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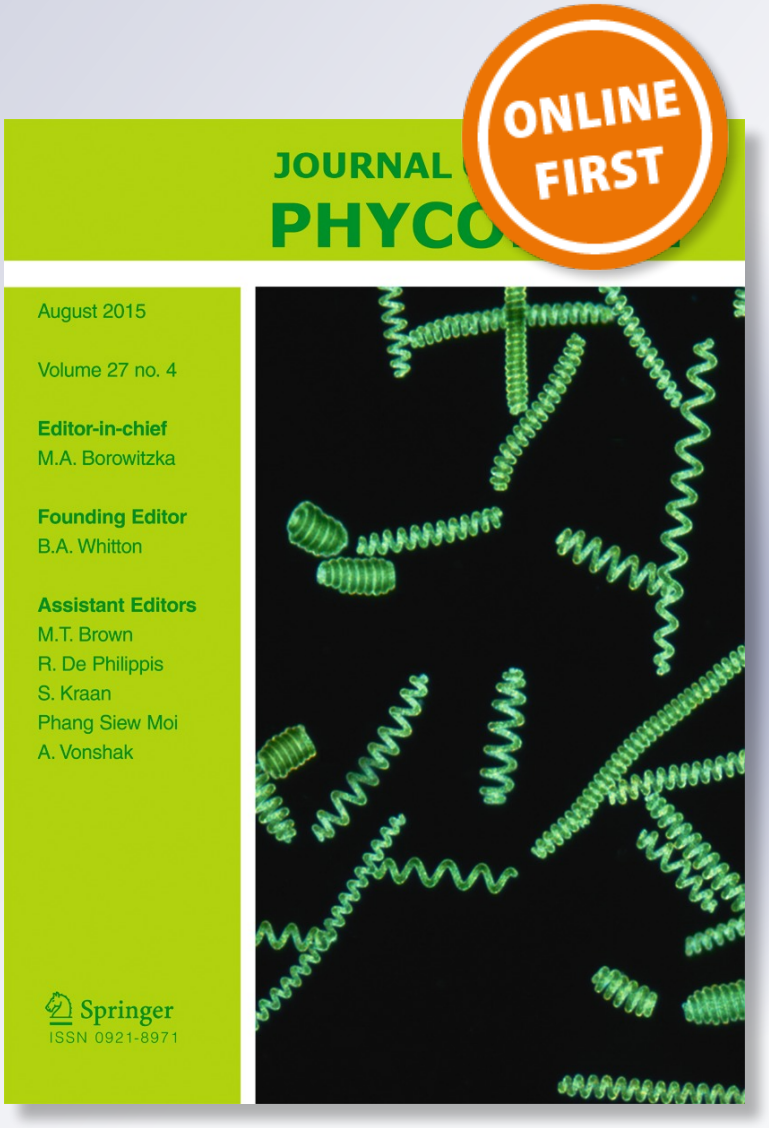

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Use of lactic acid bacteria as a biological agent against the cyanobacterium *Anabaena flos-aquae*

Yoon-Ho Kang[1,2] · **Su-Kyung Kang**[1] · **Chong-Sung Park**[1] · **Jae-Hyung Joo**[1] · **Jin-Won Lee**[1] · **Myung-Soo Han**[1]

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Abstract In the present study, we assessed whether lactic acid bacteria (LAB) with antimicrobial activity could be used to effectively control *Anabaena flos-aquae* growth. In our screening of cyanobacteriacidal bacteria from 14 LAB strains belonging to 11 species, we selected six candidate strains that could lyse 90 % or more *A. flos-aquae* cells compared with a control. Of those, *Lactobacillus paraplantarum* KCTC 5045^T had the strongest cyanobacteriacidal activity showing complete lysis of cyanobacterial cells at the initial densities of $\geq 10^4$ cells mL^{-1}. A host range assay revealed that *L. paraplantarum* strongly inhibited *A. flos-aquae*, *Anabaena crassa*, *Stephanodiscus hantzschii*, and *Peridinium bipes* but weakly inhibited or stimulated the growth of *Scenedesmus actus*, *Pediastrum* sp., *Cyclotella meneghiniana*, *Coelastrum reticulatum*, *Chlamydomonas* sp., and *Microcystis aeruginosa*. In the microcosm experiment using natural freshwater including abundant phytoplankton assemblage, moreover, the LAB under inoculation of about 10^5 cells mL^{-1} could completely terminate the co-growth of *A. flos-aquae*, *A. crassa*, *Anabaena circinalis*, and other *Anabaena* spp. (over 10^4 cells mL^{-1}) without a secondary bloom by another algal and cyanobacterial species. Our observation revealed that the cyanobacteriacidal bacterium releases two or more extracellular compounds possessing cyanobacteriacidal activity without cell-to-cell contact when the host cyanobacterium necessarily exits in surrounding water. Property study of cyanobacteriacidal substance revealed the release of heat stable and hydrophobic chemical substances that do not appear to be protein and peptide compound. Taken together, our results indicate that LAB may be a potential bio-agent for future use in controlling freshwater *Anabaena* blooms without causing the problem of pathogenicity if there is no secondary bloom by a resistant species to this bacterium.

Keywords Lactic acid bacteria · Cyanobacteriacidal bacteria · *Anabaena flos-aquae* · Biological control

Introduction

Cyanotoxins and toxic cyanobacterial blooms in eutrophic lakes, rivers, and reservoirs have been reported during the last two decades all over the world (Carmichael 1992; Park et al. 1998). Blooms of the cyanobacterium *Anabaena flos-aquae* cause serious problems in freshwater environments as well as those of *Microcystis aeruginosa* (Rubenchik et al. 1965; Sigee et al. 1999). Widespread in eutrophic lakes and reservoirs worldwide, these blooms produce toxins, which are harmful to fish, birds, wild animals, livestock, and potentially humans and cause foul odors, decreased aesthetic value, deterioration of water quality, and

Yoon-Ho Kang and Su-Kyung Kang contributed equally to this work.

