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Streptomyces neyagawaensis as a control for the hazardous biomass of *Microcystis aeruginosa* (Cyanobacteria) in eutrophic freshwaters

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Abstract

An aquatic bacterium capable of eliminating the cyanobacterium *Microcystis aeruginosa* was isolated from the sediment of an eutrophic lake (Lake Juam, Korea). On the basis of 16S rDNA sequences and biochemical and morphological characteristics, the isolate was determined to be *Streptomyces neyagawaensis*. It grew optimally at 40 °C and pH 7. In the presence of this bacterium, the biomass of cyanobacterium *M. aeruginosa* NIES-298 was strongly suppressed, by up to 84.5% in abundance compared to the control. The antialgal activity of *S. neyagawaensis* depended on the growth phase of the cyanobacterium, but not of the antialgal bacterium. The antialgal activity of *S. neyagawaensis* was effective against a wide range of algae, including the green alga *Chlorella* sp., the diatoms *Aulacoseira granulata* and *Stephanodiscus hantzschii*, and four cyanobacteria, *M. aeruginosa* NIES-44, *Anabaena cylindrica*, *Anabaena flos-aquae*, and *Oscillatoria sancta*. *S. neyagawaensis* indirectly attacked *M. aeruginosa* by secretion of extracellular antialgal substances that were localized in the bacterial periplasm and had a specific activity of 7.7 U/μg. These results suggest that indigenous bacteria isolated from sediments may have potential application in controlling harmful cyanobacterial blooms in freshwaters.

Keywords: Antialgal bacterium; *Streptomyces neyagawaensis*; *Microcystis aeruginosa*; Biological control; Freshwater; Cyanobacterial bloom

1. Introduction

Presently, the direct application of chemicals to control algal blooms harm and/or eventually disrupt the aquatic ecosystem by killing off beneficial plankton and even fish (McGuire et al., 1984; Reynolds, 1984; Sevrin-Reyssac and Pletikosic, 1990). Consistent with the panecological and environmental approaches to lake water conservation, many countries are currently seeking to develop suitable biological control agents (Barnet et al., 1981; Brabrand et al., 1983; Desjardins, 1983; Imai et al., 1993; Kim et al., 2003; Manage et al., 2000; Redhead and Wright, 1978; Reynolds and Walsby, 1975).

In particular, blooms of the cyanobacterium *Microcystis aeruginosa* (Kützing) Lemmermann f. *aeruginosa* are widespread in eutrophic lakes and reservoirs worldwide (Carmichael, 1992; Han et al., 2002; Hong et al., 2002; Vincent, 1987), and may lead to the production of microcystin, a hepatotoxin that affects fish, birds, wild animals, livestock, and humans. It is associated with allergies, irritation reactions, gastroenteritis, liver diseases, and tumors (An and Carmichael, 1994; Bell and Codd, 1994; Dawson, 1998). Historically, these cyanobacterial (algal) blooms have also caused other problems, such as foul odors, decreased aesthetic value, deterioration of water quality, and deoxygenation of water (Sigee et al., 1999).

It is generally accepted that bacteria can affect algal dynamics, either negatively or positively. In the laboratory, a number of experiments have supported the antialgal ability of bacteria (Daft et al., 1975; Kim et al., 2003; Kodani et al., 2002; Lee et al., 2000; Manage et al., 2000; Sigee et al., 1999; Yamamoto et al., 1998). Under natural conditions, levels of the antialgal gliding bacterium

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Cytophaga sp. were closely related to fluctuations and reduction of red tides caused by the flagellate *Chatonella* spp. in the Seto Inland Sea, Japan (Imai et al., 2001). In freshwater, antialgal bacteria have been reported to affect blooms of the cyanobacterium *Anabaena* sp. in the Daechung Dam, South Korea (Kim et al., 1998).

Thus, it is possible that an antialgal bacterium may be able to control blooms of the freshwater cyanobacterium, *Microcystis* sp. However, there have been no previous reports of controlling harmful algal blooms by using an anti-*Microcystis* bacterium. Here, we report the discovery of an antialgal bacterium active against *M. aeruginosa* and present the antialgal features of this bacterium in regards to its activity at various algal and bacterial growth phases and in relation to other predominant algae. We report also on the antialgal mode (direct or indirect) and the localization of the antialgal substances within the bacterial cell.

2. Materials and methods

2.1. Microorganisms and culture conditions

The cyanobacterium *M. aeruginosa* NIES-298 was obtained from The National Institute for Environmental Studies (NIES), Tsukuba, Japan, and cultured in CB (NIES, 2000) medium under an illumination of 50–80 μmol photon/m^2/s on a 12L:12D cycle, with samples rotated on an orbital shaker (150 rpm) at 25 °C. The biomass was determined by direct counting of cells using a haemocytometer after dispersing colonial cells by ultrasonic disintegration (20 kHz, 60 s) or by measurement of chlorophyll *a* by in vivo fluorometry (Turner Designs Model 10-AU, Sunnyvale, California, USA). The isolated antialgal bacterium *Streptomyces neyagawaensis* was cultured at 40 °C in nutrient broth (NB), with gentle orbital shaking of 150 rpm in the dark. The bacterial biomass was determined by dry weight (APHA, 1998).

