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ORIGINAL ARTICLE

Pseudomonas fluorescens HYK0210-SK09 offers species-specific biological control of winter algal blooms caused by freshwater diatom *Stephanodiscus hantzschii*

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Abstract

Aims: The study of an algicidal activity and mechanism of the isolated *Pseudomonas fluorescens* HYK0210-SK09 (SK09) against a winter bloomed harmful diatom, *Stephanodiscus hantzschii*.

Methods and Results: SK09 was isolated from the Paldang reservoir, Korea and used to biological control of *S. hantzschii*. The inoculation of SK09 at the final density of 5×10^6 cells ml^{-1} caused degradation of >90% of *S. hantzschii* cells within 5 days. The algal cell lysis was achieved by a direct attack of the bacteria to the diatom cells, and the algicidal compound was located in the cytoplasm of the cell. As SK09 did not suppress *Microcystis aeruginosa*, *Anabaena cylindrica*, *Coelastrum astroideum* or *Cyclotella meneghiniana*, it appeared to attack *S. hantzschii* in a species-specific manner. Testing in an indoor mesocosms confirmed that SK09 effectively reduced *S. hantzschii* cells by 88% within 9 days.

Conclusions: This bacterium is useful in regulating blooms of *S. hantzschii*. However, it should be studied in the future that their impact in shaping phytoplankton community and their activity in natural environments.

Significance and Impact of the Study: The bacterium enabled us to develop a new strategy, to understand the interaction for anthropogenic control of harmful algal blooms in nature.

Introduction

A small centric diatom, *Stephanodiscus hantzschii*, is as a representative species causing blooms in winter seasons in many rivers and reservoirs worldwide (Kilham *et al.* 1986; Kobanova and Genkal 1989; Leitao 1995; Jung *et al.* 2003). In the Han and Nakdong Rivers of the Korea, winter *S. hantzschii* blooms occur annually (e.g. Cho *et al.* 1998; Hong *et al.* 2002). The high density (3×10^4 cells ml^{-1}, Jung *et al.* 2003) of *S. hantzschii* causes low water transparency, bad odour, taste and problems in water treatment processes such as clogging or sedimentation (Oksiyuk 1965; Sakata *et al.* 1991; Kolmakov *et al.* 2002). Therefore, to control *S. hantzschii* blooms is urgently needed.

Biological controls of harmful algal blooms (HABs) using bacteria are of particular interest in marine and freshwater systems. Many algicidal bacteria have been isolated to control HABs in both marine (e.g. Fukami *et al.* 1992) and freshwater systems (e.g. Daft *et al.* 1975). Most studies in freshwaters have targeted on a few species of Cyanophyceae, which produce hazardous toxins (Daft *et al.* 1975; Burnham *et al.* 1981; Fraleigh and Burnham 1988; Yamamoto and Suzuki 1990; Yamamoto *et al.* 1993; Sigee *et al.* 1999; Manage *et al.* 2000; Kodani *et al.* 2002; Choi *et al.* 2005). There is little study on the algicidal bacteria against *S. hantzschii*. We previously reported the algicidal bacterium, *Pseudomonas putida* HYK0210-SK02 (SK02) against *S. hantzschii* (Kang *et al.* 2005). In SK02, the expression of algicidal activity required high inoculation density (over 10^7 cells ml^{-1}). Therefore, SK02 was not a suitable biological control agent in *S. hantzschii* blooms.

Several studies pointed out the prohibition of algicidal activity because of other organisms in natural environments. For example, Nagasaki *et al.* (2000) demonstrated that a bacterium prohibited the algicidal activity of *Cytophaga* sp. AA8-2 against *Heterocapsa circularisquama*. Mayali and Doucette (2002) found that bacterial assemblage co-cultured with *Karenia brevis* prevented the expression of algicidal effect of *Cytophaga* sp. 41-DBG2. Katano *et al.* (2006) demonstrated that heterotrophic nanoflagellates prohibited algicidal activity of bacterium *Pseudomonas fluorescens* HYK0210-SK09 (SK09) by grazing in certain cases. The algicidal activity should be carefully examined in various microbial assemblages to develop the biological control of algal blooms using bacteria.

The aims of this study were (i) to identify and develop a new bacterium that exhibits high algicidal activity as low inoculation density, (ii) to investigate the algicidal activity under various inoculation cell densities, algicidal host range, the algicidal mechanism and (iii) to apply the algicidal bacterium against *S. hantzschii* blooming natural waters.

Materials and methods

Algal culture

Cyanobacterial strains of *Microcystis aeruginosa* NIES 298, *M. aeruginosa* NIES 44, and *Anabaena cylindrica* NIES 19 were obtained from National Institute of Environmental System in Japan. A cyanobacterium, *M. aeruginosa* NIER 10001 and a chlorophyceae, *Coelastrum astroideum* NIER 10088 were obtained from National Institute of Environmental Research in the Republic of Korea. A bacillariophyceae of *Cyclotella meneghiniana* HYK0210-CM01 was isolated by capillary method from the Paldang Reservoir and *S. hantzschii* UTCC 267 was obtained from University of Toronto Culture Collection of Algae and Cyanobacteria in Canada.

Cyanobacterial strains of *M. aeruginosa* and *A. cylindrica*, and a chlorophyceae, *C. astroideum* were cultured in a BG11 medium at 30°C, pH 9 with shaking at 60 rpm and 40 μE m^{-2} s^{-1} under 12 : 12 (light : dark) cycles. Two diatom strains, *S. hantzschii* and *C. meneghiniana* were cultured in a DM medium (Beakes *et al.* 1988) at 15°C, pH 7 with shaking at 60 rpm and 40 μE m^{-2} s^{-1} under 12 : 12 (light : dark) cycles.