✉ Jin-Won Lee
jwl@hanyang.ac.kr

✉ Myung-Soo Han
hanms@hanyang.ac.kr

[1] Department of Life Science and Research Institute for Natural Sciences, College of Natural Sciences, Hanyang University, Seoul 04763, Republic of Korea

[2] Monitoring and Analysis Division, Wonju Regional Environmental Office, Ministry of Environment, Wonju, Gangwon-do 220-947, Republic of Korea

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deoxygenation (Sigee et al. 1999; Choi et al. 2005; Kang et al. 2012a, b).

Many pan-ecological and environmental approaches such as biological control agents (bacteria, viruses, protozoans, and fungi) are under investigation for the control of seriously toxic harmful algal blooms (Shilo 1970; Imai et al. 1993; Manage et al. 2000; Kang et al. 2005). Recent studies have focused on the identification of bacteria capable of inhibiting or degrading harmful algal blooms in water ecosystems (Kang et al. 2012a). Despite many trials in many countries, few studies have reported the control of *Anabaena* by algaecidal bacteria (Reim et al. 1974; Walker and Patrick 1998; Kim and Han 2004). Little is known, thus, about the potential of bacteria to regulate the abundance of *A. flos-aquae*. Moreover, these foreign-introduced bacteria may act as pathogens that adversely affect other economically important organisms and humans in these practical applications (Gumbo et al. 2010).

Thus, there is a continual need for more useful bacteria that can provide an environmentally friendly solution to the control of *A. flos-aquae*. Here, we examined whether lactic acid bacteria (LAB), which are generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA), can be an attractive alternative for controlling harmful algal blooms (HABs) (Curk et al. 1996; Bringel et al. 2005; Kim et al. 2009). LAB have a long history of safe use, and most strains are considered commensal microorganisms with no pathogenic potential (Soomro et al. 2002). Of the LAB strains, those of the genera *Lactobacillus* and *Lactococcus* are common and usually benign in organisms, humans, and ecosystems and are also metabolically versatile bacteria, commonly known as probiotics (Giraud et al. 1992; Gueguen et al. 1997; Leia et al. 1999; Saarisalo et al. 2007). Although the genera *Lactobacillus* and *Lactococcus* have been used in various fields as a way to prevent the growth or establishment of various pathogens (de Vuyst and Vandamme 1994; Billkova et al. 2011), we have little knowledge about their algeacidal activity (Berg et al. 2009).

In the present study, we obtained 14 strains belonging to the genera *Lactobacillus* and *Lactococcus* to identify novel *Anabaena*-lysing bacteria effective against *A. flos-aquae*. Once the best algaecidal bacterium was selected, we evaluated the following: (1) the minimum algaecidal bacterial density required for rapid lysis; (2) the bacteria-cyanobacterium population interaction; (3) the range of potential target organisms for these bacteria; and (4) the mode of cyanobacteriacidal activity and property of cyanobacteriacidal substance(s); (5) the assessment of the potential of these bacteria as a biological agent in microcosm experiment.

Materials and methods

Culture of algae and cyanobacteria *Anabaena flos-aquae* NIES 75, *Microcystis aeruginosa* NIES 298, and *Scenedesmus acutus* NIES 94 were obtained from the National Institute for Environmental Studies (NIES), Japan. *Chlorella vulgaris* KMMCC FC-109, *Chlamydomonas* sp. KMMCC FC-113, and *Coelastrum reticulatum* KMMCC FC-29 were kindly supplied by the Korea Marine Microalgae culture center (KMMCC), South Korea. *Stephanodiscus hantzschii* UTCC 267 was supplied by the University of Toronto Culture Collection of Algae and Cyanobacteria (UTCC), Canada. *Cyclotella meneghiniana* HYK0210-A1 and *Golenkinia* sp. HYK0912-A15 were isolated from the Gyeongan Stream in South Korea. *Pediastrum* sp. HYD0901-A18 and *Aulacoseira granulata* HYD0810-A8 were isolated from the Daewang reservoir, South Korea. *Peridinium bipes* HYJA0311-A2 was isolated from the Juam Stream in South Korea.

Green algae and Cyanobacteria (each 2 mL of exponential-phase culture) were cultured each in 300 mL conical flasks containing 100 mL C (pH 7) and CB medium (pH 9.0; C medium containing bicine instead of *tris*-(hydroxymethyl) aminomethane) (NIES 2004), respectively, at 25 °C under 50 μmol photons m^{-2} s^{-1} on a 12:12 (light/dark) cycle. Diatoms and dinoflagellates (each 5 mL of exponential-phase culture) were maintained at pH 7, under 50 μmol photons m^{-2} s^{-1} with a 12:12 (light/dark) cycle, in diatom medium (DM) (Beakes et al. 1988) at 15 °C.

Screening for cyanobacteriacidal bacteria For the primary screening of algaecidal bacteria, 14 strains of *Lactobacillus* and *Lactococcus* as candidate bacteria were obtained from the Korean Collection for Type Culture Center (KCTC), South Korea (Fig. 1). Each strain was then inoculated into MRS broth medium (de Man et al. 1960), grown for 24 h at 30 °C on a rotary shaker (250 rpm), harvested by centrifugation at 18,000×*g* for 10 min, and adjusted to an absorbance of around 1.0 at 660 nm (light path of 1 cm) with fresh CB medium (yielding an initial density of approximately 1×10^8 cells mL^{-1}; unless otherwise indicated). An aliquot (0.4 mL) was inoculated into a 24-well plate containing 1.6 mL of a mid-exponential-phase culture of *A. flos-aquae* (1.2×10^5 cells mL^{-1}) per well. The co-cultures were incubated under culture condition of *A. flos-aquae* for 10 days. The wells were examined for the survival of host cyanobacterial cells at 3, 7, and 10 days using a hemocytometer (Superior, Germany) under microscopic observation at a magnification of ×200. A positive well with algaecidal bacterium was indicated when 90 % or more algal cells were lysed at day 10. In this manner, we successfully identified the algaecidal bacteria. Once the algaecidal activities were verified, the bacterial strains were routinely maintained on MRS medium at 30 °C or were

