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Alga-lytic activity of *Pseudomonas fluorescens* against the red tide causing marine alga *Heterosigma akashiwo* (Raphidophyceae)

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Abstract

A bacterial strain, HAK-13, exhibited strongest activity against *Heterosigma akashiwo* and was capable of controlling this bloom forming phytoplankton. Based on 16S rDNA sequences and biochemical and morphological characteristics, the strain HAK-13 was determined to be *Pseudomonas fluorescens* on the basis of 99.9% similarity with reference strains in the DNA databases. The growth of *H. akashiwo* was strongly suppressed by HAK-13 in all growth phases, with the strongest alga-lytic activity noted against harmful bloom-forming species in the exponential stage (6–22 days). Host range tests showed that HAK-13 also significantly inhibited the growth of *Alexandrium tamarense* and *Cochlodinium polykrikoides* but could not destroy *Gymnodinium catenatum*. *P. fluorescens* HAK-13 indirectly attacked *H. akashiwo* by alga-lytic substances that might be located at the compartment of cytoplasmic membrane of the bacterium at a level of 45.86 units/mg of specific activity. The results indicated that *P. fluorescens* HAK-13 caused cell lysis and death of *H. akashiwo*, *A. tamarense*, and *C. polykrikoides* dramatically and *Prorocentrum dentatum* slightly. Therefore, *P. fluorescens* HAK-13 has potential for use as a selective biocontrol of harmful algal blooms.

Keywords: Harmful algal blooms (HABs); *Pseudomonas fluorescens*; *Heterosigma akashiwo*; Alga-lytic activity

1. Introduction

Phytoplankton blooms in marine coastal waters generally indicate eutrophication that occurs worldwide and causes mass mortalities of fish and shellfish, seriously damage aquaculture industries (Nagayama et al., 2003), and impacts environmental and human health (Jeong et al., 2000). Blooms of *Cochlodinium polykrikoides* Margalef and *Heterosigma akashiwo* (Hada) Hada ex Hara et Chihara in particular cause heavy damage almost every year in Korea and other countries. In order to solve these problems, several control techniques have been applied to manage blooms including yellow loess (Choi et al., 1998; Na et al., 1996) and clay (Sun et al., 2004). Although these methods have been found to be effective, yellow loess and clay cause secondary effects on bottom-dwelling organisms (Bricelj and Malouf, 1984; Rhoads and Young, 1970). Chemical agents such as copper sulfate (Steidinger, 1983), hydrogen peroxide (Ryu et al., 1998), and triosyn (Koji et al., 1998) are effective in controlling blooms within a short period after application, but their usage in aquatic ecosystems is potentially dangerous (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. Recent works have focused on the identification of bacteria capable of inhibiting or degrading of red tides in marine coastal regions. Marine bacteria are considered to be one of the key biological agents in the dramatic termination of phytoplankton blooms observed in coastal seawaters (Schoemann et al., 2005; van Rijssel et al., 2000).

In an effect to identify and characterize antagonistic microorganisms to control red tides, we tried to isolate

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and identify alga-lytic bacteria against to *H. akashiwo*, estimate the alga-lytic activity of the isolated bacteria, and check the cellular localization of alga-lytic substances.

2. Materials and methods

2.1. Microorganisms and culture conditions

Monoclonal axenic strain *H. akashiwo* D-075 was kindly supplied by Korea Marine Microalgae Culture Center at Pukyong National University in South Korea. The microalga was maintained as unialgal culture at 20 °C, pH 8, under an illumination of 50 μE/m^2/s on 12:12 (light: dark) cycle, with an orbital shaker set to 50 rpm. *H. akashiwo* was incubated in F/2 medium (Guillard and Ryther, 1962) adjusted to pH 8.0 ± 0.2.

2.2. Isolation of alga-lytic bacterium

Pseudomonas fluorescens HAK-13 with alga-lytic activity against to *H. akashiwo* was isolated using a modified soft-agar overlay technique (Sakata et al., 1991). Surface water samples were collected from Masan Bay in Korea (from June to August, 2002), and filtered through 5-μm Whatmann membrane filters. To develop *Heterosigma*-lawn, cells of *H. akashiwo* were cultivated in F/2 medium for 20 days and harvested by centrifugation at 10,000*g* for 20 min. The pellets were mixed with liquid F/2 soft agar (1.0% agar) to make the bottom mixture and some quantity (20 ml) of the bottom mixture was poured onto F/2 bottom agar (1.5% agar) plates and solidified for 2 days under the algal culture conditions mentioned above. A small amount (200 μl) of the surface water filtrates was spread on the *Heterosigma*-lawn, and the plates were incubated for 7–10 days under algal culture conditions mentioned above in Section 2.1. Alga-lytic bacteria that formed clear zones around the colony were isolated, incubated, and purified by serial streaking on the nutrient agar plates (Kang et al., 2005). The isolate HAK-13 showing significant alga-lytic activity against *H. akashiwo* was kept at −76 °C in nutrient broth medium (Difco™, MD, USA; beef extract 3 g, peptone 5 g, and distilled water 1000 ml) containing 20% glycerol.

