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Isolation, identification and characterization of algicidal bacteria against *Stephanodiscus hantzschii* and *Peridinium bipes* for the control of freshwater winter algal blooms

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Abstract Five strains (HYY0510-SK04, HYY0511-SK09, HYK0512-SK12, HYK0512-PK04 and HYY0512-PK05) of algicidal bacteria against the harmful bloom forming diatom *Stephanodiscus hantzschii* and dinoflagellate *Peridinium bipes*, were isolated. Among these strains, HYY0510-SK04, HYY0511-SK09 and HYK0512-SK12 have an effective algicidal activity for *S. hantzschii*, while HYK0512-PK04 and HYY0512-PK05 have an algicidal effect against *P. bipes*. Sequence analysis of 16S rDNA showed that HYY0510-SK04 and HYY0511-SK09 were closely related to *Acidovorax delafieldii* ATCC 17505^T. HYK0512-SK12, HYK0512-PK04 and HYY0512-PK05 showed high homology with *Variovorax paradoxus* IAM 12373^T (98.9%), *Hydrogenophaga palleronii* ATCC 49743^T (98.8%) and *Pseudomonas plecoglossicida* ATCC 700383^T (98.3%), respectively. HYY0510-SK04, HYY0511-SK09 and HYK0512-SK12 degraded *S. hantzschii* cells within two weeks when those bacteria were inoculated at densities of $\geq 10^7$cells mL^{-1} to the lag or logarithmic growth phase of the algal culture. HYK0512-PK04 and HYY0512-PK05 degraded more than 90% of *P. bipes* cells within 14 and 8 days, respectively, when these bacteria were inoculated at densities of $\geq 10^7$cells mL^{-1}. Among the five bacterial strains, HYK0512-SK12 and HYY0512-PK05 showed the most effective growth inhibition of all the algae and cyanobacteria tested. Biochemical assays revealed that the main algicidal substance from all isolates were likely to be extracellular substances. These results indicate that the bacterial strains isolated for this study are potential agents for the control of harmful algal blooms in eutrophic reservoirs.

Keywords Algicidal bacteria · Algal bloom · Diatom · Dinoflagellate · *Peridinium bipes* · *Stephanodiscus hantzschii*

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Introduction

In Korea, blooms of the small centric diatom *Stephanodiscus hantzschii* Grunow and the armored dinoflagellate *Peridinium bipes* Stein are regularly found every year in reservoirs and lakes during periods of low temperature (Kang and Kim 1991, Kang et al. 2005; Cho et al. 1998; Han et al. 2002; Ha et al. 2002; Kim et al. 2002; Hong et al. 2002a, b; Ki and Han 2005). The *Stephanodiscus* blooms can result in a significantly negative impact on the drinking water supply (Lee et al. 2001; Kolmakov et al. 2002; Ha et al. 2002) and expensive problems at water treatment plants (Lim et al. 2000; Lee et al. 2001). The *Peridinium* blooms also create such problems as nuisance odors in drinking water and

economically serious problems for agricultural industries (Kawavata and Hirano 1995; Ki and Han 2005).

Since these blooms usually occur during the winter, abundances of zooplankton or protists were low as compared to those in summer. Therefore, these organisms may not be suitable for use as the biological agents. In contrast, algicidal bacteria, which can have no connection with fluctuations in water temperature, may represent an important strategy (as a biological constrain) for the management of *Stephanodiscus* and *Peridinium* blooms at low temperatures. Indeed, a previously isolated algicidal bacterial strain, *Pseudomonas putida* HYK0203-SK02, showed activity against *S. hantzschii* blooms during the winter (Kang et al. 2005). *Pseudomonas putida* HYK0203-SK02 was not sufficiently stable for controlling *S. hantzschii* blooms under realistic environment exposure and these bacteria showed algicidal activity even at low water temperature (Kang et al. 2005, 2007). Hence, bacteria are superior to zooplankton or protists as biological agents for controlling these harmful algal blooms. It is, however, still very rare to isolate algicidal bacteria against the diatom *S. hantzschii* (Kang et al. 2005) as well as the dinoflagellate *P. bipes*.

In this study, we examined (1) the isolation and identification of various algicidal bacteria, (2) the algicidal activities of isolated bacteria compared to those of laboratory-generated algal blooms of the diatom *S. hantzschii* and dinoflagellate *P. bipes*, (3) the responses of important algae and cyanobacteria to the bacteria, and (4) the algicidal mode and the cellular localization of relevant algicidal substance.

Materials and methods

The diatom *Stephanodiscus hantzschii* UTCC267, obtained from the University of Toronto Culture Collection of Algae and Cyanobacteria (UTCC) in Canada, and the dinoflagellate *Peridinium bipes* HYJA0311-02, isolated from a water sample collected in the main channel of the Juam River, South Korea by S.-Y. Cho (Ki and Han 2005), were cultivated in DM medium (Beakes et al. 1988) under white fluorescent light (50 μmol photons m^{-2} s^{-1}) using a light period of 12 h at 15°C.

Isolation of algicidal bacteria against host algae

Samples were obtained from 55 sites including eutrophic lakes, reservoirs and streams, where *S. hantzschii* or *P. bipes* blooms frequently occur, from September to December of 2005. These samples were subsequently filtered through a pre-sterilized glass fiber filter (GF/F; Whatman).

Stephanodiscus hantzschii-killing bacteria were isolated using a modified soft agar over-layer technique as described by Kang et al. (2005). *Stephanodiscus hantzschii* cultures grown for three weeks were harvested by centrifugation at 7500×g for 15 min, and were mixed with 20 mL of molten DM soft agar (0.8% agar) equilibrated to 50°C. The mixture was immediately poured onto DM bottom agar (1.5% agar). After the agar had solidified for 2 days, the plates were incubated for 10 days under the diatom growth conditions described above. The surface water filtrates (200 μL) were spread on the algal lawn, and the plates were incubated for 10 days under algal growth conditions. Bacterial colonies that produced clear zones on lawns were picked, purified as described previously (Yamamoto and Suzuki 1990), and maintained on modified casitone-yeast media (MCM) agar plates containing 1.5% agar at 20°C and pH 7, or cryopreserved at −76°C in MCM medium containing 25% glycerol.