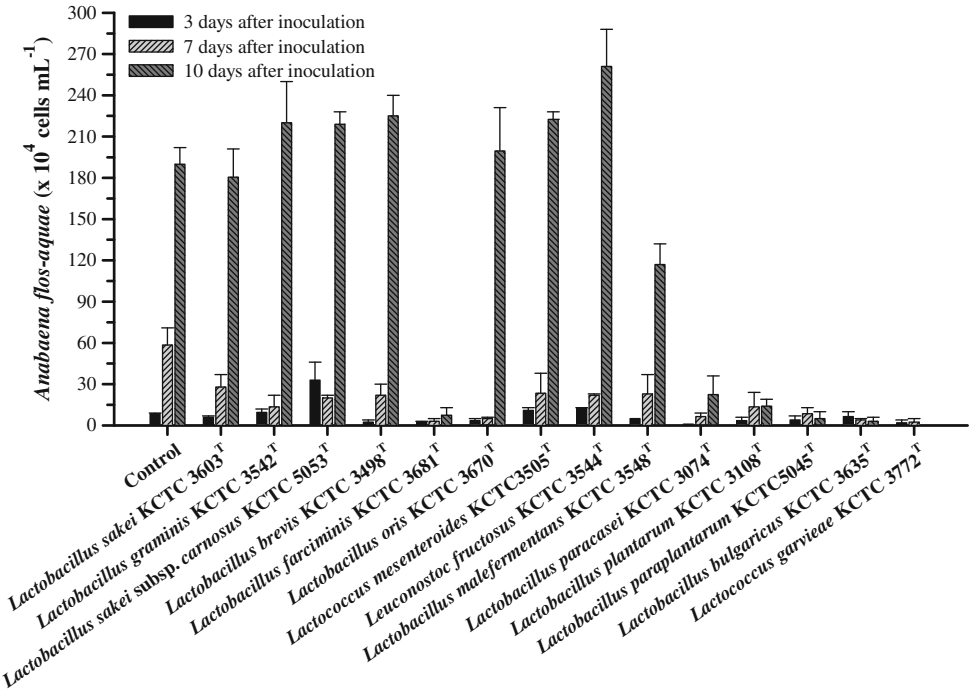

Fig. 1 Changes of *Anabaena flos-aquae* cell number (each 10^5 cells mL^{-1}) in cultures incubated with 14 candidates (each 2×10^7 cells mL^{-1}) of *Lactobacillus* and *Lactococcus* species belonging to lactic acid bacteria. Data are the mean±SD from at least three independent assays

cryopreserved at −76 °C in MRS medium containing 25 % glycerol.

Selection of algaecidal activity

To elucidate the Cyanobacteriacidal activities for different densities of the six bacteria (*Lactobacillus plantarum* KCTC 3108^T, *Lactobacillus paraplantarum* KCTC 5045^T, *Lactobacillus farciminis* KCTC 3681^T, *Lactobacillus paracasei* KCTC 3074^T, *Lactobacillus bulgaricus* KCTC 3635^T, and *Lactococcus garvieae* KCTC 3772^T) against *A. flos-aquae*, each bacterial culture was prepared as above, and then serially diluted with CB (0.2 mL) to initial densities of 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 cells mL^{-1} into a 24-well plate containing 1.8 mL of mid-exponential-phase *A. flos-aquae* culture (1.1×10^5 cells mL^{-1}) per well. The bacteria-free culture well of *A. flos-aquae* was used as control. The controls and treated samples were cultured for 10 days under the cyanobacterial growth conditions. The cyanobacteriacidal bacterium possessing the strongest algaecidal activity (by measuring the highest removal effect of the cyanobacterial cells to the least bacterial inoculum on the basis of their cell number) was selected for further analysis.

Monitoring of bacterial cell density in mixed cultures To examine the fate and behavior of *A. flos-aquae* and the bacterium (*L. paraplantarum*), bacterial culture (0.5 mL) was prepared as above and then serially diluted with CB to an initial density of 1×10^6 cells mL^{-1} into a 6-well plate containing 4.5 mL of mid-exponential-phase *A. flos-aquae* culture (1.1×10^5 cells mL^{-1}) per well. The bacterium was cultured for 10 days under the cyanobacterial growth conditions described previously and *A. flos-aquae* and the bacterial population was measured at days 0, 1, 3, 5, 7, and 10.

The monitoring of the cell density of cyanobacteriacidal bacterium was determined by a quantitative real-time PCR (qRT-PCR) assay using a new DNA-binding dye, EvaGreen, according to the method of Park et al. (2012). The specific primer sets for *L. paraplantarum* (paraF primer, 5′ TAGTGGTGCCGTTGATATTTTG 3′ and paraR primer, 5′ AGTCTTGTTCAACGTTCCGG 3′) were designed for the specific detection of each species and were described in the previous study of Lee et al. (2004). A 50-μL subsample from each well was centrifuged at 12,000×*g* for 5 min. The resultant pellet was washed twice in CB medium and resuspended in 100 μL TE buffer (pH 8.0). Total bacterial DNA from the suspension was isolated with a EZNA Bacterial DNA Kit (Omega Bio-tek, Inc., USA) and eluted in fresh distilled water (DW). Real-time PCR assays were performed in a total reaction volume of 15 μL that contained 7.5 μL of 1× SsoFast EvaGreen Supermix (Bio-Rad, USA), 0.3 μM of primer set, 2 μL of total DNA (approximately 0.1 μg) and DW to a final volume of 15 μL. qRT-PCR reactions were run using a Chromo 4 Detection System (Bio-Rad, USA) at 98 °C for 2 min, followed by 40 cycles at 98 °C for 5 s, then 62 °C for 10 s. After denaturation, melting curves were monitored from 65 to 95 °C in 0.2 °C increments or 0.5 °C increments using a 10-s hold at each step. To construct a standard curve, bacterial

number was counted by DAPI staining as described above. Serial tenfold dilutions of the DNA extracts were used to construct the standard curve.