Isolation and identification of an algicidal bacterium HYK0210-SK09

Water samples for the isolation were taken from the surface water of the Paldang Reservoir, Korea on 10 October, 2003. The samples were transferred into 1 l sterile Nalgene bottles and stored in a dark ice chest until processing.

The isolation was conducted by a modified soft-agar over-layer technique (Sakata *et al.* 1991). To create a diatomic lawn, *S. hantzschii* UTCC 267 was axenically cultured under the same condition described above. Approximately 9×10^5 cells ml^{-1} in the log phase of *S. hantzschii* cells were harvested by centrifugation at 300 ***g*** for 30 min (TNA-23, TX-160; TOMY, Fremont, CA, USA), and a portion (20 ml) of the diatom culture diluted by a DM medium poured on liquid soft agar (1·5%) was immediately spread onto the solid bottom agar (1·5% agar) and left to solidify for 1 day under the same algal growth conditions.

The filtered water sample (200 μl) with 5 μm pore polycarbonate membrane (7060-4713; Whatman, Maidstone, UK) was spread on the *S. hantzschii* algal lawn and the plates were incubated for 10 day under the same algal growth conditions. Micro-organisms forming clear zones around the colonies were gently picked with a needle, transferred and incubated at 30°C with a nutrient broth (NB) medium [D(+)-glucose 1·0 g, peptone 15·0 g, sodium chloride 6·0 g, yeast extract 3·0 g; Fluka, Steinheim, Germany] in the dark for 2 days, after which they were purified (Yamamoto and Suzuki 1990). Once purified, the bacterium was axenically maintained in the dark on nutrient agar medium (beef extract 3·0 g, peptone 5·0 g, agar 12·0 g; Difco, South Royal, Atlanta, GA, USA) plates containing 1·5% agar at 30°C and pH 7, and cryopreserved at −76°C in NB medium containing 20% glycerol.

To identify the isolate, a bacterial genomic DNA was extracted using proteinase K and phenol–chloroform–isoamylalcohol (P2069; Sigma, St Louis, MO). The 16S rDNA gene from the genomic DNA was amplified by PCR using primers 27F, 5′-AGAGTTTGATCATGGCTCAG-3′, and 1492R, 5′-GGTTACCTTGTTACGACTT-3′, as previously described by Kang *et al.* (2005). The amplicons were purified with a QiaQuick PCR purification kit (28106; Qiagen, Valencia, CA). Bacterial 16S rDNA sequences were then generated with an automated DNA sequencer using a Thermo-Sequence^{TM} 2.0 sequencing kit (USB, OH, USA) and Dual Dye automated sequencer (Model 4200; LI-COR, Lincoln, NE). BLAST search was performed to determine the closest phylogenetic type of the isolates. The data were manually aligned with *Pseudomonas* 16S rDNA sequences. PHYLIP version 3.57c (Felsenstein 1995) was used to further analyse the sequences. Genetic distances were estimated by a Kimura 2-parameter distance model; a phylogenetic tree was inferred by using the neighbour-joining algorithm of MEGA 2.0 (Charrel *et al.* 1999). The strength of the internal branches from the resulting trees was statistically tested by bootstrap analysis with 1000 replications. Referencing sequences for phylogenetic analysis

were obtained directly from GenBank. The 16S rDNA sequence of the bacterium has been deposited in the GenBank database under accession no. AY951875. The isolated bacterium was deposited in the publicly accessible culture collections of the Korean Culture Center of Microorganisms under deposit no. KFCC-11368P.

Algicidal activity of a bacterium SK09 in different conditions

To determine the optimal growth condition of the isolated bacterium, cells were grown at 10, 20, 30, 40, 50, and 60°C at pH 7 under gentle shaking in NB media. Cells were also grown at pH 4, 5, 6, 7, 8, 9, and 10 at 30°C in NB media. Bacterial growth was estimated using epifluorescence microscopic counts. Bacterial cells were counted at least 600 cells for each sample using an epifluorescence microscope (Axioplan; Zeiss, Göttingen, Germany) with DAPI (4′,6-diamidino-2-phenylindole) solution at a final concentration of 1 μg ml^{-1} (Porter and Feig 1980). The optimal condition determined from these investigations was used for all subsequent bacterial culturing.

To examine the algicidal activity of the bacterium at different densities, the bacterium was incubated at 30°C and 60 rpm in NB medium for 24 h, and was harvested by centrifugation at 5000 ***g*** for 20 min (TNA-23, TX-160; TOMY). Various inoculated densities of the bacteria were serially prepared by the dilution with a DM medium as follows, 1×10^5, 5×10^5, 1×10^6, and 5×10^6 cells ml^{-1}. The final bacterial cell density was confirmed as $5{\cdot}0 \pm 0{\cdot}4 \times 10^6$ cells ml^{-1} in the treatment of 5×10^6 cells ml^{-1}. *Stephanodiscus hantzschii* cells were previously cultured to log phase (9×10^5 cells ml^{-1}) for 10 days, and then SK09 was inoculated to the algal culture. The incubation was carried out at 15°C under 40 μE m^{-2} s^{-1} with a 12 : 12 (light : dark) cycle in a 300 ml flask containing 100 ml of *S. hantzschii* culture in triplicates. Algal cell density was monitored everyday using direct microscopic counts. A portion of sample was fixed with a glutaraldehyde solution at a final concentration of 2%. The samples were stored in the dark at 4°C until analysis. Over 500 phytoplankton cells were counted in each sample using a Sedgwick-Rafter counting chamber at ×400 magnification under a light microscope (Axiolab; Zeiss).