2.2. Screening of antialgal bacteria

Antialgal bacteria were isolated by the double-layer algal-lawn method (Sakata et al., 1991) from sediment and surface water samples collected from the following Korean locations where cyanobacterial blooms had previously occurred: Juam, Pal'tang, Seokchon, Daecheong, Murwang, Gwarim, and Dochang Reservoirs (Han et al., 1995, 2002; Jeong et al., 1998; Lee, 1999; Lee et al., 2002). Cultures of *M. aeruginosa* NIES-298 were grown in CB medium for 7 days and harvested by centrifugation at 3000*g* for 20 min. The culture pellet was mixed with molten CB soft agar medium and poured onto a CB agar plate (87 mm in diameter). After the cyanobacterial lawn was cultivated for 2–3 days under the *M. aeruginosa* culture condition mentioned above, 200 μl samples were spread on the cyanobacterial lawn. After 5–10 days incubation, the antialgal bacterium that formed clear zones of inhibition on the lawn were isolated by serial streaking onto the nutrient agar (NA) plates. The isolates were axenically maintained in the dark on NA plates and cryopreserved at −76 °C in NB medium containing 20% glycerol.

2.3. Antialgal activity test of isolated bacteria on *M. aeruginosa*

To test the antialgal activity of all isolates and/or select the most effective bacterium on *M. aeruginosa*, we tried two methods: a paper-disc test and a liquid-culture test. First, the bacteria isolated were grown at 40 °C with shaking in NB medium for 2 days. A 5 mm diameter Whatman GF/F filter (pore size = 0.7 μm) soaked with each 100 μl of cultured isolates (i.e., bacteria-coated paper) were placed on the algal lawn, and incubated 5 days. The antialgal activity was measured by the diameter of the clear zone formed in the algal lawn. Second, the antialgal bacteria grown at the above condition were inoculated into test tubes (50 ml in capacity), which included 25 ml of *M. aeruginosa* NIES-298, and cultured for 5 days. To measure the antialgal activity of isolates, the concentration of chlorophyll *a* was measured by in vivo fluorometry (Tuner Designs, Sunnyvale, California). Among 57 isolates tested, HYJ0209-MK50 showed the best antialgal activity on *M. aeruginosa* in two tests. All tests had three replications.

2.4. Optimizing the growth conditions for the antialgal bacterium HYJ0209-MK50

The isolated antialgal bacterium was grown at 25, 30, 35, 40, 45, and 50 °C at pH 7 and gentle shaking in NB. It was also grown at pH 5, 6, 7, 8, 9, and 10 at 40 °C in NB. The bacterial growth was estimated by dry weight after 24 h of incubation. The optimal condition determined from these experiments was used for all subsequent bacterial culturing. All experiments had three replications.

2.5. Biochemical characterization of the antialgal bacterium HYJ0209-MK50

To identify and characterize the most effective bacterium, HYJ0209-MK50, we incubated the isolate on several types of ISP (International Streptomyces Project) media (Atlas, 1997) at 40 °C. We also assessed the colony morphology of the isolate according to methods of Shirling and Gottlieb (1972). The analysis of 2,6-diaminopimelic acid (DAP) pattern was performed according to Staneck and Roberts (1974) using thin layer chromatography (TLC) with a methanol:water:6 M HCl:pyridine (80:26:4:10 by volume) solvent system.

2.6. Identification of the antialgal bacterium HYJ0209-MK50 by 16S rDNA sequences

DNA was prepared from the bacterial samples using the DNeasy Tissue Kit (Qiagen, Hilden, Germany), and 16S rDNA was PCR-amplified with the following primers: forward 5′-GAGTTGGATCCTGGCTCAG-3′ and reverse 5′-AAGGAGGGGATCCAGCC-3′. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by automated DNA sequencer (Bionex, Seoul, Korea) using the SequiTherm EXCEL II Labeled Primer Sequencing Kit (LI-COR, Lincoln, NE, USA) with the T3F and M13R primers. The resulting 16S rDNA sequence was aligned using the CLUSTAL W software (Nigam et al., 2000), and the distance matrices were calculated using the DNADIST program within the PHYLIP package. A phylogenetic tree was constructed by the neighbor-joining method, based on the calculated distance matrix.