Dinoflagellate *Peridinium bipes*-killing bacteria were isolated with a modified MPN method according to Imai et al. (1998). One milliliter of *P. bipes* (1500 cells mL^{-1}), cultured for 2 days, was inoculated into each well of 24-well microplates (Falcon, USA). Next, each well in the first group received 1 mL of water sample. Each well in groups two through ten received 1 mL of a specific dilution of the sample. The dilutions, from 10^{-1} through 10^{-4}, were added to groups two through ten, respectively. The inoculated microplates were incubated for at least three weeks under the conditions described above. Microplates inoculated only with autoclaved DM to *P. bipes* were used as a control. From wells in which more than 99% of the algal cells were killed, bacteria were isolated by serial streaking onto MCM agar plates. The isolated bacteria were maintained as described above.

Characterization and identification of algicidal bacteria

Morphological and physiological features of the isolated bacteria were described on the basis of Gram stain, handling drop, optimum pH, temperature for growth, cell shape, as well as the results of API 20NE and API ZYM (bioMeÂrieux, Boulogne, France). API 20NE data was compared to those in the bioMeÂrieux database (bioMeÂrieux 1990). All tests were performed in duplicate, and negative controls were obtained by using fresh medium. Their characteristics are summarized in Table 1.

The chromosomal DNA of isolates HYY0510-SK04, HYY0511-SK09, HYK0512-SK12, HYK0512-PK04 and HYY0512-PK05 was isolated using the method described by Hong et al. (2002a, b). The amplification of the 16S rDNA was conducted using two primers: 27F, 5′-AGAGT TTGATCATGGCTCAG-3′ and 1492R, 5′-GGTTAC CTTGTTACGACTT-3′. The PCR was run for 35 cycles in a DNA thermocycler (iCycler, Bio-Rad, USA), employing

Table 1 Morphophysiological characteristics of algicidal bacteria by API NE and API ZYM tests

Characteristics and Enzymes	HYY0510-SK04	HYY0511-SK09	HYK0512-SK12	HYK0512-PK04	HYY0512-PK05
Reduction of nitrates to nitrites	+	+	–	–	+
Indole production	–	–	–	+	–
D-glucose (fermentation)	–	–	+	–	–
Arginine dihydrolase	–	–	–	–	+
Urease	–	–	–	–	–
Hydrolysis (β-glucosidase)	–	–	–	–	–
Hydrolysis (protease)	–	–	–	–	+
β-galactosidase	–	–	–	–	–
Assimilation glucosidase	–	–	–	–	+
Assimilation arabinose	W[a]	W	–	–	–
Assimilation mannose	W	W	–	–	+
Assimilation mannitol	W	W	–	–	–
Assimilation *N*-acetyl-glucosamine	W	W	–	–	+
Assimilation maltose	–	–	–	–	–
Assimilation gluconate	–	–	–	–	+
Assimilation caprate	W	W	–	–	+
Assimilation adipate	–	–	–	–	–
Assimilation malate	–	–	–	–	+
Assimilation citrate	W	W	–	–	+
Assimilation phenyl-acetate	–	–	–	–	–
Oxidase	–	–	–	+	–
Alkaline phosphatase	+	+	+	+	+
Esterase	+	+	+	+	+
Esterase lipase	+	+	+	+	+
Lipase	+	+	+	–	+
Leucine arylamidase	+	+	+	+	+
Valine arylamidase	–	–	+	–	+
Crystine arylamidase	–	–	+	–	–
Trypsin	–	–	–	–	+
α-chymotrypsin	–	–	–	–	–
Acid phospatase	+	+	+	+	+
Naphtol-AS-BI-phosphohydrolase	+	+	+	+	+
α-galactosidase	–	–	–	–	–
β-galactosidase	–	–	–	–	–
β-glucuronidase	–	–	+	–	–
α-glucosidase	–	–	+	–	–
β-glucosidase	–	–	+	–	–
N-acetyl-β-glucosaminidase	–	–	–	–	–
α-mannosidase	–	–	–	–	–
α-fucosidase	–	–	–	–	–

[a] W, weak reaction; +, positive reaction; −, negative reaction

the thermal profile according to Yoon et al. (1997). Identification of isolated bacteria was obtained by comparing the full-length sequence of the bacteria with a collection of 16S rDNA obtained from the EMBL/DDBJ/Genbank database. The resulting 16S rDNA sequences were aligned and distance matrices were calculated using CLUSTAL X software (Thompson et al. 1997). A phylogenetic tree was constructed using the neighbor-joining method (N-J plot program, Saitou and Nei 1987), based on the calculated distance matrix.

The genomic sequences for HYY0510-SK04, HYY0511-SK09, HYK0512-SK12, HYK0512-PK04 and HYY0512-PK05 were registered into the GenBank database under accession numbers AB269773, AB269774, AB269775, AB269772, and AB269776.

Effect of media on algicidal activity

Algicidal bacteria were incubated with three different media types, including nutrient broth (NB; beef extract

0.3% and peptone 0.5%), peptone yeast extract (PYE; bacteriological peptone 0.2% and yeast extract 0.2%) and modified casitone-yeast medium (MCM; casitone 1%, yeast extract 0.1% and $MgSO_4 \cdot 7H_2O$ 0.1%.).

Each bacterium was harvested by centrifugation at 7500 g for 15 min (TX-160, TOMY), adjusted to an OD_{660} of 1.2 with sterilized DM to remove nonspecific effect of bacterial culture media itself, yielding an initial density of about 1×10^9 cells mL^{-1}, of which 1 mL was inoculated into 100 mL of *S. hantzschii* (1.3×10^6 cells mL^{-1}) and *P. bipes* (2×10^3 cells mL^{-1}) cultures, respectively. Flasks containing the same amount of *S. hantzschii* or *P. bipes* alone served as controls for this experiment. *S. hantzschii* fixed with glutaraldehyde at 1% final concentration and *P. bipes* fixed with 1% Lugol's solution were counted by use of a hemocytometer and a Sedgwick-Rafter chamber, respectively.