To examine the algicidal activities of SK09 against *S. hantzschii* in different growth phases such as lag ($1{\cdot}05 \times 10^5$ cells ml^{-1}), log ($9{\cdot}40 \times 10^5$ cells ml^{-1}), and stationary phase ($1{\cdot}96 \times 10^6$ cells ml^{-1}), the bacterium was inoculated at density of $5{\cdot}0 \pm 0{\cdot}4 \times 10^6$ cells ml^{-1} into a 300-ml flask containing 100 ml of *S. hantzschii* culture. The algicidal activity was also tested on several algae in log phase, including *M. aeruginosa* NIES 298 ($1{\cdot}3 \times 10^7$ cells ml^{-1}), *M. aeruginosa* NIES 44 ($1{\cdot}3 \times 10^7$ cells ml^{-1}), *M. aeruginosa* NIER 10001 ($1{\cdot}3 \times 10^7$ cells ml^{-1}) and *A. cylindrica* NIES 19 ($4{\cdot}4 \times 10^6$ cells ml^{-1}) in cyanophyceae; *C. astroideum* NIER 10088 ($1{\cdot}6 \times 10^6$ cells ml^{-1}) in chlorophyceae and *C. meneghiniana* HYK0210-CM01 ($4{\cdot}2 \times 10^5$ cells ml^{-1}) and *S.hantzschii* UTCC 267 ($9{\cdot}0 \times 10^5$ cells ml^{-1}) in bacillariophyceae. In this test, SK09 cells were inoculated at a final cell density of $5{\cdot}0 \times 10^6$ cells ml^{-1} into each 300 ml culture flask containing 100 ml algal culture. Incubation was carried out under the same conditions in triplicates for 10 days. Algal cell densities were monitored using direct microscopic counts.

Under the light microscope and scanning electron microscope (SEM), the algal morphology and bacterial attraction in the presence of the bacteria were observed. For the analysis with SEM, the specimen was subsampled at 4 and 12 h after inoculation. Subsamples were fixed in 2% glutaraldehyde for 24 h at 4°C, and postfixed in 1% osmium tetroxide at 4°C for 30 min, followed by rinsing with phosphate buffer (pH 7·2) after fixation. Specimens were then dehydrated by an ethanol series (30, 50, 70, 90, 100 and 100%; each stage for 30 min) and dried using a Critical-Point Dryer (SPI-DRY CPD, SPI, West Chester, PA). Finally, specimens were coated with gold for 3 min and examined under the SEM (JSM-5600 LV; JEOL, Tokyo, Japan).

Algicidal mode and location of algicidal substance of an algicidal bacterium HYK0210-SK09

To determine whether direct contact between algal and bacterial cells was required or not for the expression of algicidal activity, dialysis bag test was carried out according to Doucette *et al.* (1999). In the test, SK09 cells contained within the dialysis bags (300 000 MWCO, Spectra/Por CE sterile dispodialyzer; Spectrum, Laguna Hills, CA, USA) were added to the *Stephanodiscus* culture in log phase at a density of $9{\cdot}0 \times 10^5$ cells ml^{-1} (treatment). Final cell density of the bacterium to the total volume was 5×10^6 cells ml^{-1}. *Stephanodiscus* cell densities were compared with the control (no addition of SK09) for 6 days in triplicate.

To evaluate the algicidal activity of each cellular fraction of SK09 against *S. hantzschii*, the bacterial cells grown in 1 l of NB media was centrifuged at 10 000 ***g*** for 15 min (A500S-6, Supra 25K, Hanil Science, Lucheon, Korea). The supernatant was filtered through a 0·2-μm syringe filter and used as cell-free extract. The remaining bacterial pellet (5·0 g) was re-suspended with 20 ml of a freshly prepared isotonic buffer solution (TMS) containing Tris–HCl (50 mmol l^{-1}, pH 8·0), $MgCl_2$ (16 mmol l^{-1}), and sucrose [66% or, occasionally, 33% (w/v)], lysozyme (final concentration, 100–200 μg ml^{-1}) and phenylmethlysulfonyl fluoride (final concentration,

0·1 mmol l^{-1}). The cell suspension was incubated at 37°C for 30–60 min and centrifuged at 21 000 ***g*** at 4°C for 15 min. Then, the supernatant was carefully collected to obtain periplasmic proteins. The remained pellet was re-suspended in lysis buffer containing Tris–HCl (50 mmol l^{-1}, pH 8·0) and 5 mmol l^{-1} $MgSO_4$, followed by repeated sonication (VibramCell; Sonic & Materials Inc, Newtown, CT) at 50 A and 4°C for 5 min. The solution was centrifuged at 21 000 ***g*** at 4°C for 1 h to obtain the cytoplasm in supernatant. The lastly remained pellet was immediately re-suspended in TMS and acquired cytoplasmic membrane (Merchante *et al.* 1995). We quantified the protein content of all fractions and cell-free extracts according to the Bradford method (Bradford 1976) using bovine serum albumin (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as the protein standard. Three millilitre of *S. hantzschii* UTCC 267 culture containing 9×10^5 cells ml^{-1} was poured into 24-well plates. Six-hundred microlitre of each fraction was added to wells and incubated at 15°C with shaking at 60 rev min^{-1} and 40 μE m^{-2} s^{-1} under 12 : 12 (light : dark) cycles for 24 h. The experiment was carried out in triplicate, and cells of *S. hantzschii* were counted after the incubation. A unit of enzyme was defined as decreased algal cells (10^4 cells) within 24 h (Choi *et al.* 2005).