2.7. Range of antialgal activity of HYJ0209-MK50

The bacterium was cultured at 40 °C in nutrient broth (NB), with orbital shaking at 150 rpm in the dark for 24 h. After harvest by centrifugation at 14,000*g* for 20 min, the concentration was adjusted to an initial density of 1% (w/v, dry weight) of total volume of the culture system of each algal species. Samples of each alga were collected from exponential-phase cultures of the following: cyanobacteria *M. aeruginosa* NIES-44, *Anabaena macrospora* (Lyngbye) Brebisson ATCC22664, *Anabaena cylindrica* Lemmermann NIES-19, *Anabaena affinis* Lemmermann KCTC-AG10008, *Anabaena flos-aquae* Brebisson ex Bornet et Flahault 1886 KCTC-AG10011, *Oscillatoria sancta* (Kutzing) Gomont, NIER-10027, *Synechococcus bacillaris* Butcher 1952 CCAP1479/7; green alga *Chlorella* sp.; diatom *Stephanodiscus hantzchii* Grunow UTCC267, *Aulacoseira granulata* (Ehrenberg) Simonsen 1979 CCAP 10021/1, and *Cyclotella* sp. (Table 3). The green algae and cyanobacteria were cultured in BG11 medium at 30 °C, pH 9, with gentle shaking, under 50 μmol photons/m^2/s on a 12L:12D cycle. The diatom medium (DM) was used for two diatoms under the same conditions as above for the algae.

2.8. Preparation of cell-free extracts and culture supernatants

The HYJ0209-MK50 isolate was cultivated in 1 L NB and centrifuged (Supra 25K, Hanil Science, South Korea) for 15 min at 450,000*g*. The supernatant was filtered through 0.2-μm syringe filter and used as bacterial culture supernatant, i.e., monoculture filtrate. The bacterial pellet (4.7 g) was washed twice with phosphate buffer (pH 7) and resuspended in 4.5 ml of 10 mM phosphate buffer (pH 7). The bacterial cells were sonicated (VibramCell, Sonic & Materials, USA) at 50A for 5 min at 5 °C, and the cell debris was removed by centrifugation at 900,000*g* for 2 h. The supernatant was used as a cell-free extract. We also prepared the supernatant from the bacterial culture co-cultivated in CB medium with the cyanobacterium *M. aeruginosa*. To obtain the mixed-culture filtrate, the bacterial culture was centrifuged for 15 min at 17,000*g* and the supernatant was sterilized by syringe-filtering through a 0.2-μm pore membrane. We did not obtain the cell-free extract from the mixed-culture filtrate due to the co-culture of the cyanobacterium.

2.9. Preparation of cell fractions

To obtain cell fraction (Rafael et al., 1995), 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 17,000*g* for 30 min at 4 °C. To obtain the cytoplasm and cytoplasmic membrane, the spheroplast, without cell wall, was homogenized by a mortar and pestle for 90 min in 10 mM phosphate buffer (pH 7) at 5 °C and centrifuged at 21,000*g*. We obtained the supernatant, the cytoplasm fraction, by centrifugation at 900,000*g* for 5 h at 4 °C. The remaining pellet was resuspended in 10 mM phosphate buffer (pH 7) and used as the cytoplasmic membrane fraction.

2.10. Analysis of enzyme activity

We quantified the protein content of all fractions and culture supernatants according to the Bradford method (Bradford, 1976) using bovine serum albumin (Amersham–Pharmacia Biotech, Piscataway, NJ, USA) as the protein standard. *M. aeruginosa* NIES-298 was treated with the various protein fractions (20% v/v) in 24-well plates (Falcon, USA), and the cells were counted with a haemocytometer after 24 h. A unit of enzyme was defined as the amount of enzyme that reduced 10^4 algal cells after 24 h.

3. Results

3.1. Screening of bacterial isolates and identification of the most effective isolate

The antialgal bacterium, HYJ0209-MK50, isolated from a sample taken in the sediment of Juam Reservoir on September 6, 2002, showed the highest activities from both the paper-disc and liquid-culture tests. The optimal growing conditions for this bacterium were pH 7 and 40 °C. The bacterium showed typical characteristics of *Streptomyces*, such as aggregation of mycelium

Table 1
Characterization of the antialgal bacterium HYJ0209-MK50 by culturing at 40 °C on ISP (International Streptomyces Project) media

ISP media	Substrate mycelium	Aerial mycelium	Pigment
Tryptone-yeast extract (ISP 1)	++ Gray	++ White	–
Yeast extract-malt extract (ISP 2)	+ White	+ White	–
Glycerol-asparagine (ISP 5)	++ White	–	–
Peptone-yeast extract-iron (ISP 6)	+ White	–	Olive brown
Tyrosine (ISP 7)	++ Yellow	–	Olive brown
Nitrate (ISP 8)	+ White	++ White	–
ISP 9	++ Yellow	+ White	–