Relationship between bacterial cell density and algicidal activity

The bacteria were cultured at 25°C in MCM with orbital shaking at 150 rpm in the dark for 5 days. Bacterial cells had been harvested by centrifugation at 7500 g for 15 min at 4°C. The prepared bacterial pellet was serially diluted with DM to initial densities of 10^7, 10^8, 10^9 and 10^{10} cells mL^{-1}. Next, 1 mL of bacterial suspension was mixed for about 30 days with 100 mL cultures containing an initial cell density of host algae [*S. hantzschii* (1.3×10^6 cells mL^{-1}) and *P. bipes* (3×10^3 cells mL^{-1})] in mid-exponential phase. Algae were counted daily as described above.

The effect of algal growth phases on the algicidal activity

The algicidal activities of bacteria during different growth phases of algae were studied. The bacterial culture was prepared as described above. One milliliter of a bacterial suspension of 10^9 cells mL^{-1} adjusted with sterilized DM was inoculated into 250 mL flasks containing 100 mL of lag [*S. hantzschii* (3×10^5 cells mL^{-1}) and *P. bipes* (5×10^2 cells mL^{-1})], exponential [*S. hantzschii* (1.6×10^6 cells mL^{-1}) and *P. bipes* (3×10^3 cells mL^{-1})] or stationary phases [*S. hantzschii* (2.6×10^6 cells mL^{-1}) and *P. bipes* (4.2×10^3 cells mL^{-1})] of algae. *Stephanodiscus hantzschii* and *P. bipes* were counted daily for a period of 25 and 49 days, respectively, as described above.

The relative sensitivity of algae to algicidal bacteria

The responses of 13 important algae and cyanobacteria to algicidal bacteria was examined: cyanobacteria (*Microcystis aeruginosa* NIER 10001, *M. aeruginosa* NIES 101, *M. aeruginosa* NIES 44, *M. viridis* NIES 102, *Anabaena macrospora*, *A. cylindrica* NIES 19, *A. flos-aquae* AG 10064); diatoms (*Cyclotella meneghiniana* HYK0210-A1, *Stephanodiscus* sp. HYK0210-A2, *Stephanodiscus hantzschii* UTCC 267, *Aulacoseira granulata* CCAP 1002-1); Chlorophyta (*Coelastrum astroideum* NIER 10088); and dinoflagellate (*Peridinium bipes* HYTG0601-01) (Table 2). The cyanobacteria and chlorophyte were cultured in Cyanobacteria Medium (CBM; Watanabe et al. 2000) at 25°C, pH 9, under 50 μmol photons m^{-2} s^{-1} with a 12:12 light:dark cycle. The diatoms and dinoflagellates were cultured in DM medium at 15°C and 20°C, respectively. Bacterial culture was prepared as described above. Bacteria were adjusted to a final concentration of 10^7 cells mL^{-1}. Algae were counted daily for a period of 10 days, as described above.

Preparation of cell extracts and culture media

Cell extracts were separated according to the method of Niviere et al. (1986). For the preparation of cell extract, cells were incubated in MCM for 5 days at 25°C and pH 7, then harvested by centrifugation at 7500 g for 30 min. The supernatant was filtered through a 0.2 μm syringe filter and then used as bacterial culture medium. The bacterial pellet was washed twice with phosphate buffer (pH 7) and suspended in 10 mL of 10 mM phosphate buffer (pH 7). The bacterial cells were sonicated at 50A for 10 min at 5°C, and the cell debris was removed by centrifugation at 30,000 g for 40 min. The supernatant was used as a cell extract.

Analysis of algicidal activity

The protein content of cell extracts and culture media was quantified according to the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as the protein standard. A 16 mL culture of host algae (*S. hantzschii* or *P. bipes*) was mixed with 4 mL of cell extracts and spent media (20% v/v) in 50 mL test tubes, and the cells were enumerated after 24 h. A unit of algicidal activity was defined as the amount of substrate that reduced 10^4 cells of *S. hantzschii* and 10^2 cells of *P. bipes* after 24 h, respectively.

Results

In an effort to identify useful indigenous algicidal bacteria, the algal lawn and MPN methods were used to identify 224 bacteria in relevant water and sediment samples. Of these,

five isolates showed algicidal activity against *Stephanodiscus hantzschii* or/and *Peridinium bipes*. The *Stephanodiscus*-killing bacteria included strains HYY0510-SK04, HYY0511-SK09 and HYK0512-SK12 and *Peridinium*-killing bacteria included HYK0512-PK04 and HYY0512-PK05 that exhibited the strongest algicidal activity and were used for further analysis.

All isolates were Gram-negative rods and their optimum pH for growing was pH 7, while temperature optimums ranged from 15 to 30°C (data not shown). The examined strains were classified by similarity of morphophysiological and enzymatic characteristics (Table 1). At a level of similarity ≥80%, the *Peridinium*-killing bacterium HYK0512-PK04 was distinguished, while the other isolates could not be identified with the API 20NE system; on the basis of their numerical profile, HYK0512-PK04 were homologous with *Pasteurella multocida* (95.7%) (API bioMeÂrieux database). In addition, the API ZYM profile of this isolate was almost identical to *P. multocida* strains AY683485 through AY683530. Since there is no information obtained from the identical performance of the API 20NE system about any strains except HYK0512-PK04, the five bacterial isolates were identified using 16S rDNA gene sequencing as a rapid and useful technique. 16S rDNA gene sequencing based on the 97% similarity criterion (Stackebrandt and Goebel 1994) revealed that strain HYY0512-PK05 was matched to *Pseudomoas plecoglossicida* ATCC 700383^T (98.3%) (Fig. 1d) with the highest homology in the GenBank. The bacterial isolates HYY0510-SK04 and HYY0511-SK09 were 98.5% and 98.8% similar to *Acidovorax delafieldii* ATCC 17505^T (Fig. 1a), respectively. Analogously, strains HYK0512-SK12 and HYK0512-PK04 were virtually identical with *Variovorax paradoxus* IAM 12373^T (similarity; 98.9%) (Fig. 1b) and *Hydrogenophaga palleronii* ATCC 49743^T (98.8%) (Fig. 1c), respectively. The identification of HYK0512-PK04 differed between the two methods used above.