Algicidal activity of a bacterium HYK0210-SK09 against natural diatom-blooming water

To test the algicidal activity against naturally occurring *Stephanodiscus* bloom, we applied SK09 to a natural water sample. Bacterial cells grown in NB media were then harvested by centrifugation at 17 000 ***g*** for 20 min (A500S-6, Supra 25K, Hanil Science) and washed two times with phosphate buffer (pH 7·2) prior to experimental use. The natural water was taken from the Paldang reservoir on 6 January 2004 when *S. hantzschii* bloom was occurred ($1{\cdot}43 \times 10^4$ cells ml^{-1}). Four enclosures, which were made of cylindrical, nontransparent plastic (height: 0·4 m, width: 0·5 m, length: 0·5 m), were prepared in a culture room maintained at 15 ± 2°C. Water circulation in these mesocosms was controlled using an electronic circulator at 3 rpm. SK09 cells were inoculated on day 1 at a final density of 5×10^6 cells ml^{-1} to two enclosures containing 100 l of natural water. The experiment was carried out in duplicate (control and treatment) for 10 days. Samples were taken every day at 10.00 hours. To determine chlorophyll *a* concentrations, 300 ml of samples were immediately filtered through a 47 mm Whatman GF/F filter under low vacuum pressure. The filter was soaked in 15 ml cold 90% acetone-distilled water (v/v), then sonicated to break cell walls and extracted for 24 h in the dark at 4°C. Finally, chlorophyll *a* concentrations were measured using a spectrophotometer (8453; HP, Palo Alto, CA) according to the equation of Humphrey and Jeffrey (1997). To enumerate phytoplankton cells, 100 ml of samples were immediately fixed, and phytoplankton cells were counted.

Data analysis

The algicidal effect of SK09 on *S. hantzschii* was calculated using the following equation: algicidal effect (%) = $(1–Tt/Ct) \times 100$, where T was the algal density in the presence of bacteria (treatment group), C was the algal density in the absence of bacteria (control group) and *t* was time. To determine significant differences among the treatments, one-way ANOVA tests were applied. Correlation coefficients among the experiments were analysed using SAS Systems v. 8.12 (SAS Institute Inc., Cary, NC).

Results

An algicidal bacterium, *Pseudomonas fluorescens* HYK0210-SK09

During screening using the algal lawn and liquid tests, seven strains among 29 isolates displayed inhibitory activity against strain *S. hantzschii* UTCC 267. Of these seven, a strain HYK0210-SK09 showed the greatest anti-algal activity among isolated strains in this study. The strain was identified as *Ps. fluorescens*, (hereafter SK09) by 16S rDNA sequence (Fig. 1). When analysed the physiological tests, SK09 is Gram negative, rod type, nonpigmentation and swarming in NB medium. The optimal range of growth temperature for SK09 was between 10 and 30°C, while the optimal pH value was between 5 and 9.

Algicidal activity of *Pseudomonas fluorescens* HYK0210-SK09

Algicidal activities at inoculated density of 1 and 5×10^6 cells ml^{-1} were 90% and 99% for 4 days respectively, and then *S. hantzschii* cells were perfectly destructed by SK09 within 8 and 5 days respectively. Consequently, *S. hantzschii* did not occur after 8 days in these treatments. When SK09 was inoculated at 1×10^5 and 5×10^5 cells ml^{-1}, cells of *S. hantzschii* were not lysed (Fig. 2). When the inoculation density was 5×10^5 cells ml^{-1}, *S. hantzschii* cells decreased during the first 2 days, but then returned to its initial concentration. In the case of 1×10^5 cells ml^{-1}, results were negligible, and were similar to those of the control group (Fig. 2).