in broth medium and formation of hard colony surfaces on agar plates. The isolate grew on ISP media 1, 2, 5, 6, 7, 8, and 9, forming an olive brown pigment on ISP 6 and 7 (Table 1). The isolate was Gram-positive, and whole-cell hydrolysates had positive LL-diaminopimelic acid (LL-DAP) activity and consequently was classified to have cell wall type I. All biochemical characteristics were consistent with the genus *Streptomyces* (Table 2). Specifically, the aerial mycelium observed growing on ISP 1 began as yellow but progressed to black, white, and then gray according to incubation time, which is characteristic of *S. neyagawaensis* (Shirling and Gottlieb, 1972). This identification was confirmed by comparing the 16S rDNA sequence of HYJ0209-MK50 with the sequences of various type strain of *Streptomyces* strains. The HYJ0209-MK50 16S sequence was most similar to that of *S. neyagawaensis* ATCC27449^T (99.3%), prompting us to identify our isolate as *S. neyagawaensis* HYJ0209-MK50 (Fig. 1). *S. neyagawaensis* HYJ0209-MK50 has been deposited in the Korean Culture Center of Microorganisms (KCCM-10463).

Table 2
Taxonomic and physiological characteristics used to identify the isolate HYJ0209-MK50 as *S. neyagawaensis*[a]

Characteristics	Strain HYJ0209-MK50
Spore chain morphology	Spiral
Cell wall constituent	LL-DAP
Hydrolysis of starch	–
Liquefaction of gelatin	–
Coagulation of skim milk	–
Hydrolysis of casein	+
Tolerance to NaCl	Below 4%
Carbon utility	
L-Arabinose	+
D-Fructose	+
Galactose	+
Myo-inositol	+
D-Mannitol	+
Raffinose	+
L-Rhamnose	+
Salicine	–
Sucrose	+
Cellulose	–
D-Xylose	+

[a] This comparison was performed to validate the results of 16S rDNA and phylogenic tree analysis (Fig. 1).

3.2. Antialgal effect of *S. neyagawaensis* HYJ0209-MK50 on *M. aeruginosa*

The cyanobacterium *M. aeruginosa* was clearly suppressed by treatment with the isolated *S. neyagawaensis* (Fig. 2). After 2 days of bacterial treatment, the cyanobacterial biomass was suppressed by 34.1%, and after 7 days, by 84.5%, compared to the control. Dry weight of the antialgal bacterium was determined by direct measurement of the difference between the total dry weight of the cyanobacterium co-cultured with the antialgal bacterium minus the dry weight of cyanobacterium at the same density. In contrast, the biomass of the antialgal bacterium showed no distinct change. To determine the effect of reciprocal growth phases, we examined antialgal activity during different rates of growth of both the bacterial isolate and the cyanobacterium (Fig. 3). After 3 days of incubation, the lag and log phase *M. aeruginosa* NIES-298 were suppressed 32.9 and 49.2%, respectively, by treatment with *S. neyagawaensis*. After 7 days, the cyanobacterium at lag and log phases were suppressed up to 96.9 and 41.5%, respectively. In contrast, stationary algae were not inhibited by the bacterium ($t = -0.731$, $P = 0.475$).

3.3. Effect of bacterial growth phase on its antialgal activity

When cultures of *S. neyagawaensis* at the lag, log, and stationary growth phases were inoculated into log-phase cultures of *M. aeruginosa*, after 3 days of incubation, the antialgal activities of lag, log, and stationary phase bacteria were 44.3, 46.9, and 38.3%, respectively (Fig. 4). After 7 days, these activities were 41.5, 43.6, and 47.5%, respectively, and these were not statistically different ($F = 0.019$, $P = 0.981$, one-way ANOVA).

3.4. Host range of antialgal activity of *S. neyagawaensis* on other algae

The antialgal bacterium *S. neyagawaensis* suppressed not only *M. aeruginosa* NIES-44, but also *A. flos-aquae* KCTC-AG10011 and *O. sancta* NIER-10027 (Table 3); these algae produce toxic substances and, along with *Microcystis*, dominate many freshwaters in the summer. In addition, *S. neyagawaensis* suppressed the cyanobacterium *A. cylindrica*