2.3. Identification of alga-lytic bacterium HAK-13 by phylogenetic analysis

The chromosomal DNA was isolated using a method described by Yoon et al. (1997). The amplification of the 16S rDNA was conducted using two primers (Stackebrandt and Liesack, 1993), 5′-GAGTTTGATCCTGGCTCAG-3′ and 5′-AGAAAGGAGGTGATCCAGCC-3′. A PCR was run for 35 thermal cycles of denature for 1 min for 94 °C , annealing for 2.5 min at 55 °C, extension for 2.5 min for 72 °C, and within a final elongation step of 7 min at 72 °C in a DNA thermal cycler, (Genetic analyzer 377; Perkin–Elmer, Boston, MA, USA), employing the thermal profile. The PCR products were purified with the QIA quick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by automated DNA sequencer (ABI PRISM 310 Genetic Analyzer, Amersham Pharmacia Biotech., Uppsala, Sweden) using the SequiTherm EXCELTh II Labeled Primer Sequencing Kit (EPICENTRE Biotechnologies, Madison, WI, USA). The 16S rDNA sequence of the bacterial isolates HAK-13 was aligned using CLUSTAL W software (Nigam et al., 2000). The evolutionary distance matrices were calculated with the DNADIST program within the PHYLIP package (Felsenstein, 1993). The sequence of representative species of the genus *Pseudomonas* and related taxa were cited using the GenBank Database. The values of 16S rDNA similarity were calculated from the alignment, while the evolutionary distances were calculated using a Kimura two-parameter correction. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) based on the calculated distance matrix.

2.4. Bacterial density and alga-lytic effect on H. akashiwo

To investigate the alga-lytic activity of the different concentrations of the bacterium HAK-13 against *H. akashiwo*, the bacterial strain HAK-13 was cultured and then serially diluted with F/2 (5 ml) medium to initial concentration of 1×10, 1×10^2, 1×10^3, 1×10^4, 1×10^5, or 1×10^6 cfu/ml in 100 ml of *H. akashiwo* culture (1.3×10^6 cells/ml) at mid-exponential phase (a 15-day culture following inoculation). Algal cells were counted daily for 40 days.

2.5. The influence of algal and bacterial growth phases on the alga-lytic activity of the bacterium HAK-13

The alga-lytic activity of HAK-13 against different growth phases of *H. akashiwo* was examined. The bacterium was cultured and the cells were adjusted to a concentration of 1×10^6 cfu/ml. An aliquot (2.5 ml) of the bacterial suspension was inoculated into 250 ml-flasks containing 100 ml of lag, exponential, and stationary phases of *H. akashiwo* (1.5, 12.7, and 25.6×10^5 cells/ml, respectively). Also, the alga-lytic activity of HAK-13 in different growth phases such as lag, exponential, and stationary phases, against to *H. akashiwo* in mid-exponential growth phase was tested. Aliquots of HAK-13 were cultivated for 3 (lag phase), 24 (exponential phase), and 36 h (stationary phase) in 100-ml flasks containing 50 ml of nutrient broth medium, centrifuged at 18,000*g* for 20 min, and adjusted to 1×10^6 cfu/ml in initial concentration. The bacterial suspensions (2.5 ml) were inoculated into 250-ml flasks containing 100 ml of mid-exponential phase of 15 days old *H. akashiwo* (about 1.6×10^6 cells/ml) cultures. Algal cells were counted daily.

2.6. Analysis of alga-lytic activity of the bacterium HAK-13

Cells of *H. akashiwo* were calculated with the aid of a hemocytometer under a light microscope (BX51, Olympus, Japan) and the bacterium was quantified as colony forming

units (cfu). The alga-lytic activity of isolate HAK-13 was estimated by the flowing equation: Alga-lytic activity (%) = $(1-T_t/C_t)\times 100$, where T (treatment) and C (control) are the cell concentration of *H. akashiwo* with and without HAK-13, respectively, and t is the inoculation time. After the cells of HAK-13 were harvested by centrifugation at 18,000*g* for 20 min and resuspended into fresh F/2 medium. A suitable volume of the suspension was inoculated to the cell cultures of *H. akashiwo* in the treatment. In the case of control, the equal volume of fresh F/2 medium was added to the *H. akashiwo* cultures in the treatment instead of bacterial inocula. All experiments were repeated in triplicate and all results are shown as means and standard deviations.