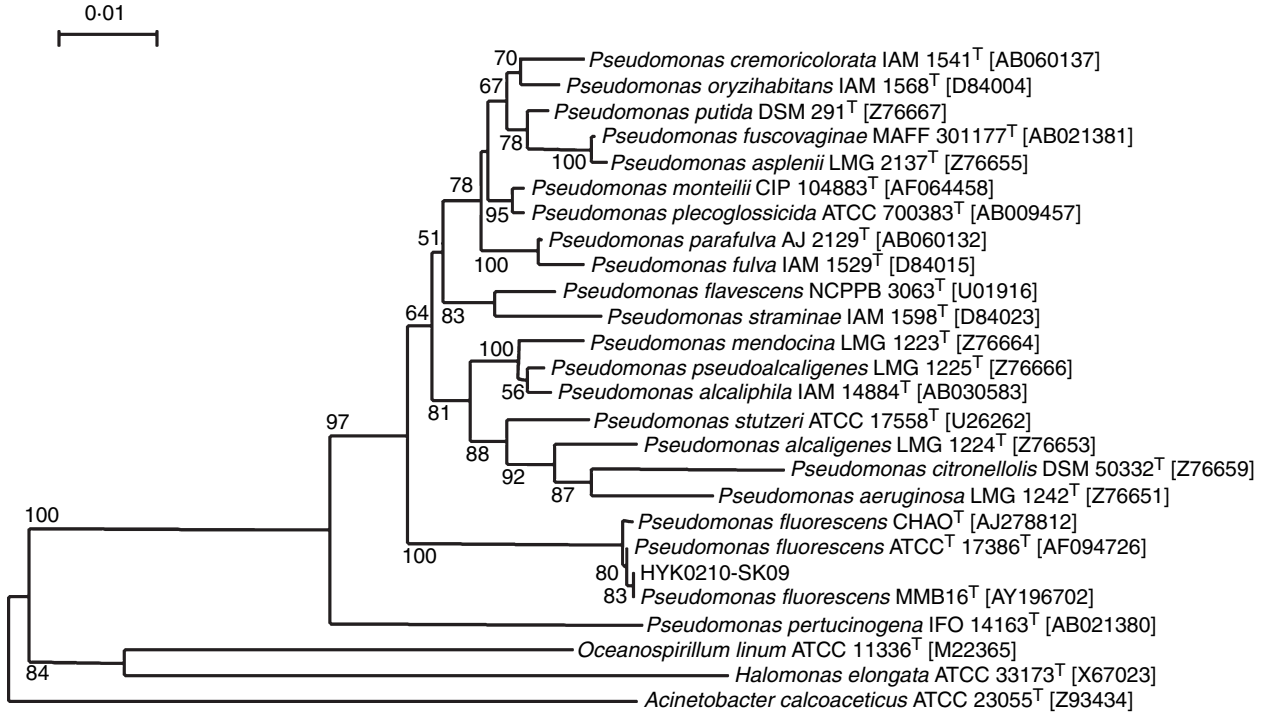

Figure 1 Phylogenetic tree based on 16S rDNA sequences showing the relative positions of the isolated *Pseudomonas fluorescens* HYK0210-SK09. Scale bar represents 0·01 substitutions per nucleotide position.

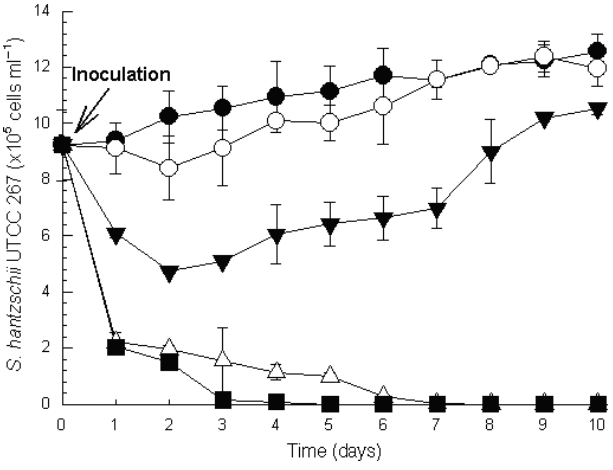

Figure 2 Algicidal effects of *Pseudomonas fluorescens* HYK0210-SK09 with different inoculation densities on *Stephanodiscus hantzschii* UTCC 267. Inoculation densities of *Ps. fluorescens* HYK0210-SK09: (●) control; (○) 1×10^5 cells ml^{-1}; (▼) 5×10^5 cells ml^{-1}; (□) 1×10^6 cells ml^{-1}; (■) 5×10^6 cells ml^{-1}. The arrow indicates inoculation of the bacterium. Data are mean ± SD for three replications.

We confirmed the algicidal activity against an each different growth phase of *S. hantzschii* such as lag (1 day old at density of $1{\cdot}05 \times 10^5$ cells ml^{-1}), log (10 day old, $9{\cdot}40 \times 10^5$ cells ml^{-1}) and stationary-phase (16 day old, $1{\cdot}96 \times 10^6$ cells ml^{-1}). Algicidal effects were significantly different among the three phases ($P < 0{\cdot}05$, ANOVA). When SK09 was inoculated in lag phase, although the growth of *S. hantzschii* had been suppressed during the first 16 days, the algal cells after 14 days rapidly increased from $0{\cdot}8 \times 10^5$ to $18{\cdot}4 \times 10^5$ cells ml^{-1} at 30 days. When SK09 was inoculated in the log phase, the diatom cells continuously increased for 3 days after the inoculation of SK09, but in 7 days after of the inoculation, 99% of *S. hantzschii* cells were degradated, and then the host alga completely were controlled within 10 days. When SK09 was inoculated in stationary phase, *S. hantzschii* cells were not degradated (or lysed) but suppressed their growth by SK09 (Fig. 3).

Change in cell densities of the bacterium are shown in Fig. 4. The growth rate of SK09 was low for the first 2 days after the inoculation. However, after 8 days of the inoculation, the algal cells decreased to 91% of the cell density when compared with those in the control. The bacterial cell density increased more than eightfold at 8 days after inoculation and exhibiting a density of $4{\cdot}3 \times 10^7$ cells ml^{-1}.

Observation using SEM revealed that SK09 rapidly covered and attached to the girdle view (side part) of the diatom cells within 4 h (Fig. 5a) using pili. Within 12 h, the side of *S. hantzschii* cells was breached (Fig. 5b). Therefore,

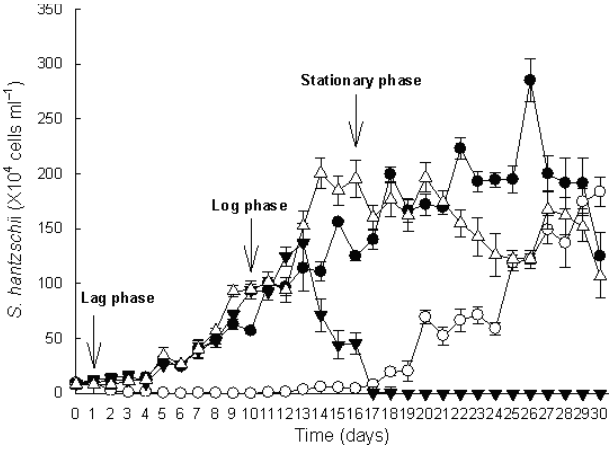

Figure 3 Changes in cell density of *Stephanodiscus hantzschii* UTCC 267. Algicidal bacterium, *Pseudomonas fluorescens* HYK0210-SK09 was inoculated to the *S. hantzschii* culture in different growth phase at a final cell density of 5×10^6 cells ml^{-1}. Inoculation times of *Ps. fluorescens* HYK0210-SK09: (●) control; (○) Lag phase; (▼) log phase; (△) stationary phase. The arrows represent the inoculation of *Ps. fluorescens* HYK0210-SK09 to each growth phase of *S. hantzschii* cells. Data are mean ± SD from experiments for three replications. Control was not inoculated with the bacterium in the algal culture.