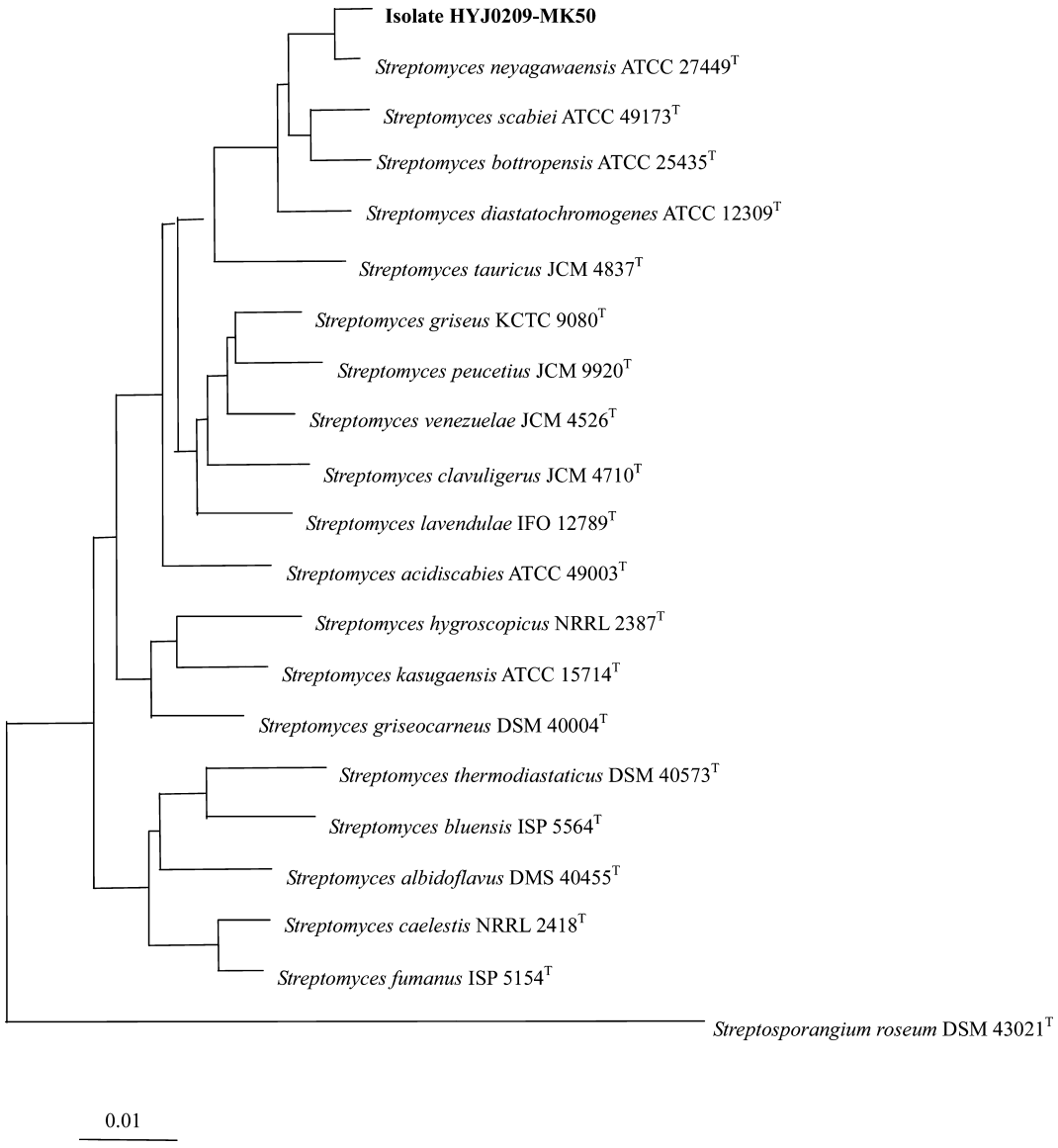

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the relative positions of isolate HYJ0209-MK50, the type strains of some *Streptomyces* species, and the type strain of *Streptosporangium roseum*. Scale bar represents 0.01 substitutions per nucleotide position.

NIES-19 but not *A. macrospora* NIES-ATCC22664, *A. affinis* KCTC-AG10008, and *S. bacillaris* CCAP1479/7. The centric diatoms, *S. hantzschii* UTCC267 and *A. granulata* CCAP10021/1, which predominate in cooler waters, were effectively inhibited, but *Cyclotella* sp. was not. The green alga *Chlorella* sp. was also suppressed effectively. Thus, *S. neyagawaensis* HYJ0209-MK50 is able to suppress several toxic cyanobacteria and has activity also against certain cyanobacteria, *Chlorella* sp., and diatoms tested.

3.5. Localization and activity of antialgal substances

To determine the antialgal mode of activity, we analyzed the total proteins and the specific activities of bacterial culture filtrates, co-culture filtrates, and cell-free extracts (Table 4). The cell-free extracts contained 9913.8 g/L of total protein and conferred 1141.4 U of antialgal activity. The monoculture filtrate of *S. neyagawaensis* contained 30 μg/L of total protein and no antialgal activity, and the co-culture filtrate of *S. neyagawaensis* and *M. aeruginosa* contained 780 μg/L of total protein and 44,000 U of antialgal activity. These results indicate that *S. neyagawaensis* releases an antialgal substance when it encounters a target alga such as *M. aeruginosa.*

The periplasmic and cytoplasmic membranes and cytoplasmic fractions contained 699.1, 253.7, and 3912.3 μg/L of total protein, respectively, with antialgal activities of 5392.1, 483, and −398 U, respectively, and specific activities of 7.7, 1.9, and −0.1 U, respectively (Table 5). These results suggest that the antialgal substance originates in the bacterial periplasm, and is secreted when the bacterium meets the algal target *M. aeruginosa.*