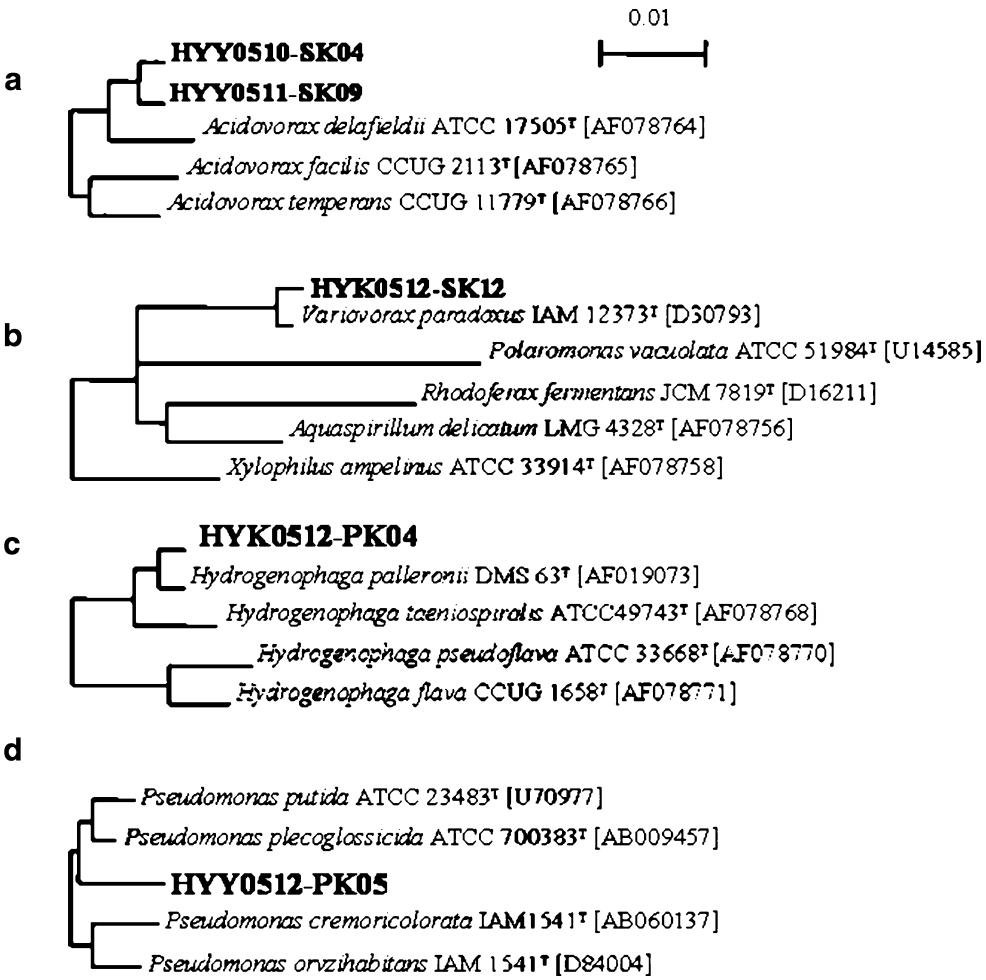

Fig. 1 Phylogenetic trees based on 16S rDNA sequences showing the positions of the five algicidal bacteria, the type strains of algal species, and representatives of some other related taxa. The scale bar represents 0.01 substitutions per nucleotide position