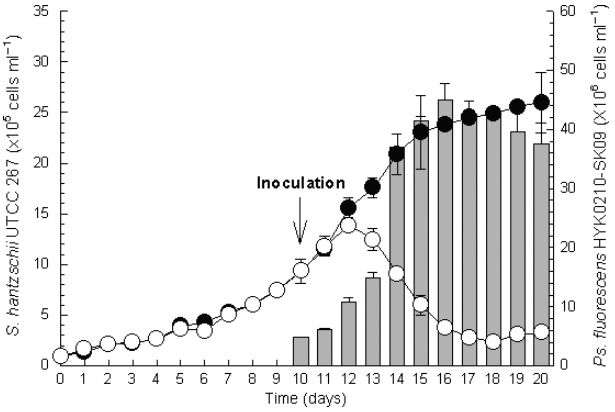

Figure 4 The growth of *Pseudomonas fluorescens* HYK0210-SK09 cells (□) and changes in *Stephanodiscus hantzschii* cell densities cultured with (treatment, ○) or without (control, ●) *Ps. fluorescens* HYK0210-SK09 cells. The arrow indicates the inoculation of *Ps. fluorescens* HYK0210-SK09 (5×10^6 cells ml^{-1}) to log phase of *S. hantzschii* cells. Data are mean ± SD for three replications.

we assumed that SK09 directly attached to girdle view for the destruction of *S. hantzschii*. After the cell walls were breached, other bacteria swam towards the lysed cells from outside to a breached target alga (swarming behaviour).

SK09 did not inhibit or suppress the growth of other algae such as *M. aeruginosa* NIER 10001 (−2·9%), NIES 44 (−11·4%), and NIES 298 (−7·0%), and *A. cylindrica* NIES 19 (−32·4%), *C. astroideum* NIER 10088 (8·0%)

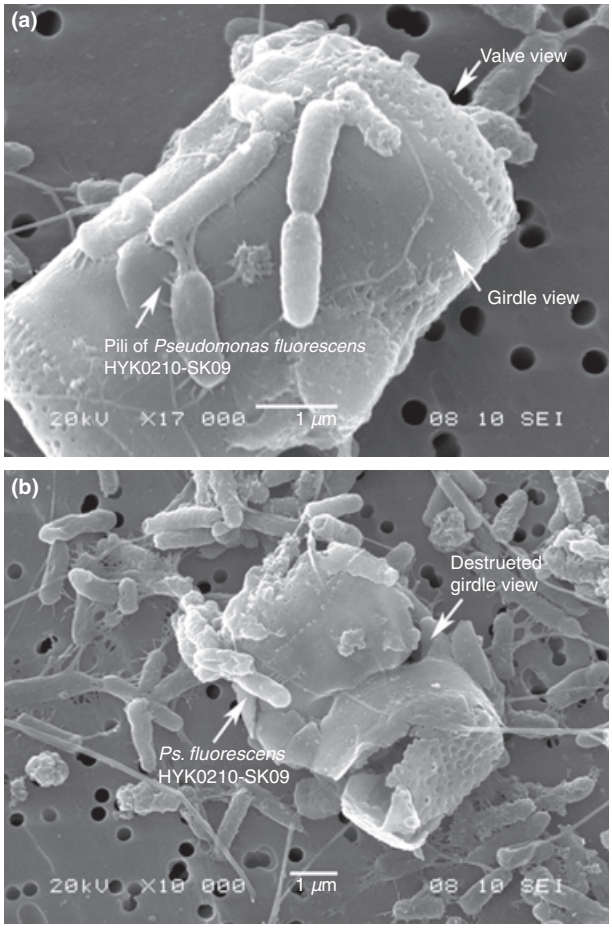

Figure 5 Scanning electron microscopic observations of *Stephanodiscus hantzschii* UTCC 267 attacked by *Pseudomonas fluorescens* HYK0210-SK09: (a) a cell of *S. hantzschii* at 4 h after inoculation; *Ps. fluorescens* HYK0210-SK09 mainly attached on girdle view by released the bacterial pili, while the bacteria were barely attached on valve view: (b) a cell of *S. hantzschii* at 12 h after inoculation; the cell was destroyed on girdle view more than on valve view.

and *C. meneghiniana* HYK0210-CM1 (8·3%). Notably, the growth of *C. meneghiniana* HYK0210-CM1 which has a similar morphological structure to *S. hantzschii* was not inhibited; rather it was only suppressed (Table 1).

Algicidal mode and location of algicidal substances of *Pseudomonas fluorescens* HYK0210-SK09

During 6 days of incubation, cell density of *Stephanodiscus* increased from $7{\cdot}95 \pm 0{\cdot}78 \times 10^5$ to $13{\cdot}30 \pm 0{\cdot}01 \times 10^5$ in the control and to $11{\cdot}70 \pm 1{\cdot}41 \times 10^5$ cells ml^{-1} in the treatment. Significant difference was not detected between them ($P > 0{\cdot}05$, *t*-test). This result shows that algicidal mode of SK09 is direct attack. To determine the location of algicidal substances, we analysed the cell-free extracts

Table 1 Algicidal effects of isolated *Pseudomonas fluorescens* HYK0210-SK09 against several algae