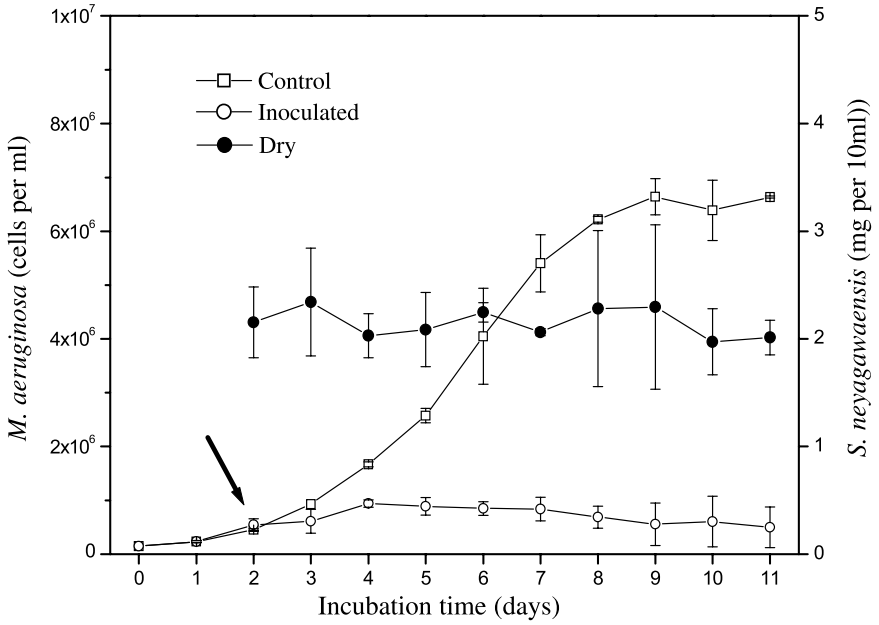

Fig. 2. Antialgal effect of *S. neyagawaensis* HYJ0209-MK50 on *M. aeruginosa* and changes in cell density of the bacterium after inoculation. The arrow represents the inoculation time of the bacterium. Each value is means ± SEM of three replications. Cell densities of *S. neyagawaensis* and *M. aeruginosa* were determined by dry weight and direct counting under microscope (see Section 2 for details). Control, cell density of *M. aeruginosa* without treatment of *S. neyagawaensis*; inoculated, cell density of *M. aeruginosa* when inoculation of *S. neyagawaensis*; and dry, dry weight of *S. neyagawaensis* in the presence of *M. aeruginosa*.

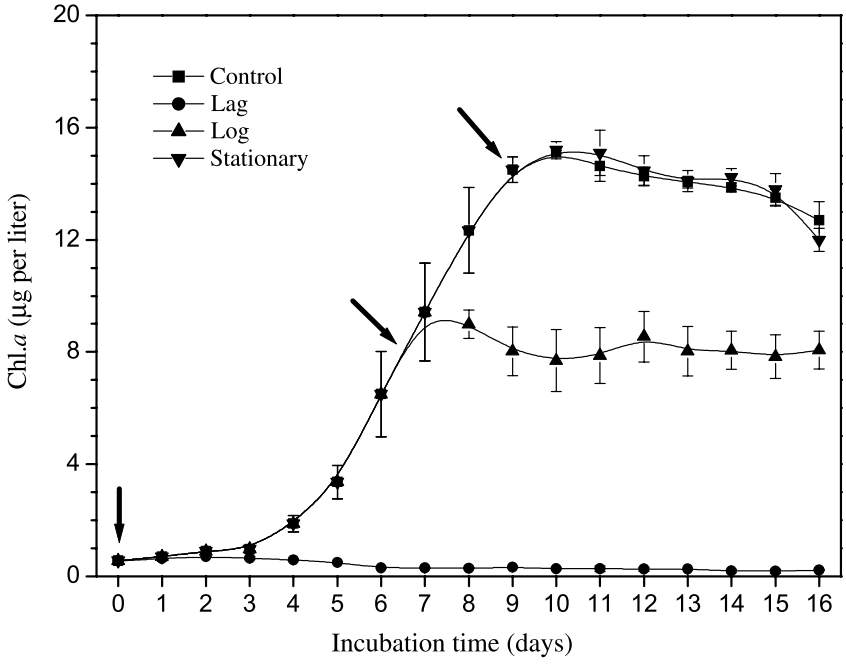

Fig. 3. Antialgal effect of *S. neyagawaensis* HYJ0209-MK50 on the growth of *M. aeruginosa* NIES-298. The arrows represent the time at which *S. neyagawaensis* cells were inoculated to lag, log, and stationary phase algae. Each value is means ± SEM of three replications. Control algal culture was not inoculated with the bacterium.