2.7. Preparation of cell-free extracts and culture supernatants

Cell-free extracts were separated according to the method of Niviere et al. (1986). For preparation of cell-free extract, cells were incubated in NB medium for 24 h at 30 °C and pH 7.0, harvested by centrifugation at 75,000*g* for 30 min, and suspended in 10 mM phosphate-citrate buffer (pH 7.0). The bacterial cells were homogenized with a mortar and pestle for 40 min at 4 °C. The cellular debris was removed by centrifugation at 140,000*g* for 2 h at 4 °C and bacteria were completely removed through 0.2-μm polycarbonate filters to prepare cell-free extracts and supernatants. Finally, acetone was added to the clear supernatant (crude extract) at 10% (v/v) and the mixture was immediately assayed for degradation activity.

2.8. Preparation of cell fractions

Periplasm fraction was prepared according to the method of van der Western et al. (1978), and the other cell fractions were separated according to the method of Niviere et al. (1986). A bacterial pellet (7 g; prepared as above) was suspended in phosphate buffer (pH 9) containing 50 mM EDTA and incubated at 40 °C for 30 min. The periplasm fraction was obtained by centrifugation at 18,000*g* for 30 min at 4 °C. The pelleted spheroplast fraction was homogenized with a mortal and pestle for 90 min in 10 mM phosphate buffer (pH 7) at 4 °C. The homogenate was centrifuged at 30,000*g* and the supernatant further centrifuged at 150,000*g* for 5 h at 4 °C. The supernatant was used as the cytoplasmic fraction. The pellet was resuspended in 10 mM phosphate buffer (pH 7), and represented the cytoplasm membrane fraction.

2.9. Preparation of crude cell extracts of H. akashiwo

Mid-exponential phase *H. akashiwo* cells were harvested by centrifugation at 8000*g* for 30 min at 4 °C, washed twice with sterilized water and dried at 4 °C. Dried cells (2.0 g) were suspended in 200 ml of 4% sodium dodecyl sulphate (SDS), boiled at 100 °C for 30 min, harvested by centrifugation at 120,000*g* for 30 min, and cooled to room temperature. This process was repeated five times and then the *H. akashiwo* cell extracts were suspended in 400 ml of boiled distilled water, harvested, and cryopreserved at 20 °C until they were used for measurement of specific activity.

2.10. Assay of protein content and degradation activity

The concentration of protein was determined by the Bradford method Bradford (1976), using bovine serum albumin as the standard. For the measurement of the ability of the bacterium to degrade the alga, the HAK-13 cell fractions and cell free-extract were adjusted to 1.2 at A_{660} in 25 mM Tris–HCl buffer (pH 8.0), inoculated into crude cell extracts of *H. akashiwo*, and incubated at 40 °C for 30 min. The assay of protein content, degradation activity and specific activity were determined by modified method of Kim et al. (2002). The degradation activity was calculated by comparison of absorption at 660 nm before and after the reaction. One unit of degradation activity indicates the ability to decrease absorbance 0.001 in 1 min of reaction time. The specific activity of HAK-13 was calculated by the following equation: specific activity (unit/mg) = the degradation activity (unit)/the protein concentration (mg).

3. Results and discussion

3.1. Isolation and identification alga-lytic bacteria

Ninety-five bacterial isolates were screened from surface seawater from Masan Bay in South Korea and approximately 10% of the isolated alga-lytic bacteria showed algal-lytic activity against to *H. akashiwo* (data not shown). About 5.15% (5 isolates) of the total number of the isolated bacteria had strong activity. Of these, the isolates HAK-13 showed the strongest algal-lytic activity when 5 ml (1×10^5 cfu/ml) of these isolates were inoculated into 100 ml (1×10^6 cells/ml) of *H. akashiwo* culture for 10 days. This experiment was carried out in duplicate. This alga-lytic bacterium HAK-13 was gram-negative, rod-shaped, and nonpigmented in Nutrient broth medium with 1.5% agar. The optimum temperature and pH for the growth ranged between 14 °C and 30 °C, and 5 and 10, respectively (data not shown).

The similarity of HAK-13 to *P. fluorescens* in the 95 carbon-source utilization profiles was found to be 0.923, which was the highest of the profiles of other microorganisms (data not shown), indicating that HAK-13 matched best with *P. fluorescens*. These results were confirmed by the 16S rDNA sequence analysis of the isolate HAK-13 (Fig. 1). The sequences of HAK-13 exhibited a high homology to the sequence of *P. fluorescens*, having 99.9% similarity to the *P. fluorescens* DNA databases such as EMBL, GenBank, and DDBJ. As the value of DNA–DNA homology is a conclusive factor in identifying bacterial strains, with strains having values higher than 70% being included