Host alga	Algicidal effect (±SD)
Bacillariophyceae	
Stephanodiscus hantzschii UTCC267	90·2 ± 3·7*
Cyclotella meneghiniana HYK0210-CM1	8·3 ± 12·4*
Cyanophyceae	
Microcystis aeruginosa NIER 10001	−2·9 ± 0·6*
Microcystis aeruginosa NIES 298	−7·0 ± 20·8*
Microcystis aeruginosa NIES 44	−11·4 ± 28·8*
Anabaena cylindrica NIES 19	−32·4 ± 18·2*
Chlorophyceae	
Coelastrum astroideum NIER 10088	8·0 ± 8·7

Inoculation density: 5×10^6 cells ml^{-1}, and cultured for 10 days.
UTCC, University of Toronto Culture Collection of Algae and Cyanobacteria in Canada; NIER, National Institute of Environmental Research in the Republic of Korea; NIES, National Institute for Environmental Studies in Japan
*Paired *t*-tests were analysed to determine significant differences ($P < 0{\cdot}01$) between treatment and control groups.

Table 2 Algicidal activities and protein assay of each cellular fraction of *Pseudomonas fluorescens* HYK0210-SK09 by the Bradford method

Cell compartment	Alga-lytic activity (units)	Protein concentration (mg ml^{-1})	Specific activity (units mg^{-1})
Cell-free extract	0·00	0·36	0·00
Cell extract	40·85	11·72	3·49
Periplasm	2·38	3·21	0·74
Cytoplasm	49·83	10·39	4·80
Cytoplasmic membrane	0·09	1·01	0·09

A unit of enzyme: the amount of enzyme that reduced 10^4 algal cells after 24 h.
Specific activity (unit mg^{-1}) = degradation activity (unit) protein concentration (mg)$^{-1}$.

and cell extracts (periplasm, cytoplasm and cytoplasmic membrane). In cell extracts of SK09, they showed algalytic activity (40·85 units) and specific activity was 3·49 units mg^{-1}. Notably, in cellular fractions of the periplasm, cytoplasm and cytoplasmic membrane in cell extracts, cytoplasm showed higher activity (specific activity of 4·8 units mg^{-1}) indicating that algicidal materials were mostly in the cytoplasm (Table 2).

Application of *Pseudomonas fluorescens* HYK0210-SK09 to naturally occurring *Stephanodiscus* bloom

Before the inoculation of SK09 into mesocosms, the phytoplankton communities (density of $1{\cdot}4 \pm 0{\cdot}0 \times 10^4$ cells ml^{-1}) were predominated by *S. hantzschii* population,

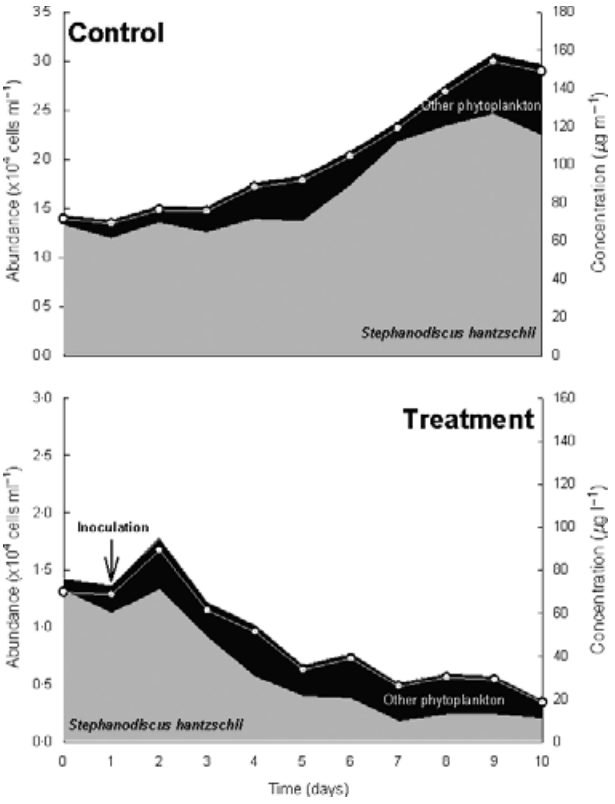

Figure 6 The algicidal effects of *Pseudomonas fluorescens* HYK0210-SK09 against *Stephanodiscus hantzschii* blooming natural water: (▨) The variation of *S. hantzschii* cells, (■) other phytoplankton populations except *S. hantzschii*, (○) chlorophyll *a* concentrations. The arrow represents the inoculation of *Ps. fluorescens* HYK0210-SK09 at a cell density of 5×10^6 cells ml^{-1} in treatment group (panel B).

(89% to the total phytoplankton cells, Fig. 6). In the control group, cell densities of total phytoplankton and *S. hantzschii* increased from $1{\cdot}4 \times 10^4$ to $2{\cdot}9 \times 10^4$ cells ml^{-1} and $1{\cdot}3 \times 10^4$ to $2{\cdot}2 \times 10^4$ cells ml^{-1} respectively. In the treatment, *S. hantzschii* cells rapidly decreased from $11{\cdot}3 \times 10^4$ to $0{\cdot}2 \times 10^4$ cells ml^{-1} within 10 days, while other phytoplankton populations which mainly occupied with diatoms such as *Cyclotella*, *Asterionella* and *Fragilaria* were not varied by attack of SK09. The ratio of *S. hantzschii* cells *vs* total phytoplankton abundances decreased from 93% on day 0 to 56% on day 4 (Fig. 6). Chlorophyll *a* concentrations also showed similar results as those of phytoplankton abundances, and significantly decreased by 90% compared with the concentration in the control group.