4. Discussion

Bacteria with antialgal activity against *Mycrocystis* have been previously reported, and the list includes *Pseudomonas* sp. (Kodani et al., 2002), *Alcaligenes denitrificans* (Manage et al., 2000), and *Streptomyces phaeofaciens* (Yamamoto et al., 1998). Indeed, several members of the genus *Streptomyces* have been previously reported as having cyanobacteria-killing activity (Safferman and Morris, 1962; Yamamoto et al., 1998).

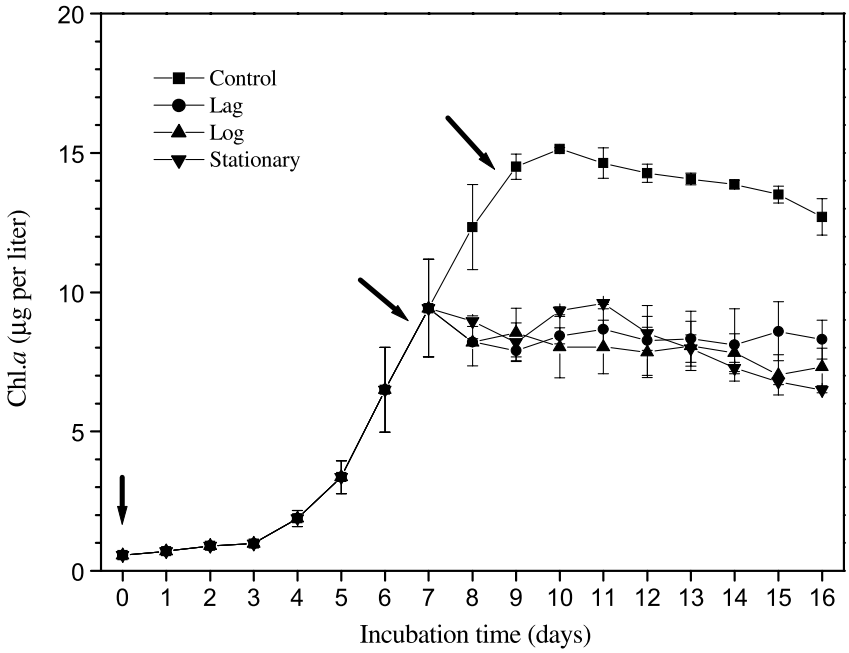

Fig. 4. Effect of growth stage of *S. neyagawaensis* HYJ0209-MK50 on its antialgal activity on *M. aeruginosa*. The arrow represents the time of inoculation of the bacterium with a log-phase culture of the alga (see Section 2 for details). Each value is means ± SEM of three replications.

Table 3
The antialgal effect of the bacterium *S. neyagawaensis* HYJ0209-MK50 on algal species[a]

Algal strains	Antialgal activity
Microcystis aeruginosa NIES-44[b]	+
Microcystis aeruginosa NIES-298[b]	++
Anabaena macrospora ATCC2266-4[c]	–
Anabaena affinis KCTC-AG10008[d]	–
Anabaena flos-aquae KCTC-AG10011[d]	++
Anabaena cylindrica NIES-19[b]	+
Oscillatoria sancta NIER-10027[e]	++
Synechococcus bacillaris CCAP1479/7[f]	–
Chlorella sp. Isol.[h]	++
Stephanodiscus hantzschii UTCC267[g]	+
Aulacoseira granulata CCAP 10021/[f]	+
Cyclotella sp. Isol.[h]	–

[a] Antialgal activity was determined by measurement of chlorophyll *a* concentration after 48 h of co-culture. ++, >50% inhibition of total; +, >20%; and –, no inhibition and/or stimulation.

The algal cultures were obtained from:

[b] NIES, National Institute for Environmental Studies, Japan.

[c] ATCC, American Type Culture Collection, Manassas, Virginia, USA.

[d] KCTC, Korean Collection for Type Cultures, Korea.

[e] NIER, National Institute of Environmental Research Culture Collection of Environmental Microorganisms, Korea.

[f] CCAP, Culture Collection of Algae and Protozoa, UK.

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

[h] Isolated from Kyungan, South Korea by H.J. Choi.