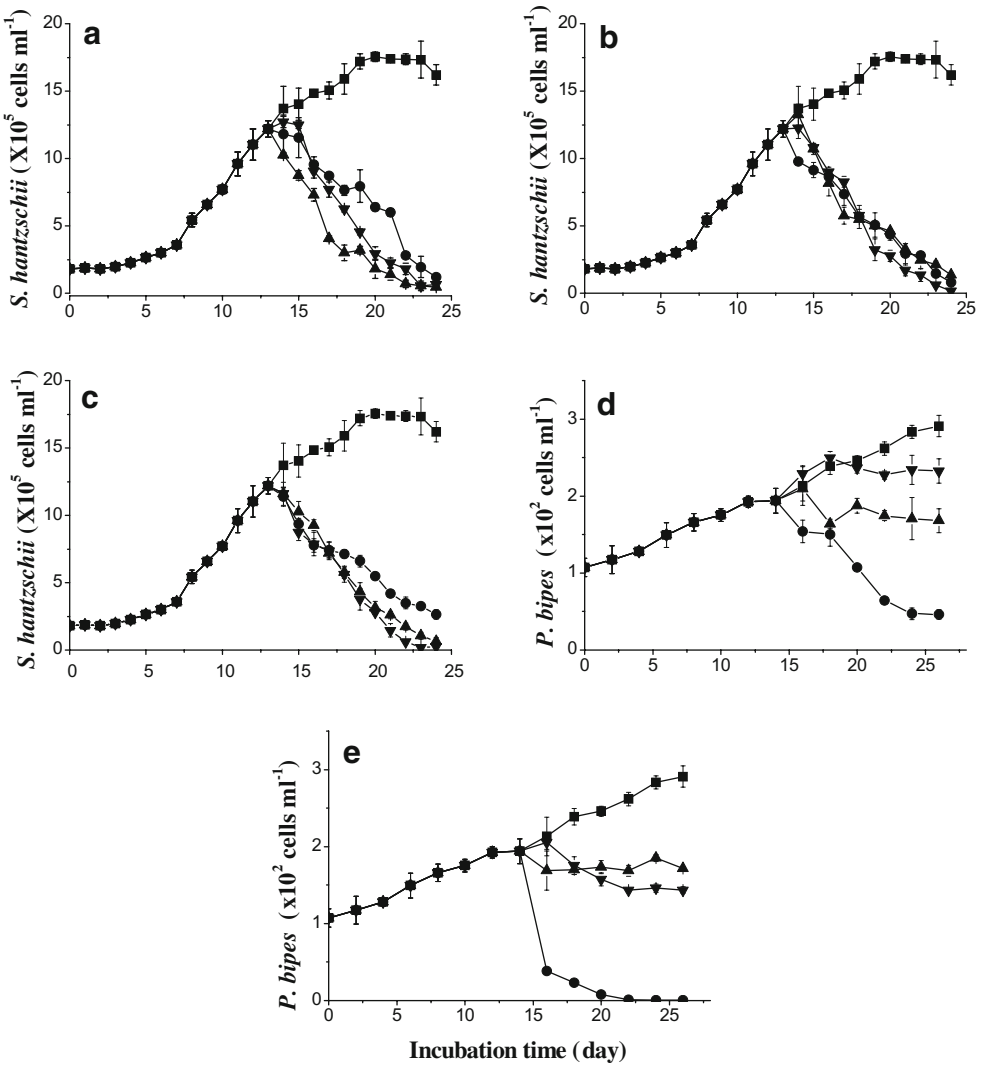

Fig. 2 Growth of *S. hantzschii* in cultures inoculated with (**a**) HYY0510-SK04, (**b**) HYY0511-SK09 and (**c**) HYK0512-SK12 and growth of *P.bipes* in cultures inoculated with (**d**) HYK0512-PK04 and (**e**) HYY0512-PK05, respectively, incubated in different bacterial media as (■: control, ●: PYE, ▲: NB and ▼: MCM)

Algicidal effects of bacteria on Stephanodiscus hantzschii and Peridinium bipes

All bacteria tested showed very similar growth pattern and rate in among PYE, NB, and MCM media (data not shown). The algicidal activities of *Stephanodiscus*-killing bacteria (HYY0510-SK04, HYY0511-SK09 and HYK0512-SK12) cultured in different bacterial media (MCM, PYE, and NB) did not show a marked difference but did show effective algicidal activity of similarly decreasing patterns (Fig. 2a, b, c). There are, however, marked contrasts among the three different bacterial media in the case of the *Peridinium*-killing bacteria (HYK0512-PK04 and HYY0512-PK05). *Peridinium*-killing bacteria cultured in MCM showed the strongest algicidal activity, while algicidal activities of *Peridinium*-killing bacteria incubated in PYE or NB medium did not show a significant disparity (Fig. 2d, e).

Stephanodiscus-killing bacteria (HYY0510-SK04, HYY0511-SK09 and HYK0512-SK12) showed algicidal activity. More than 90% of *S. hantzschii* cells were killed by *Stephanodiscus*-killing bacteria within 14 days of inoculation with bacteria of a final concentration of $\geq 10^7$ cells mL^{-1}, although the concentrations below $\leq 10^6$ cells mL^{-1} showed mild algicidal activity during the experimental period (Fig. 3a, b, c). *Peridinium*-killing bacteria also showed algicidal activity, although the algicidal activity of HYY0512-PK05 was much higher than that of HYK0512-PK04 (Fig. 3d, e).

Algicidal activity of bacteria on algal growth phase

Stephanodiscus-killing bacteria (HYY0510-SK04, HYY0511-SK09 and HYK0512-SK12) exhibited effective algicidal activity against *S. hantzschii* during lag or log diatom growth phases (Fig. 4a, b, c). The responses of algal cultures from different growth phases to bacteria could be ranked in the following order of sensitivity: logarithmic ≥ lag ≫ stationary phase. Strain HYK0512-