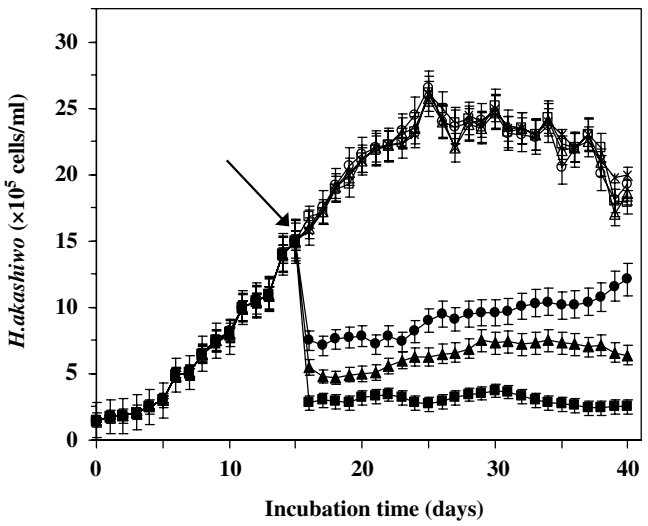

Fig. 1. Growth inhibition of *Heterosigma akashiwo* in cultures incubated with various concentrations of alga-lytic bacterium *P. fluorescens* HAK-13. (×: without the bacterium, ○: 1×10^1, △: 1×10^2, □: 1×10^3, ●: 1×10^4, ▲: 1×10^5 and ■: 1×10^6 cfu/ml. The arrow indicates the inoculation time of the bacterium HAK-13. Data are the mean ± standard deviation from at least tree independent assays.

in the same species (Wayne et al., 1993). According to the reports on the correlation between the DNA–DNA homology and 16 S rDNA sequence homology (Guha and Jaffe, 1996; Kobayashi and Ritmann, 1982), strains reveal DNA–DNA homology higher than 70% when strains show sequence homology higher than 99.5%. Consequently, the isolate HAK-13 was considered identical to *P. fluorescens* and designated as *P. fluorescens* HAK-13. This strain has been deposited in the Korean Culture Center of Microorganisms (KFCC-11354P).

Fluorescent pseudomonads have been known to produce effective antibiotic-substances against to harmful algal blooms in eutrophic marine environments (Wang et al., 2005; Yoshinaga et al., 1997) and *P. fluorescens* plays an important role in protecting plants from soilborne diseases. Strains of *P. fluorescens* are known to produce antifungal substances to control phytopathogenic fungi such as *Rhizoctonia solani*, *Pyricularia oryzae*, *Monilinia fructicola*, *Botrytis cinerea*, *Altenaria kikuchiana*, *Fusarium oxysporum*, *F. solani*, and *Phytophthora capsici* (Andrade et al., 2000; Brimecombe et al., 1998; Jung and Kim, 2005; Nakata et al., 2000; Schoonbeek et al., 2002).

3.2. Alga-lytic effect of *P. fluorescens* HAK-13 against to *H. akashiwo*

To determine the effective alga-lytic threshold concentration of *P. fluorescens* HAK-13 against to *H. akashiwo*, the alga was inoculated with six different concentrations of the bacterium (1×10^1, 1×10^2, 1×10^3, 1×10^4, 1×10^5, and 1×10^6 cfu/ml). The bacterial concentration of $<1\times10^3$ cfu/ml showed little or no alga-lytic activity against to *H. akashiwo*, and in the initial bacterial concentration of 1×10^4, 1×10^5, and 1×10^6 cfu/ml showed algal-lytic activity of 39.6%, 68.0%, and 87.5%, respectively (Fig. 2).

The population dynamics of alga-lytic bacteria has a close relationship to the blooms of the phytoplankton, in marine ecosystems. The alga-lytic bacteria sometimes increase in abundance concurrently with the decline of algal blooms, suggesting that they may affect algal bloom dynamics (Mayali and Azam, 2004). Since alga-lytic activity from field isolates was also influenced by strain and environmental variations, it is important to test selected strains at threshold cell concentration that will influence the algal-lytic activity (Doucette et al., 1999). The threshold density of alga-lytic bacteria was studied because it is independent of the influence of inorganic nutrient concentration or host density (Fraleigh and Burnham, 1988). Our data suggested that the threshold concentration at which *P. fluorescens* HAK-13 had algal lytic effects on *H. akashiwo* was 1×10^4 cfu/ml; naturally a higher alga-lytic activity was observed at concentrations over the threshold (Fig. 2).

However, the growth of *H. akashiwo* was effectively inhibited by *P. fluorescens* HAK-13 during all the three algal growth phases, decreasing the algal biomass by 75.9% at lag phase, 88.9% at exponential phase, and 83.8% at stationary phase for *H. akashiwo* (Fig. 3), which they were significantly different at the 95% level. *P. fluorescens* HAK-13 of three different growth phases consistently suppressed growth of *H. akashiwo* at mid-exponential phase, with the biomass of *H. akashiwo* deceased to 88.6% at lag phase, 87.0% at exponential phase, and 83.3% by *P. fluorescens* HAK-13 at stationary phase (Fig. 4).

