile (Baker and Herson, 1978) or heat-tolerant (Skerratt *et al*., 2002); some putative algicides are tolerant to autoclaving and are thus unlikely to be enzymes (Skerratt *et al*., 2002).

In general, indirect attacks occur through chemical mediators and are species-specific (Yoshinaga *et al*., 1995, 1997). The strain *Bacillus* sp. AB-4 showed algicidal activity against *Chattonella marina* as an indirect attacker, but this strain did not show species-specificity. This strain showed a wide host range against most HAB species tested. *Bacillus* sp. AB-4 showed comparatively strong activity against *A. sanguinea*, *F. japonica*, *H. akashiwo*, and *S. trochoidea*; however, the strain did not show algicidal effects on the growth of livefood organisms, with the exceptions of *N. elegan* and *I. galbana*. Consequently, the culture filtrate of *Bacillus* sp. AB-4 is toxic against HAB species and nontoxic against livefood organisms. It is useful, that *Bacillus* sp. AB-4 possesses only species-specific algicidal activity.

Therefore, our results show that the algicidal bacterium *Bacillus* sp. AB-4 can function as a red-tide controller. Recent work has been focusing on algicidal bacteria as controllers of water blooms, and so the identification and characterization of the molecules involved and responsible should us help understand the phenomenon within an ecosystem. In future study, we will work to identify released algicides, to understand better how algicidal bacteria kill their prey.

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