Host range of the cyanobacteriacidal bacteria We investigated the algaecidal and cyanobacteriacidal spectrum of the bacterium against 16 strains belonging to 13 algal and cyanobacterial species in abundance in single-species tests; 0.5 mL (1×10^8 cells mL^{-1}) of bacterial culture was added to a 6-well plate containing 4.5 mL of exponential-phase culture per well of *M. aeruginosa*, *Microcystis wesenbergii*, *A. flos-aquae*, *Anabaena crassa*, *Scenedesmus actus*, *C. vulgaris*, *Chlamydomonas* sp., *C. reticulatum*, *Gorenkinia* sp., *Pediastrum* sp., *A. granulata*, *C. meneghiniana*, *S. hantzschii*, or *P. bipes*. Algae and cyanobacteria were incubated for 10 days under their respective growth conditions described above and counted at days 0, 1, 3, 5, 7, and 10. The algaecidal and cyanobacteriacidal activity (%) of the bacteria on each algal or cyanobacterial species was calculated by the following equation: algaecidal and cyanobacteriacidal activity (%)=$(1-Tt/Ct\times100$, where T (treatment) and C (control) are the algal cell densities with and without the bacterium, respectively, and t is the inoculation day.

Filtrate and preparation We first determined whether direct contact was necessary between *A. flos-aquae* and the cyanobacteriacidal bacterium for algaecidal activity. Bacterium was inoculated into MRS broth medium, grown for 24 h at 30 °C on a rotary shaker (250 rpm), harvested by centrifugation at 18,000×*g* for 10 min, and inoculated into 100 mL fresh CB medium. After incubation for 6 h at 250 rpm, 50 mL of the culture (1×10^8 cells mL^{-1}) was filtered through a 0.2-μm filter (Millipore Corp, USA) and the resultant filtrate was termed "mono-culture filtrate." In the case of co-culture filtrate, 50 mL (1×10^8 cells mL^{-1}) was co-cultured in a 250-mL flask containing 50 mL exponential-phase *A. flos-aquae* (1×10^6 cells mL^{-1}) under the cyanobacterial culture condition. The co-culture, after 2 days when 90 % or more of the cyanobacterial cells were lysed, was filtered through a 0.2-μm filter and the resultant filtrate was termed the "co-culture filtrate." Different subsamples of 0.5, 1.0, and 2.5 mL from each filtrate were each inoculated into 6-well plates containing 4.5, 4.0, and 2.5 mL exponential-phase *A. flos-aquae* culture (1×10^5 cells mL^{-1}) per well, respectively. The co-cultures were incubated under the culture conditions of *A. flos-aquae* for 10 days. Cyanobacterial cells were counted at days 0, 1, 3, 5, 7, and 10.

Properties of cyanobacteriacidal substance(s) To examine the major properties of the algaecidal substance(s) originating from the bacterium, the following treatments were carried out on the co-culture filtrates prior to use in the cyanobacteriacidal activity test. (i) For heat treatment, one (15 mL) aliquot of co-culture filtrate was placed into a test tube with a silicon stopper and autoclaved at 121 °C for 15 min. (ii) To determine whether the cyanobacteriacidal substance was protein like, another (15 mL) aliquot of co-culture filtrate was incubated at 55 °C in a water bath for 3 h after mixing with 1 μL (because its protein concentration was <0.03 μg mL^{-1}) of proteinase-K solution (Sigma-Aldrich, USA) and was then autoclaved at 121 °C for 15 min. The protein content was quantified according to the Bradford (1976) method using bovine serum albumin (Amersham-Pharmacia Biotech, USA) as the protein standard. Then, the filtrates (0.5, 1.25, and 2.5 mL) were each inoculated into 6-well plates (Falcon Inc., USA) containing 4.5, 3.75, and 2.5 mL of exponential-phase *A. flos-aquae* culture (1×10^5 cells mL^{-1}), respectively, to adjust the final filtrate concentrations to 10, 25 and 50 % (*v*/*v*). The cultures were incubated for 10 days and counted at days 0, 1, 3, 5, 7, and 10. Fresh CB medium was used as a control for each test. The cyanobacteriacidal activity (%) of bacterium on *A. flos-aquae* was calculated as described above. (iii) To better characterize the nature of the cyanobacteriacidal compound(s), we carried out a range of solvent partitioning between co-culture filtrate and petroleum ether, diethyl ether, chloroform, ethyl acetate, or *n*-butanol, followed by a bioassay to determine the distribution of cyanobacteriacidal compound(s) as follows. The other aliquot (400 mL) of co-culture filtrate was added equivalently to petroleum ether (400 mL) and transferred to a 1000-mL separation funnel. The mixture was shaken vigorously for 15 min and allowed to stand for 1 h at 4 °C. Then, the aqueous phase was re-added to the equivalent diethyl ether, chloroform, ethyl acetate, and *n*-butanol, successively as described above. The five organic and one aqueous phases (at final step) were finally obtained. Each phase was concentrated and dried at 45 °C under reduced pressure in a rotary evaporator (EYELA, Tokyo, Japan), and the bottom layer was redissolved with methanol up to the final concentration of 1 mg mL^{-1}, approximately. For bioassay, a 5-mm diameter Whatman GF/F filter (pore size=0.7 μm), which was soaked with a 20-μL aliquot of each extract, was placed onto *A. flos-aquae* algal lawn plate that was made up of 1.5 % CB bottom agar overlaid with 0.8 % top agar containing 1 mL of *A. flos-aquae* (10^9 cells mL^{-1}) under sterile air being blown for 2 h. The plate was incubated at 25 °C under a 12:12 (light/dark) cycle for 2 days. The cyanobacteriacidal activity of each extract was measured by the diameter of the clear zone formed in the algal lawn.