Discussion

SK09 exhibits a stronger algicidal effect than previous isolates of SK02 (Kang *et al.* 2005). SK09 degradated 99% of

S. hantzschii cells at the inoculation density of 5×10^6 cells ml^{-1}, while SK02 degradated 80% of the cells at a density of 2×10^7 cells ml^{-1}. The inoculation density (5×10^6 cells ml^{-1}) of SK09 was also lower than those of others (Doucette *et al.* 1999; Mayali and Azam 2004). It is an advantage of SK09 in its application to control natural *S. hantzschii* blooms.

Most algicidal bacteria belong to the genus *Cytophaga* or *Saprospira* and directly attack the host algal cells, while other bacteria from the genus *Alteromonas* or *Pseudoalteromonas* indirectly kill the target algae by releasing algicides (Mayali and Azam 2004). SK09 requires direct attack to kill its host algal cells. The algicidal substances of SK09 were located mainly in the cytoplasm (Table 2). In contrast, the algicidal substances of SK02 were found in the cytoplasmic membrane (Kang *et al.* 2005). Thus, although two bacteria are same genera of *Pseudomonas* (SK09 and SK02), their algicidal substances are different. This is one possible reason to explain the strong algicidal activity of SK09 over SK02.

In addition to this, SK09 has some observable differences in physiological characteristics with SK02. Scanning electron microscopic observation revealed that none of the surviving *S. hantzschii* had SK09 attached by pili to the diatom cell. After attachment of SK09 to *S. hantzschii*, the host alga may be degradated by lysozyme-like enzymes (Baker and Herson 1978; Lee and Park 1998; Kitaguchi *et al.* 2001). When comparing SK09 and SK02, elongated pili from SK02 were easily dissociated from the cell surface of destroyed *S. hantzschii* (Kang *et al.* 2005), while those of SK09 remained (Fig. 5a,b). Moreover, SK09 possessed numerous thin and sticky pili on the cell surface, which may make it easier for the bacteria to attach to the girdle view of *S. hantzschii* [a relatively weak structural component on the side, rather than to the valve view (front part) of *S. hantzschii*]. This probably plays a crucial role in determining bacterial algicidal ability against *S. hantzschii*.

Results of this experiment lead us to propose a six stages conceptual model to describe interactions between an algicidal bacterium and its target algal species, (i) requirement of optimum cell densities of the algicidal bacterium, SK09 to express the algicidal activity, (ii) SK09 swarming behaviour allows itself to identify and attach to the target algal cells, (iii) attachment of the bacterium to the surface of the target alga, *S. hantzschii*, followed by cell lysis and release of intracellular substances, (iv) increased bacterial density in response to degradation of algal-derived nutrients (in preparation), (v) continuous attack of the algicidal bacterium on varying temporal and spatial scales during the bloom, (vi) the bloom begins to decline, accompanied by a reduction in levels of *S. hantzschii*-derived organics. This algicidal mechanism of SK09 is similar to that reported from the previous research of Doucette *et al.* (1999), who isolated bacteria that showed algicidal effects on *Gymnodinium breve*. The hypothetical model, which we propose here, will serve as a testable framework for ensuring field studies (Doucette *et al.* 1999).

In the application of algicidal bacteria to control the naturally occurring algal bloom, the host range of the bacterium is important. SK09 showed narrow host range (Table 1). SK09 could not cause cell lyses of *C. meneghiniana,* which has a similar morphology to *S. hantzschii* (Table 1). Growth of *Microcystis* and *Anabaena,* both nuisance species, were stimulated by the inoculation of SK09, indicating that application of SK09 could cause bloom formation of these cyanobacteria. However, these cyanobacteria generally occur in summer season, while *Stephanodiscus* predominate in winter season. For example, Jung *et al.* (2003) reported that *Stephanodiscus* cells ($1{\cdot}3 \times 10^4$ cells ml^{-1}) occupied more than 90% of total phytoplankton ($1{\cdot}5 \times 10^4$ cells ml^{-1}) in terms of cell density during its bloom in the Han River in December 2001. At that time cyanobacterial cells were not detected. Thus, SK09 may not cause cyanobacterial blooms. Indeed, our indoor mesocosm experiment demonstrated that inoculation of SK09 did not cause other algal growth (Fig. 6).

Another specific problem for the application of SK09 against *Stephanodiscus* blooms is the low water temperature. During the bloom of the *Stephanodiscus*, water temperature was below 13°C (Jung *et al.* 2003). Therefore, low temperature is an essential requirement for the expression of algicidal activity of SK09. In this study, we confirmed that SK09 grows well between 10 and 30°C. Thus, it is possible to apply this bacterium to naturally occurring *Stephanodiscus* bloom.

Loss of the applied bacterium by grazing is another factor to be considered in the application to natural algal blooms. Katano *et al.* (2006) reported that grazing by flagellates on algicidal bacteria strongly affected the expression of algicidal activity in natural microbial communities. In addition, interaction between other organisms in natural environments should also be considered. Nagasaki *et al.* (2000) demonstrated that a bacterium prohibited the algicidal activity of *Cytophaga* sp. AA8-2. Mayali and Doucette (2002) also reported that bacterial assemblage co-cultured with *Karenia brevis* prevented the expression of algicidal effect of *Cytophaga* sp. 41-DBG2. Therefore, we have to carefully examine the algicidal activity in natural environments to develop the biological control of algal blooms using bacteria.

In conclusion, this study clearly demonstrated that SK09 is an effective bio-agent to control blooms of *S. hantzschii*. The indoor mesocosm experiment also revealed that SK09 effectively controlled the *S. hantzschii* bloom in natural waters without any other algal bloom

(Fig. 6). Therefore, we propose the strain SK09 as a possible bio-agent in the biological control of *Stephanodiscus* bloom during the winter season.

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