Alga-lytic activity depends on different growth stages of *H. akashiwo*, increasing exponential>stationary>lag phase (Fig. 3). It suggests that the cell wall of the alga at the exponential growth phase was easily destroyed by bacterial attack since the algal cell division is most active at this phase than at other phases (Cole, 1982). In contrast, the alga-lytic activity of *P. fluorescens* HAK-13 was consistently exhibited regardless of the growth stage of the bacterium (Fig. 4).

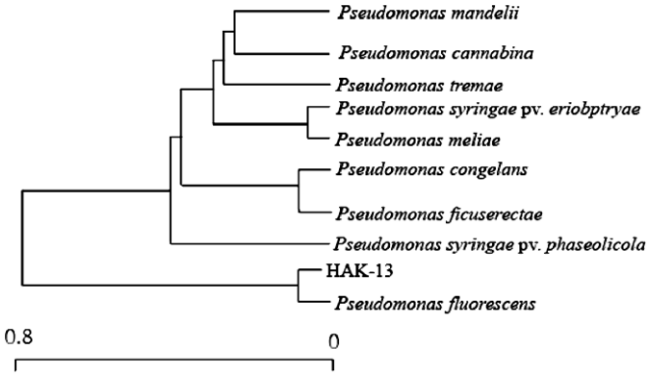

Fig. 2. Phylogenetic tree based on 16S rDNA sequences showing the positions of strain HAK-13, and representatives of pseudomonad. The scale bar represents 0.8 substitutions per nucleotide position.

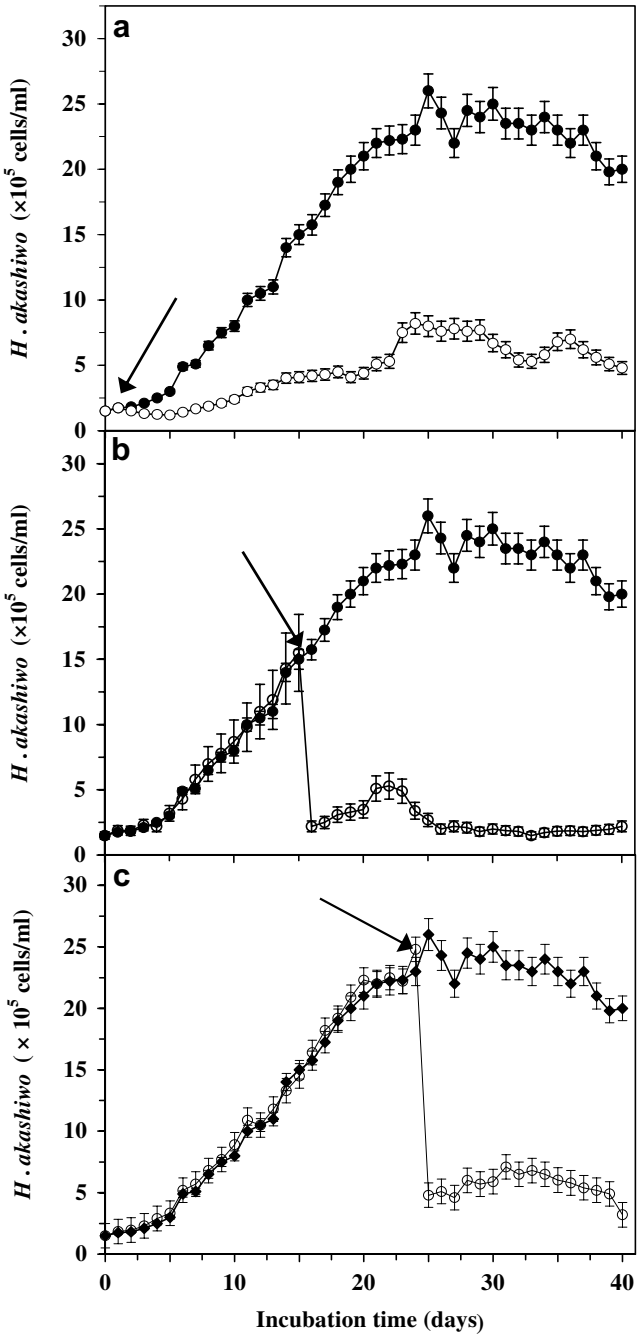

Fig. 3. Cell concentration changes of *H. akashiwo* in culture inoculated with (○) alga-lytic bacterium *P. fluorescens* HAK-13 at three different growth stages of *H. akashiwo* (a, lag phase; b, mid-exponential phase; c, stationary phase) and without (●) HAK-13 as control. The arrow indicates the time of inoculation. Data are the mean ± standard deviation from at least three independent assays.