Cyanobacteriacidal bacteria application in microcosm experiments Each microcosm which was comprised of a transparent tetragonal glass tank (40 cm long, 40 cm wide, 25 cm deep) was filled with 30 L natural water collected from Uiam dam (37° 50′ 14″ N, 127° 40′ 32″ E) on the Uiam Rservoir, South Korea. The microcosm was incubated at 25 °C under a 12:12 (light/dark) cycle in a culture room equipped with a

thermo-hygrostat. Artificial light was provided by two fluorescent lamps set at 30 cm above the water surface, with a light intensity of 80 μmol photons m^{-2} s^{-1} on the surface. It was mixed continually, by use of small pumps, to re-suspend bottom-dwelling microorganisms and to maximize our chances of stimulating the growth of natural *Anabaena* assemblage. Sampling began two days before the introduction of each cyanobacteriacidal bacterium (day −2). On day 0, the following experimental control and treatments were initiated in the microcosms: microcosms without any inoculation (control) and with *L. paraplantarum* KCTC 5045^T, containing 1.5×10^{10} cells (yielding a final density of approximately 10^5 cells mL^{-1}): bacterial culture was prepared as above. After incubation, 100 mL of the culture was inoculated into 3 L conical flask containing 1 L MRS broth, incubated at 30 °C under 150 rpm for 48 h, and harvested by centrifugation at 18, 000×*g* for 20 min. The pellet was twice washed and adjusted to 100 mL (1.5×10^{10} bacterial cells) with sterilized DM.

Samples for enumeration of phytoplankton was fixed with glutaraldehyde (final concentration 2 %) in 50 mL polypropylene bottles and stored in the dark at 4 °C until analyzed. Phytoplankton enumeration was carried out in water samples (2 mL) placed in a Sedgwick–Rafter counting chamber and observed directly at ×9400 magnification by use of an IX71 inverted microscope.

Data analysis All experiments were repeated in triplicate and the results are shown as the mean and standard deviation of the raw data. Analysis of covariance (ANCOVA) was used to determine if there were statistically significant differences ($P<0.05$) through time in responses between control and treatment. All statistical analyses were performed using the SPSS 17.0 software (SPSS Inc., USA).

Results

The screening of cyanobacteriacidal bacteria

In the primary screening of cyanobacteriacidal bacteria, we identified six (*Lactobacillus. farciminis* KCTC 3681^T, *L. paracasei* KCTC 3074^T, *L. plantarum* KCTC 3108^T, *L. paraplantarum* KCTC 5045^T, *L. bulgaricus* KCTC 3635^T, and *L. garvieae* KCTC 3772^T) out of fourteen strains of LAB that could lyse 90 % or more *A. flos-aquae* cells as compared with the control, 10 days after inoculation (Fig. 1). In contrast, eight other bacteria (*Lactobacillus sakei* KCTC 3603^T, *Lactobacillus graminis* KCTC 3542^T, *L. sakei* subsp. *carnosus* KCTC 5053^T, *Lactobacillus brevis* KCTC 3498^T, *Lactobacillus oris* KCTC 3670^T, *Leuconostoc mesenteroides* KCTC 3505^T, *Lactobacillus fructosus* KCTC 3544^T, and *Lactobacillus malefermentans* KCTC 3548^T) inhibited *A. flos-aquae* by approximately 0 to 40 % compared with the untreated control over the same period.

The six LAB strains were subjected to a second screening to evaluate their cyanobacteriacidal activities at different cell densities against *A. flos-aquae* (Fig. 2). Of those, *L. plantarum* and *L. paraplantarum* had the strongest algaecidal activity, showing lysis of all *A. flos-aquae* cells at the initial densities of $\geq10^5$ and $\geq10^4$ cells mL^{-1}, respectively, although the cell densities $\leq10^4$ and $\leq10^3$ cells mL^{-1} of the two bacteria showed mild algaecidal activity during the experimental period (Fig. 2a, b). However, the other LAB strains displayed little or no cyanobacteriacidal activity even at the initial density of 1×10^7 cells mL^{-1} (Fig. 2c–f) in contrast to the primary screening step at 2×10^7 bacterial cells mL^{-1} (Fig. 1). Overall, *L. paraplantarum* KCTC 5045^T had higher *L. plantarum* KCTC 3108^T and was thus chosen for further study.

Fate and behavior of *Anabaena* and cyanobacteriacidal bacteria in co-culture

To determine the change in density of cyanobacteriacidal bacterium, *L. paraplantarum* that can lyse *A. flos-aquae*, we incubated *A. flos-aquae* with the bacterium at an initial density of 1×10^5 cells mL^{-1} (Fig. 3). *A. flos-aquae* cell numbers sharply decreased from 1×10^5 cells mL^{-1} on day 0 and were completely lysed on day 5. In contrast, *A. flos-aquae* in all controls increased sharply to 2.0×10^6 cells mL^{-1} on day 10. Cyanobacteriacidal bacteria increased slightly to a peak of 2.2×10^5 cells mL^{-1} on day 1 and then declined rapidly to 1.8×10^3 cells mL^{-1}, towards the end of the experiment. The growth curve of treatment showed a faster and higher depletion during the last experimental period. In the treatment, cyanobacterial cell density had a significantly negative relationship with the abundance of bacterium ($P<0.01$).