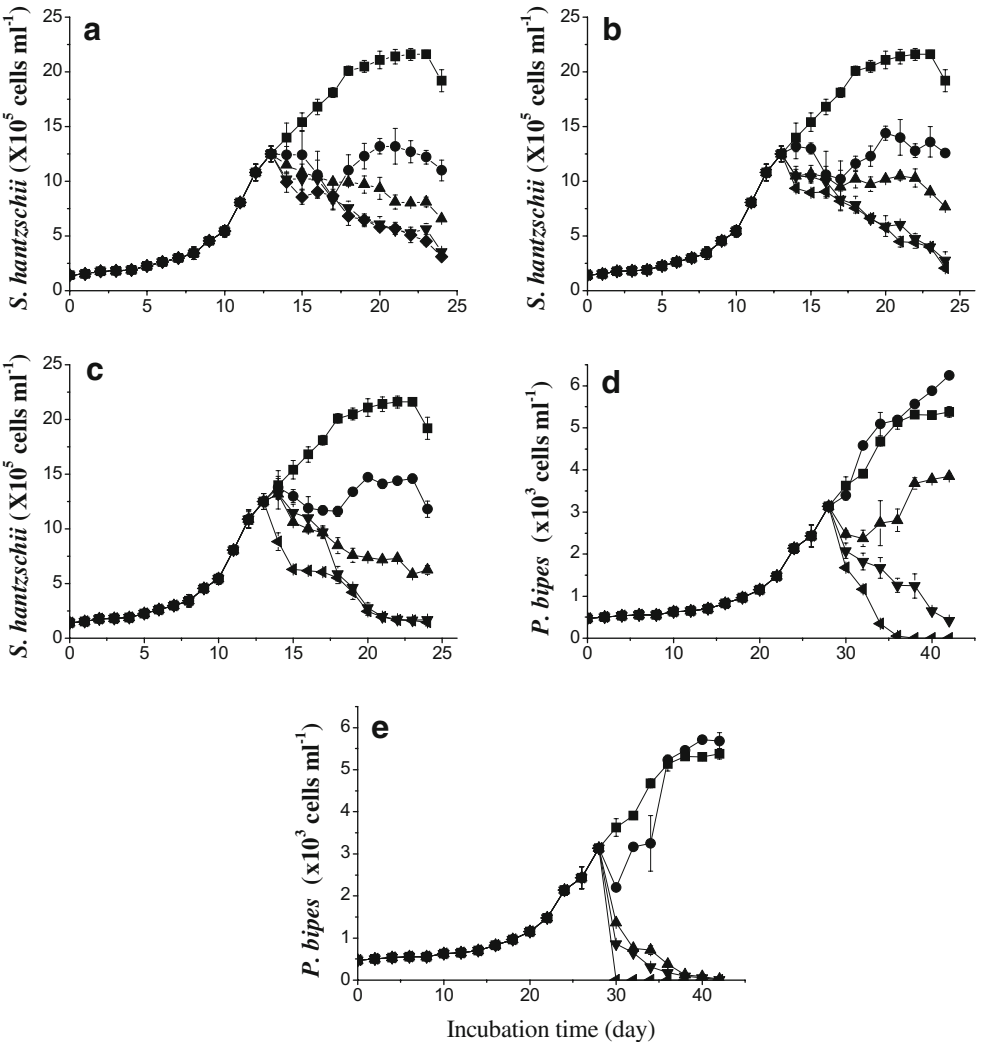

Fig. 3 Growth of *S. hantzschii* in cultures inoculated with various concentrations (■: control, ●: 10^5, ▲: 10^6, ▼: 10^7 and □: 10^8 cells ml^{-1} of bacteria) of (**a**) HYY0510-SK04, (**b**) HYY0511-SK09 and (**c**) HYK0512-SK12 and growth of *P. bipes* in cultures inoculated with various concentrations (**d**) HYK0512-PK04 and (**e**) HYY0512-PK05, respectively

SK12 showed the strongest algicidal activity, and complete lysis of *S. hantzschii* in the lag and logarithmic phases. In the case of *Peridinium*-killing bacteria, strains HYK0512-PK04 and HYY0512-PK05 exhibited complete lysis of *P. bipes* in the lag and logarithmic phases (Fig. 4d, e). Of the two strains, HYY0512-PK05 has much stronger algicidal activity, showing complete lysis during all dinoflagellate growth phases.

Microscopic observation of algicidal mode

The light micrographs of HYK0512-PK04 and HYY0512-PK05 on dinoflagellate *P. bipes* show the serial process of the algal-lytic mode (Fig. 5). HYK0512-PK04 and HYY0512-PK05 first caused the cell wall to breach partially, swam toward the breached cells within 1 day (Fig. 5b, f), and continuously stimulated the algal cells to shed their theca at 2 days (Fig. 5c, g). The dinoflagellate was completely lysed by HYK0512-PK04 and HYY0512-PK05 by 5 and 3 days after inoculation, respectively (Fig. 5d, h).

The relative algicidal effect of bacteria to algae

Various responses of the 13 important strains of algae and cyanobacteria to the five isolates of algicidal bacteria were observed (Table 2). HYK0512-SK12 and HYY0512-PK05 showed effective growth inhibition of most algae and cyanobacteria tested. However, the rest of the bacteria isolated showed no effective inhibition on the growth of *Microcystis aeruginosa* and *Anabaena cylindrica*, which dominate during high water temperature, whereas all bacteria effectively suppressed both the diatom *S. hantzschii* and dinoflagellate *P. bipes*, which dominate during low water temperature.

To investigate the localization of relevant algicidal substances, the culture media separated from the bacteria and bacterial pellet were tested against target algae

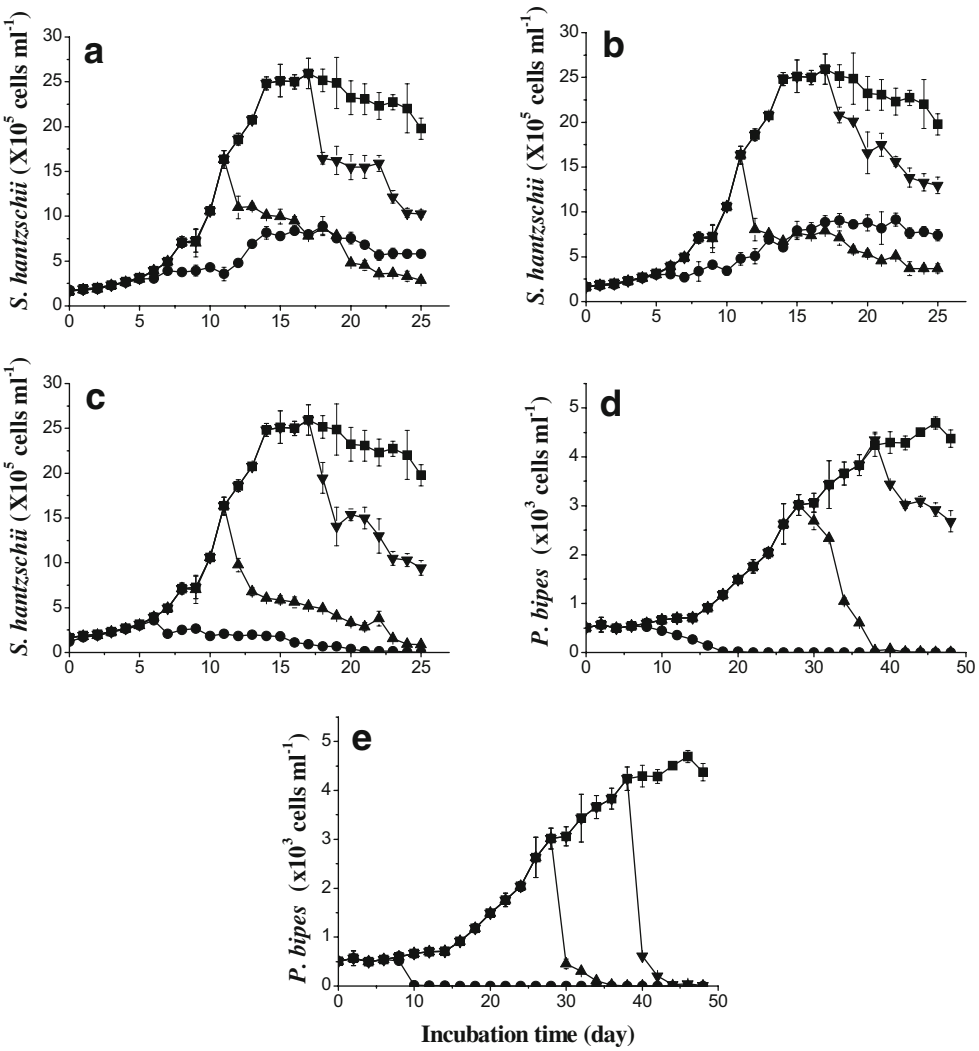

Fig. 4 Growth of *S. hantzschii* at lag (●), logarithmic (▲) and stationary (▼) growth phases in cultures inoculated with (**a**) HYY0510-SK04, (**b**) HYY0511-SK09 and (**c**) HYK0512-SK12 and growth of *P. bipes* at lag, logarithmic and stationary growth phases in cultures inoculated with (**d**) HYK0512-PK04 and (**e**) HYY0512-PK05, respectively. Controls were algal cultures without bacterial inoculation (■)