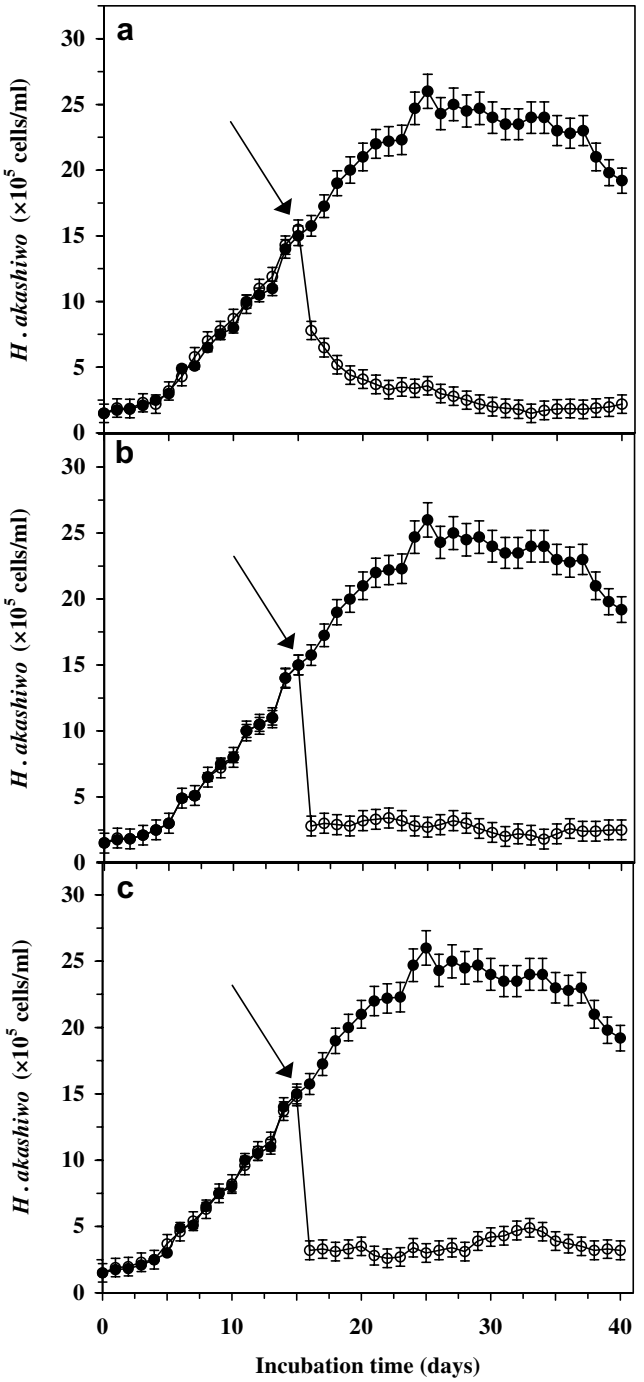

Fig. 4. Changes in the cell concentration of *H. akashiwo* inoculated with (○) alga-lytic bacterium *P. fluorescens* HAK-13 at three different growth stages of the alga-lytic bacterium (a, lag phase; b, mid-exponential phase; c, stationary phase) and without (●) HAK-13 as control. The arrow indicates the time of inoculation of alga-lytic bacterium. Data are the mean ± standard deviation from at least three independent assays.

Microscopic examination revealed that the motility of algal cells also was badly damaged. No immediate morphological change was observed in the algal cells when the bacterium HAK 13 was inoculated into *H. akashiwo* culture (Fig. 5a). Bacterial concentration of 1×10^5 cfu/ml induced all *H. akashiwo* cells at mid-exponential growth phases to lose motility after 1 h (Fig. 5b), whereas the early exponential growth phage lost motility earlier under the same treatment. At low cell concentration of HAK-13 (1×10^4 cfu/ml), although all of the cells of *H. akashiwo* were still motile, their speed of motility (monitored by an automatic biotest system (ECOTOX, Real Time Computer, Möhrendorf, Germany) decreased markedly within 2 h (data not shown). The cell wall of *H. akashiwo* was breached within 2 h (Fig. 5c) and subsequently algal chloroplast and cytoplasm