The relative sensitivity of algae and cyanobacteria to cyanobacteriacidal bacteria

The responses of 16 dominant/abundant algae and cyanobacteria in South Korea water bodies to the bacterium were examined (Table 1). This strain showed high algaecidal and cyanobacteriacidal activities against *A. flos-aquae* (97.5 %), *A. crassa* (97.5 %), *S. hantzschii* (82.1 %), *P. bipes* (77.3 %), *S. actus* (52.3 %), and *Pediastrum* sp. (42.2 %) but had mild or no activity against the other algae and cyanobacteria.

Microscopic observation of cyanobacteriacidal mode

No immediate morphological change was observed in the cyanobacterial cells when *L. paraplantarum* was inoculated into an *A. flos-aquae* culture (Fig. 4a). At 1 h after inoculation, one cell was decolored and expanded (Fig. 4b). Over time,

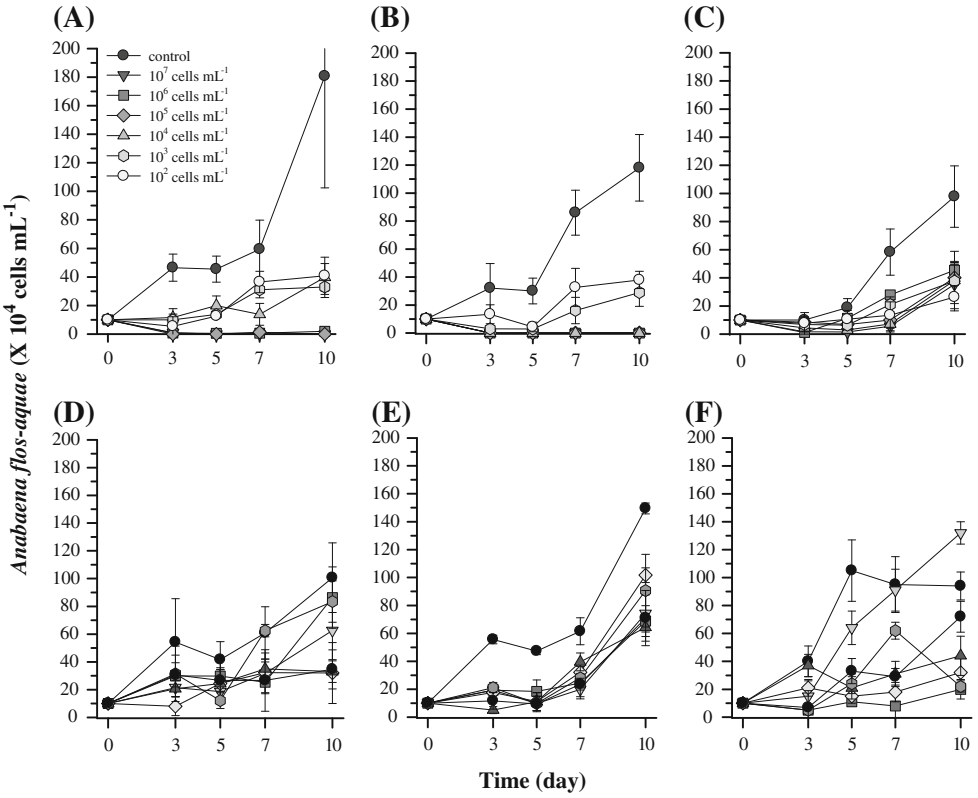

Fig. 2 Changes of *Anabaena flos-aquae* cell number (each 10^5 cells mL^{-1}) in cultures incubated with various concentrations (10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 cells mL^{-1}, respectively) of six *Lactobacillus* species: **a** *Lactobacillus plantarum*, **b** *Lactobacillus paraplantarum*, **c** *Lactobacillus paracasei*, **d** *Lactobacillus bulgaricus*, **e** *Lactococcus garvieae*, and **f** *Lactobacillus farciminis*. Data are the mean±SD from at least three independent assays

most cells became circular and swollen (Fig. 4c). Finally, several cells burst, and as a consequence the filament fell apart within 12 h (Fig. 4d). At 6 h, a few cells remained intact. However, the remaining cells were all destroyed within 12 h and their structure was completely unrecognizable (Fig. 4e).

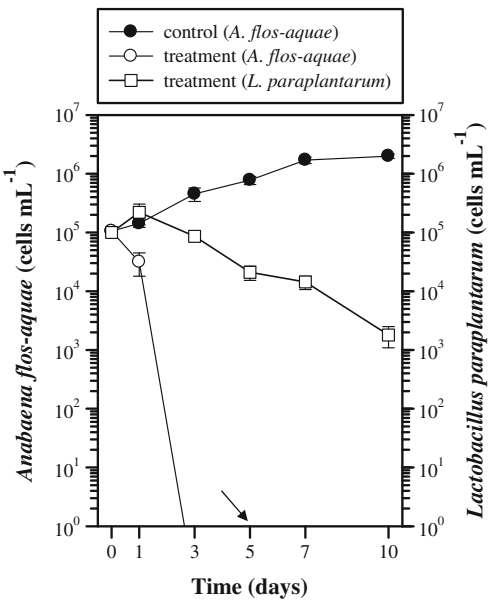

Fig. 3 Changes of *Anabaena flos-aquae* cell number (10^5 cells mL^{-1}) and *Lactobacillus paraplantarum* at initial bacterial density of 10^5 cells mL^{-1} in co-culture. The *arrow* indicates the time for complete lysis of *A. flos-aquae* cells. Data are the mean±SD from at least three independent assays

Properties of cyanobacteriacidal substance(s)