(Table 3). The significantly higher activity was observed in the spent medium of all strains versus their cell extracts (34.7, 33.8, 23.1, 1.5, and 9.4 units mg^{-1} for HYY0510-SK04, HYY0511-SK09, HYK0512-SK12, HYK0512-PK04, and HYY0512-PK05, respectively). The spent media of all isolates appeared to have high activities of 1866.1, 447.8, 157.8, 11635.8, and 1047.5 units mg^{-1} for HYY0510-SK04, HYY0511-SK09, HYK0512-SK12, HYK0512-PK04, and HYY0512-PK05, respectively, when compared with the control. Moreover, the algicidal activities of spent media treated with heat were even higher than those of the media themselves (data not shown). These results indicate that, to

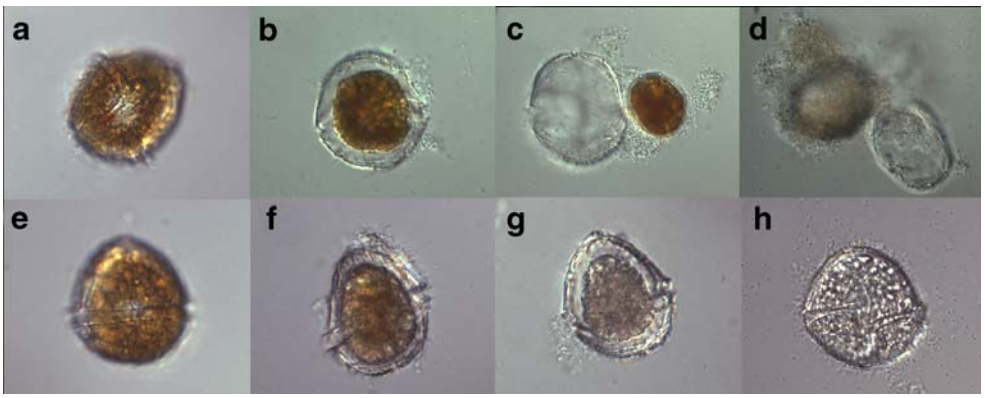

Fig. 5 Light microscopic observations of *P. bipes* in cultures with the absence (**a**, **e**) and presence (**b**-**d**) of algicidal bacteria. HYK0512-PK04 (**b**, 1 day; **c**, 2 day; and **d**, 5 day) and HYY0512-PK05 (**f**, 1 day; **g**, 2 day; and **h**, 3 day)

Table 2 Algicidal effect of bacteria against several algae and cyanobacteria[a]

Host strains	HYY0510-SK04	HYY0511-SK09	HYK0512-SK12	HYK0512-PK04	HYY0512-PK05
Stephanodiscus hantzschii UTCC[b] 267	++	++	++	++	++
Cyclotella meneghiniana HYK0210-A1	O	+	+	O	++
Aulacoseira granulata CCAP[c]1002-1	O	+	O	O	++
Peridinium bipes HYJA0311-02	++	++	++	++	++
Peridinium bipes HYTK0601-01	+	+	++	+	++
Microcystis aeruginosa NIER[d] 10001	–	O	++	–	++
Microcystis aeruginosa NIES[e] 101	+	+	++	+	++
Microcystis aeruginosa NIES 44	–	–	++	–	++
Microcystis viridis NIES 102	–	O	O	–	++
Anabaena macrospore ATCC[f] 22664	–	O	++	O	O
Anabaena cylindrica NIES 19	–	O	++	++	++
Anabaena flos-aquae AG[g]10064	–	–	O	–	++
Coelastrum astroideum NIER 10088	+	O	O	+	++

[a] Algicidal activity was determined by the following equation: after 10 days; algicidal activity (%) = (1-*T*/*C*) × 100, where *T* (treatment) and *C* (control) are the cell densities with and without algicidal bacteria, respectively. ++, >50% inhibition of total; +, 20~50%; O, −20~20%; and −, <−20% *Cyclotella meneghiniana* HYK0210-A1 and *Cyclotella* sp. HYK0210-A2 were isolated from the Gyeongan stream in Korea. *Peridinium bipes* HYJA0311-02 and *Peridinium bipes* HYTK0601-01 were isolated from the Juam stream and Togyo Reservoir in South Korea

[f] ATCC, American Type Culture Collection, Manassas, VA, USA

[c] CCAP, Culture Collection of Algae and Protozoa, USA

[e] NIES, National Institute of Environmental Studies, Japan

[d] NIER, National Institute for Environmental Research, Korea

[b] UTCC, University of Toronto Culture Collection of Algae and Cyanobacteria, Canada

[g] AG, Korean Collection for Type Cultures, South Korea

inhibit algal growth, algicidal bacteria secrete a heat stable chemical substance which may not be a protein compound.

Discussion

Recently, many algicidal bacteria have been isolated from various freshwater regions (Kitaguchi et al. 2001; Kim and Han 2003; Choi et al. 2005). However, only two bacteria having algicidal activity against the diatom *Stephanodiscus hantzschii* have been previously reported (Kang et al. 2005, 2007). In particular, for the dinoflagellate *Peridinium bipes*, no report has been made to date regarding the isolation and identification of algicidal bacteria with the potential to lyse this algal species. In an effort to isolate a bio-agent capable of controlling species of *S. hantzschii* and *P. bipes*, 3 isolates (HYY0510-SK04, HYY0511-SK09 and HYK0512-SK12) of *Stephanodiscus*-killing bacteria and 2 isolates (HYK0512-PK04 and HYY0512-PK05) of *Peridinium*- killing bacteria were identified in this study, and their effective algicidal activities were confirmed in the lab.