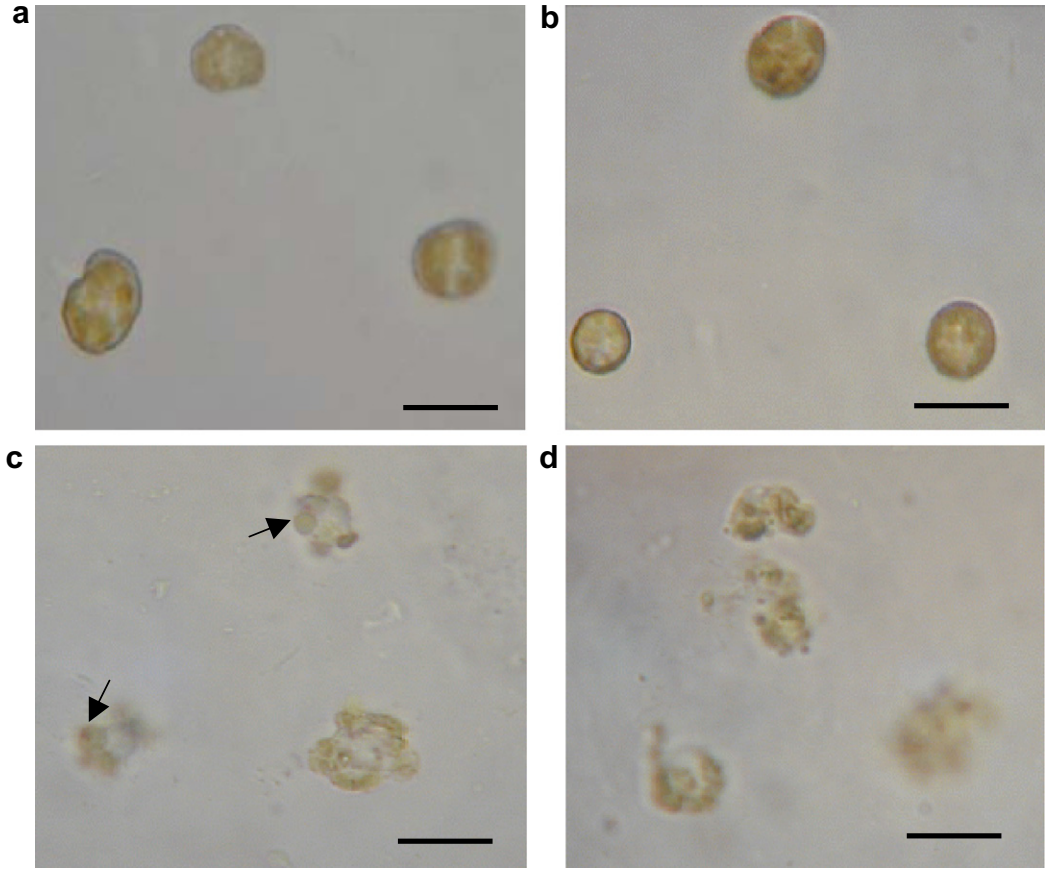

Fig. 5. Light microscopic observation of *H. akashiwo*: (a) untreated control cells, (b) round and expanded cells after 1 h of inoculation with *P. fluorescens* HAK-13, severely damaged cells after 2 h (c) and 4 h (d) after inoculation. *Heterosigma akashiwo* was used in the mid-exponential phase. Arrows show the chloroplast. Scale bar = 20 μm.

also lost their integrity (Fig. 5d). The physiological status of the host alga is important to the success of bacterial biocontrol of water blooms (Manage et al., 2001) and is related to the lytic effects of bacteria (Toncheva-Panova and Ivanova, 2000). In the present study, physiological status of the alga was a key factor in growth inhibition of *H. akashiwo* since the cells of *H. akashiwo* lack cell walls and the cytoplasm is only surrounded by the cell membrane, which may be the main reason for the quick lysis after swelling observed by Lovejoy et al. (1998). Our results suggest that the alga-lytic activity of the bacterial strain HAK-13 is potentially useful for controlling outbreaks of *H. akashiwo*.

3.3. Cellular localization of the enzymes related to algal-lytic activity

Cell-free extract had 6.99 units/mg of *H. akashiwo*-lytic activity, while culture filtrates had none (Table 1). These results suggest that *P. fluorescens* HAK-13 possesses alga-lytic substances on the bacterial surface or the inside of membrane. Alga-lytic activity of the periplasmic, cytoplasmic, and cytoplasmic membrane fractions was tested against *H. akashiwo* (Table 1). Although there were very slight differences in protein concentrations among the three cell fractions, significant differences were observed in the algal degradation activity of the periplasm (0.35 unit), cytoplasm (308.21 units), and cytoplasmic membrane (0.25 units) fractions. In terms of specific activity, the cytoplasm had higher specific activity (45.86 units/mg) than cytoplasmic membrane (0.05 units/mg) and periplasm (0.08 units/mg). The periplasmic fraction showed 0.08 units/mg of specific activity, but enzymatic degradation activity was markedly lower than that of the cytoplasmic fraction. It seemed that the periplasmic fraction was contaminated with proteins from the cytoplasmic fraction when the cell was treated with EDTA. Therefore, the strong alga-lytic activity exists in cytoplasmic fraction, confirming that the alga-lytic substance acting against *H. akashiwo* is localized in the cytoplasm. A rhamnolipid biosurfactant produced by *P. aeruginosa* (Wang et al., 2005) and sophorolipid released from *Candida bombicola* (J.F.T. Spencer, Gorin & A.P. Tulloch) S.A. Meyer & Yarrow (Baek et al., 2003) are lethal to several harmful bloom-forming species including *H. akashiwo* Table 2.