To confirm whether the presence and absence of target cyanobacterium (*A. flos-aquae*) can have an important impact on the release of cyanobacteriacidal substance(s) from the LAB, both mono-culture and co-culture filtrates of the *Lactobacillus* were tested against *A. flos-aquae* (Fig. 5). At concentrations of 10, 25, and 50 % (*v*/*v*) the co-culture filtrates showed strong cyanobacteriacidal activities, with typical concentration-dependant algaecidal activity (45 to 100 %). In contrast, the mono-culture filtrates at all concentrations displayed little or no activity (6 to 12 %). The co-culture filtrates (10 to 50 %, *v*/*v*) after heat (45 to 100 %) or proteinase-K treatment (51 to 100 %) showed effective concentration-dependant cyanobacteriacidal activity, 10 days after treatment. Moreover, the values did not significantly differ among all treatments except for mono-culture filtrate. When five organic solvent extracts were subjected to halo assay on *A. flos-aquae* culture plates, the diethyl ether- and ethyl acetate-extracted co-culture filtrates of *L. paraplantarum* showed clear lytic zones, as shown in Fig. 6. However, other clear lytic zone was not observed around the disk containing other extracts placed on the lawn plate.

Table 1 The algaecidal and cyanobacteriacidal activity of cyanobacteriacidal bacterium against a range of abundant algae and cyanobacteria found in Korean freshwater

Strain	Algaecidal and cyanobacteriacidal activity[a] *Lactobacillus paraplantarum*
Microcystis aeruginosa WREO 3	0
Microcystis aeruginosa WREO 4	0
Microcystis wesenbergii WREO 5	0
Anabaena flos-aquae CCAP 1403/13B	++**
Anabaena flos-aquae WREO 1	++**
Anabaena crassa WREO 8	++
Anabaena crassa WREO 7	++
Scenedesmus actus NIES 94	+**
Chlorella vulgaris KMMCC FC-109	0
Coelastrum reticulatum KMMCC FC-29	0
Golenkinia sp. HYK0912-A15	0
Pediastrum sp. HYD0810-A9	+*
Aulacoseira granulata HYD0810-A8	0
Cyclotella meneghiniana HYK0210-A1	+*
Stephanodiscus hantzschii UTCC 267	++**
Peridinium bipes HYJA0311-A2	++**

"++" >60 % inhibition of total, "+" >30 %, "0" no inhibition, *ANCOVA* analysis of covariance (used to determine if there were statistically significant differences in response between control and treatment over time)

*$P<0.05$; **$P<0.01$

[a] Algaecidal and cyanobacteriacidal activity (for 6 days after day 4) was determined by the following equation: algaecidal and cyanobacteriacidal activity (%)=$(1-Tt/Ct)\times 100$, where treatment (T) and control (C) are the cell densities with and without algaecidal bacteria, respectively, and t is the incubation time. The value for the reduction ratio (%) is the mean±SD (n=9)

The application of cyanobacteriacidal bacteria in microcosm experiments

Figure 7 shows the result of population changes in natural phytoplankton assemblage involving genus *Anabaena* in the microcosm after the application of the LAB as a bio-agent. In the control, *Anabaena* bloom was observed, while the phytoplankton abundance showed a lag phase (~day 2) followed by a rapid increase in cell density (1.47×10^4 cells mL^{-1} on day −2 to 4.32×10^4 cells mL^{-1} on day 6) (Fig. 7a). The *Anabaena* group (mean composition ratio, 78.7~90.0 %) of the total phytoplankton assemblage was composed mainly of *A. crassa* (60.5~71.9 %), *Anabaena circinalis* (5.4~15.5 %), *A. flos-aquae* (3.4~8.5 %), other *Anabaena* (1.0~3.0 %), and others representing 10.0~21.3 % during the whole experimental period. The level and order of phytoplankton composition were consistently maintained throughout the study period; the Cyanobacteria (including *Anabaena*), Bacillariophyta and Chlorophyta exhibited the first- (84.9~91.0 %) second- (3.3~12.1 %), and third-highest dominance (2.0~5.5 %), respectively, while the rest contributions (0.4~2.7 %) remained very low.

In contrast, the microcosm inoculated with the bacterium showed significant decline of total phytoplankton (1.47×10^4 cells mL^{-1} on day −2 to 0.01×10^4 cells mL^{-1} on day 8) and *Anabaena* in cell density (1.24×10^4 cells mL^{-1} on day −2 to 0 cells mL^{-1} on day 8) (Fig. 7b). At the beginning of the study period (on day −2), 84.8 % of the total phytoplankton assemblage was made up of *Anabaena* community (*A. crassa*, 60.5 %; *A. circinalis*, 15.5 %; *A. flos-aquae*, 7.8 %; the other *Anabaena*, 1 %) in the treatment. However, *Anabaena* accounted for only 0.7 % (*A. crassa*, 0.7 %) on day 6 and 0 % on day 8. At the beginning of study (day −2), the Cyanobacteria was the dominant group (85.8 %) in the treatment. Subsequently, the Bacillariophyta (67 to 93.5 %) rapidly gained dominance from 4 days after inoculation while the