However, there is no report demonstrating the in situ application of antialgal bacteria against a cyanobacterial bloom. One of the major reasons is probably the unpredictability of the antialgal effects of bacteria on other members of the freshwater ecosystem (EPA, 2002). Before application of an antialgal agent to freshwater systems, there should be information on (1) the antialgal activity against the target alga, (2) the effects on the other organisms in the freshwater ecosystem, and (3) a forecast of the algal dynamics after the removal of the target alga. Hence, we tested the effects of the antialgal bacterium *S. neyagawaensis* on several dominant algae in the Paltang, Juam, Daechung Reservoir, and Naktong River (Han et al., 1995; Hong et al., 2002; Jeong et al., 1998; Lee, 1999; Lee et al., 2002). *S. neyagawaensis* had an effect on some species of a genus but not others. For example, it controlled *A. flos-aquae* and *A. cylindrica* but not *A. macrospora* and *A. affinis*. It also affected strains within a species differently. For example, the antialgal activity was 38.8% on *M. aeruginosa* NIES-44 and 70.2% on *M. aeruginosa* NIES-298. The difference of activity could be due to physiological differences of the strains (Walker and Higginbotham, 2000; Yasuno et al., 2000). The antialgal bacterium *S. neyagawaensis* did not affect all of the algal species tested, which is an advantage of biological agents. However, although certain algal species may be removed by antialgal bacteria, the resulting loss of species could theoretically provide niches for other algal species to colonize and thrive.

Streptomyces neyagawaensis suppressed the growth of the *M. aeruginosa* biomass (Fig. 2) but the bacterial biomass did not increase. There are two possible explanations: (1) the culture conditions utilized in this experiment were not suitable for bacterial growth and (2) *M. aeruginosa* exudates may suppress bacterial growth. The first explanation is based on the difference between optimum culture conditions for the antialgal

Table 4
Protein assay of *S. neyagawensis* HYJ0209-MK50 by the Bradford method

Fraction	Protein concentration (μg/L)	Total activity (units)	Specific activity (units/μg)[c]
Cell-free extract	9913.8	1141.4	0.2
Monoculture filtrate[a]	30	0	0
Mixed-culture filtrate[b]	780	44000	56.4

[a] Monoculture filtrate of *S. neyagawaensis* in CB medium.
[b] Mixed-culture filtrate of *S. neyagawaensis* and *M. aeruginosa.*
[c] The specific activity was calculated as follows: specific activity (units/μg) = Total activity/total protein.

Table 5
Protein assay of *S. neyagawaensis* cell fractions according to the Bradford method

Fraction	Total protein (μg/L)	Total activity (units)	Specific activity (units/μg)[a]
Periplasm	699.1	5392.1	7.7
Cytoplasmic membrane	253.7	483	1.9
Cytoplasm	3912.3	−398	−0.1

[a] The specific activity was calculated as follows: specific activity (units/μg) = Total activity/total protein.

bacterium and the cyanobacterium. The bacterium did not grow well at pH 9 and 25 °C. The second explanation is that the toxicity of microcystin, from *M. aeruginosa*, is known to inhibit growth of organisms such as cladocerans, copepods, and mosquito larvae (Sathiyamoorthy and Shanmugasundaram, 1996; Singh et al., 2003). Kasumigamide, another *M. aeruginosa* exudate, is a peptide that is allelopathic against the green alga, *Chlamydomonas neglecta* (Keish and Masahiro, 2000). Other cyanobacterial secondary metabolites, such as the cyclic tridecapeptides tolybyssidins A and B, have been reported to have antifungal activity (Jaki et al., 2001). However, there are no reports on the bacteriolytic activity of *M. aeruginosa.* Thus, we think the culture condition is the main reason for the poor growth of the bacterium.

Kodani et al. (2002) reported that harmane (1-methyl-β-carboline), an ethyl acetate-soluble factor produced by *Pseudomonas* sp. K44-1, is lethal to several types of cyanobacteria, including *M. aeruginosa*. However, we found that ethyl acetate, methanol, and acetone extracts of *S. neyagawaensis* HYJ0209-MK50 showed little antialgal activity on *M. aeruginosa* (data not shown), which suggests that the antialgal substance from *S. neyagawaensis* HY0209-MK50 could be an enzyme. The serine protease of *Pseudoalteromonas* sp. strain A28 can kill the diatom *Skeletonema costatum* (Lee et al., 2000), and secreted enzymes such as cellulases, cutinases, and proteases are related to fungal pathogenicity on plants (Fan and Wolfram, 1998; Francis et al., 1996; Fric and Wolf, 1994; Nicholson et al., 1993). In relation to the current study, *Streptomyces* was reported to produce several enzymes such as proteases and cellulases (Francis et al., 1996; Jeong et al., 1988; Jung et al., 1986).

Our results indicate that *S. neyagawaensis* did not secrete the antialgal substance until the bacterium met the target alga (Table 4) and that the antialgal substance was present in the periplasm fraction (Table 5). This fraction is generally composed of periplasmic proteins, cell wall-bound proteins, and proteins secreted across the periplasm (Rafael et al., 1995). That the cytoplasm fraction had no antialgal activity implied that the proteins secreted across the periplasm were not antialgal, and that the antialgal proteins are contained within the periplasmic or cell-wall-bound proteins. The small amount of specific activity observed in the cytoplasmic membrane fraction may be due to contamination by traces of periplasmic fraction. Further study is required for identification of antialgal substance from the periplasm.

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