We could not find any compound showing algal lytic effects in culture broth of *P. fluorescens* HAK-13 (data not shown) when protease treated, which suggests that substances possessing the alga-lytic effects could be enzymes

Table 1
Alga-lytic activity of cell filtrate, cell-free extract, and various fractions of cell-free extract of the bacterial strain *Pseudomonas fluorescens* HAK-13 against *Heterosigma akashiwo*

Cell component	Cell fraction	Lytic activity (unit)	Protein concentration (mg/ml)	Specific activity (unit/mg)
Cell filtrate		0.00	0.02	0.00
Cell-free extract		68.24	9.76	6.99
	Periplasm	0.35	4.81	0.08
	Cytoplasm	308.21	16.72	45.86
	Cytoplasmic membrane	0.25	4.84	0.05

Table 2
Alga-lytic activity of the bacterial strain HAK-13 against several harmful algal species

Harmful algae[a]	Alga-lytic activity (%)
Alexandrium tamarense	70.8 ± 12.2
Cochlodinium polykrikoides	68.2 ± 10.8
Gymnodinium catenatum	2.0 ± 2.0
Prorocentrum dentatum	43.7 ± 4.8

The alga-lytic activity of *P. fluorescens* HAK-13 was estimated by the following: Alga-lytic activity (%) = $(1-T_t/C_t) \times 100$, where T (treatment) and C (control) are the cell concentration of *H. akashiwo* with and without HAK-13, respectively, and t is the inoculation time ($t = 2$ days).

[a] *Alexandrium tamarense*, *Gymnodinium catenatum*, *and Prorocentrum dentatum* were supported by Korea Marine Microalgae Culture Center at Pukyong National University and *Cochlodinium polykrikoides* by National Fisheries Research and Development Institute in South Korea.

(Table 1). This speculation is supported by the finding that *Pseudoaltomonas* sp. strain A28 produces a serine-type protease to kill *Skeletomena costatum* (Greville) Cleve (Lee et al., 2000) which causes marine diatom blooms. The activity of extracellular enzyme beta-glucosidase, secreted by *Bacillus megaterium* and *B. halmapulus* is reported to inhibit the growth of *Alexandrium tamarense* (Lebur) Balech (Su et al., 2005). Our results suggest that alga-lytic enzymes are present in the cytoplasmic fraction of *P. fluorescens* HAK-13 and that they may be released when the alga-lytic bacterium encounters its targets, the harmful marine alga.

3.4. Lytic effects of HAK-13 on other harmful algal bloom-forming species

The alga-lytic activity of the bacterial strain *P. fluorescens* HAK-13 on other harmful algal bloom species such as *Alexandrium tamarense* and *Cochlodinium polykrikoides* Margalef was investigated. The growth of *A. tamarense* was strongly inhibited in co-culture with HAK-13; moreover, the bacterial strain HAK-13 showed strong lytic activity toward *C. polykrikoides*. In addition, the algal lytic effect of *P. fluorescens* HAK-13 on the growth of *Gymnodinium catenatum* Graham and *Prorocentrum dentatum* Stein, two kinds of harmful bloom-forming species, was also tested. Compared with the remarkable algal lytic effect on *H. akashiwo*, the cells of *P. dentatum*, were moderately inhibited or lysed when 1×10^5 cfu per ml of the bacterial cells were co-cultured with *H. akashiwo*. The cells of *G. catenatum* were not suppressed at the same treatment, indicating that the alga-lytic *P. fluorescens* HAK-13 had the potential for the selective biocontrol of harmful algal blooms.

In conclusion, control of harmful marine algal blooms requires not only the prevention of blooms but also the early curtailment of the excess algal propagation to maintain the cell density under a reasonable concentration. In the present study, the bacterial strain *P. fluorescens* HAK-13 significantly destroyed the population of *H. akashiwo* when they were in the lag to mid-exponential growth phase with treatment of 1×10^5 cfu/ml of the bacterial cells. Although the relationship between the cell structure of harmful algal bloom species and alga-lytic substances must be assessed in more detail during degradation of the harmful algal blooms, the development of a new strategy for the mitigation of harmful algal blooms may be possible since *P. fluorescens* HAK-13 is potentially useful for controlling outbreaks of algal blooms.

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