Bacterial strains HYY0510-SK04, HYY0511-SK09, HYK0512-SK12, HYK0512-PK04, and HYY0512-PK05 were identified as *Acidovorax delafieldii*, *Acidovorax delafieldii*, *Variovorax paradoxus*, *Hydrogenophaga palleronii* and *Pseudomonas plecoglossicida*, respectively, using 16S rDNA gene sequencing to effectively identify many of the bacteria to the genus level (Fig. 1). However, the isolate HYK0512-PK04 identified as *H. palleronii* were distinguished into *Pasteurella multocida* by the API 20NE

Table 3 Algicidal activity of cell spent media and cell extract of algicidal bacteria

Cell compartments	Specific activity (units [a] mg^{-1}) [b]				
	HYY0510-SK04	HYY0511-SK09	HYK0512-SK12	HYK0512-PK04	HYY0512-PK05
Spent media	1866.1	447.8	157.8	11635.8	1047.5
Cell extract	34.7	33.8	23.1	1.5	9.4

[a] One unit of degradation activity indicates the ability to decrease 10000 or 100 cells of *S. hantzschii* or *P. bipes*, respectively

[b] The specific activity of isolate bacteria was calculated by the following equation: specific activity (unit mg^{-1}) = the degradation activity (unit)/ the protein concentration (mg)

system. Moreover, the enzyme activity of HYK0512-PK04 using the API ZYM profile was almost identical to 33 isolates of *Pasteurella multocida* reported by Grehn et al. (1991), but was not identical to *H. palleronii*. Although the API 20NE system using the limited number of environmental strains in databases has the limitation of poor corroboration of identification when compared with 16S rDNA gene sequencing, the accuracy of identification can increase dramatically when results from API 20NE systems are used to support the 16S rDNA gene sequencing results (Song et al. 2000). The data in this study indicate that use of only the 16S rDNA gene sequencing method may not be sufficient to distinguish bacterial species in some cases as described by Forney et al. (2004); more reliable identifications can be achieved by coupling two or more techniques.

Yamamoto and Suzuki (1990) reported that different algicidal activity was observed according to media composition. HYY0510-SK04, HYY0511-SK09 and HYK0512-SK12 exhibited high algicidal activities against *S. hantzschii* with no difference among media tested. However, HYK0512-PK04 and HYY0512-PK05 may exert algicidal activities against *P. bipes* only in the MCM medium. Therefore, this result indicates that the composition of growth media might play a crucial role in the success of bacterial attack upon specific algae. According to this result, MCM was used as a bacterial culture medium for achieving more efficient control of target algae in all experiments in this study.

Previous studies have identified the bacterial inoculum levels (Doucette et al. 1999; Sigee et al. 1999; Manage et al. 2000) and the physiological status of the host algae (Mayali and Doucette 2002; Kang et al. 2005) as primary determinants of algicidal activity. The greater bacterial concentration can exert more effective algicidal activity and, at the same initial levels, bacteria HYK0512-SK12 and HYY0512-PK05 induced more rapid and higher algicidal effects than the other *Stephanodiscus*- and *Peridinium*-killing bacteria, respectively (cf. Fig. 3). Moreover, all strains could easily destroy host algal cells of the lag and logarithmic phases due to relatively weak cell walls and more active cell division than those of the stationary phase, which is in agreement with the results of Manage et al. (2000). Thus, it is thought that inoculation of algicidal bacteria into the initial or developmental natural blooms with a threshold concentration can maximize the algicidal activity.

Among the strains isolated in this study, HYK0512-SK12 and HYY0512-PK05 have a relatively wide host range from cyanobacteria to algae. Thus, it is thought that HYK0512-SK12 and HYY0512-PK05 can extensively cover blooms caused by the 13 tested species existing mostly in freshwater ecosystems. Daft et al. (1985) considered a wide host range as one of the characteristics of a good predator (algicidal bacteria). An advantage of more general predators, such as bacterial strains HYK0512-SK12 and HYY0512-PK05, is the excellent ability to survive, due to a broad range of alternative prey, given a low abundance of their primary prey (Symondson et al. 2002; Koss and Synder 2004) and the applicable possibility to control various host algal blooms (Walker and Higginbotham 2000; Manage et al. 2000). This result indicates that HYK0512-SK12 and HYY0512-PK05 can also prevent blooms of other competitive species from increasing in abundance in response to the extinction of the blooms of *Stephanodiscus* or *Peridinium*.

Mitsutani et al. (2001) reported that the supernatant of *Pseudoalteromonas* sp. culture showed high protease activity against the diatom *Skeletonema costatum*. In the present study, culture media of all strains showed high algicidal activity, whereas cell extracts had little activity. Additionally, spent media treated with heat showed enhanced algicidal activity (data not shown). This result indicates that the extracellular substance of all bacteria associated with algicidal activity which breached the cell wall and to degrades most of the algal cells may be related to a chemical substance released into the surrounding water from *Peridinium*-killing bacteria, as previously reported in *Anabaena*-lysing bacteria (Kim and Lee 2006). In general, bacteria that inhibit host algae are effective through either a direct or indirect attack (Lovejoy et al. 1998; Doucette et al. 1999; Sigee et al. 1999). In this study, it was found that *P. bipes* was rapidly lysed indirectly by the release of extracellular compounds which besiege the algal cell. However, we could not identify the substances responsible for the algal cell lysis. Therefore, the isolation and identification of algicidal substances remains to be further studied.

In summary, algicidal activities of five bacteria isolated from indigenous freshwater were compared to obtain the best algicidal bacteria as agents for algal bloom control. HYK0512-SK12 and HYY0512-PK05 are important candidates for the effective management of *Stephanodiscus* or *Peridinium* blooms due to their strong algicidal activity and wide host range. However, algicidal bacteria themselves may trigger serious environmental consequences including major economic disadvantages and the environmental impacts (Jeong et al. 2001; Kang et al. 2007). Additional studies will be required to exterminate harmful effects caused by algicidal bacteria. Of the most alternative methods, powder of the active algicidal products extracted from bacteria would be helpful to provide a practical method for effectively controlling these common algal blooms with minimal adverse effects on the freshwater ecosystem during the winter.

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