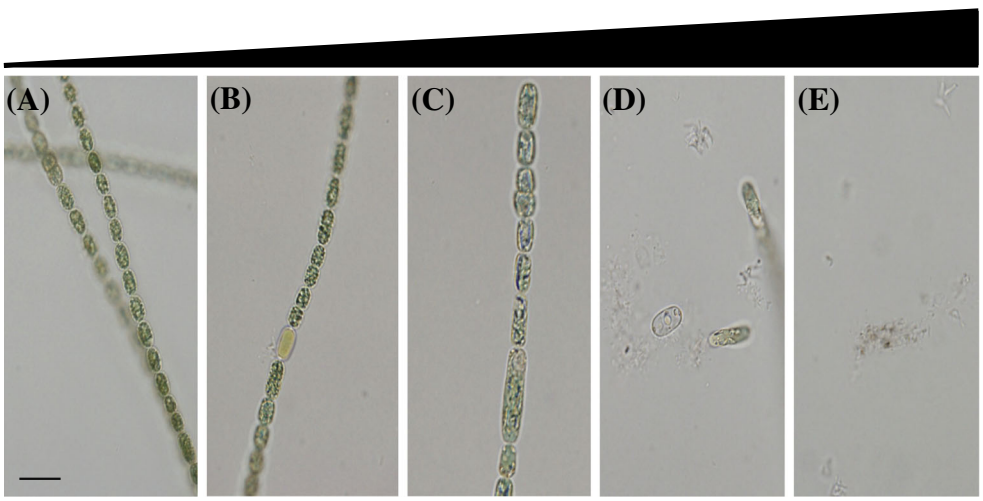

Fig. 4 Light microscopic observation of *Anabaena flos-aquae* in the presence (**a**–**e**) of *Lactobacillus paraplantarum* (**a** 0, **b** 1, **c** 4, **d** 8, and **e** 12 h). *Scale bar*=10 μm

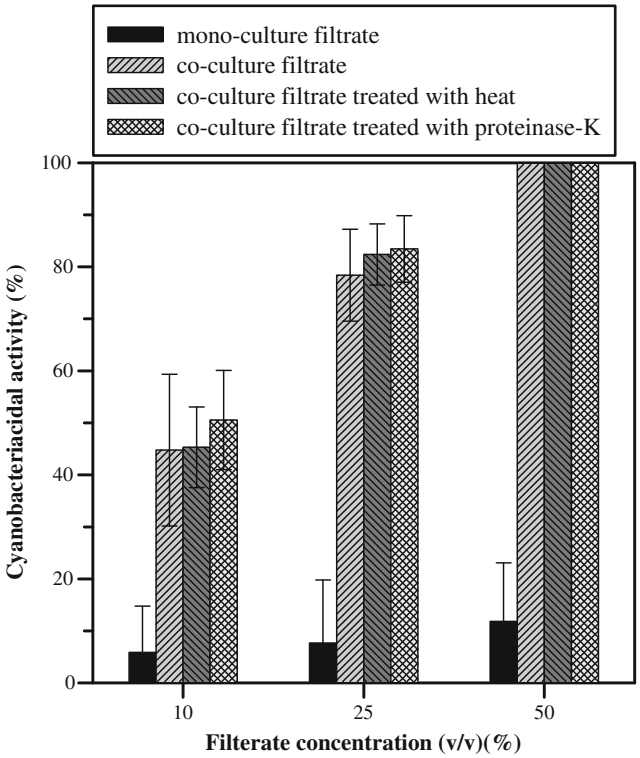

Fig. 5 Cyanobacteriacidal activity (%) against *Anabaena flos-aquae* (1×10^5 cells mL^{-1}) in cultures incubated with the mono- and co-culture filtrates of *Lactobacillus paraplantarum* and treated with two conditions, for 10 days as follows: (1) mono-culture filtrate; (2) co-culture filtrate; (3) co-culture filtrate treated with heat (121 °C, 15 min); and (4) co-culture filtrate treated with proteinase-K. Data are the mean±SD from at least three independent assays

cyanobacteria composition ratios diminished on day 8. The Bacillariophyta on day 0 increased sharply to a peak value on day 6. In addition, the Chlorophyta and the rest in the treatment enclosure showed greater increases in their densities versus the control up to day 4; thereafter, all microcosms (the control and treatment) showed similar growth pattern from day 6 to the final experimental day (day 10).

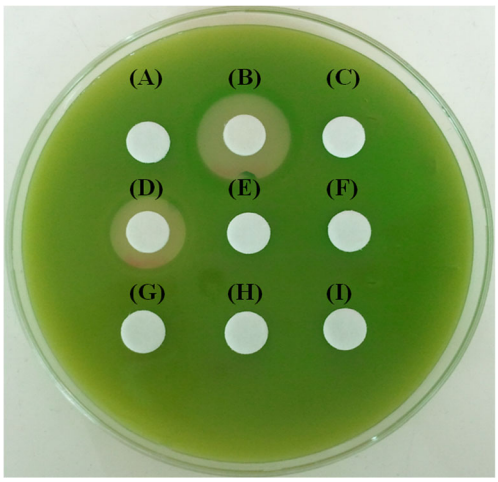

Fig. 6 Cyanobacteriacidal activities of several organic solvent-extracted co-culture filtrates of *Lactobacillus paraplantarum*. A 20-μL aliquot of each extract was spotted onto a paper disk on an *Anabaena flos-aquae* lawn plate. **a** Petroleum ether, **b** ethyl ether, **c** chloroform, **d** ethyl acetate, and **e** *n*-butanol extracts; **f** aqueous phase; **g** CB medium; and **h** ethyl ether and **i** ethyl acetate solvents

Discussion

Fourteen strains of genera *Lactobacillus* and *Lactococcus* were tested for cyanobacteriacidal activity against *A. flos-aquae*, and six (40 %) of the strains could effectively lyse cyanobacterial cells. Of the LAB tested, *L. paraplantarum* showed the strongest cyanobacteriacidal activity. The inoculation of greater than 10^5 cells mL^{-1} resulted in the complete lysis of *A. flos-aquae* cells, while its cyanobacteriacidal activities were sharply diminished below these densities. This is in agreement with the results of Kang et al. (2011), which demonstrated that the initially inoculated density of algaecidal bacteria is one of the important factors influencing the changes of algal and cyanobacterial cell abundance. At the density of 10^5 cells mL^{-1}, moreover, the LAB could completely control the growth of natural *Anabaena* species in microcosm, indicating that the threshold density of *L. paraplantarum* may be 10^5 cells mL^{-1}. Thus, the threshold density (10^5 cells mL^{-1}) of the LAB tested may be a prerequisite for achieving successful termination of natural *A. flos-aquae* blooms with a minimum initial inoculation density.

Moreover, the cyanobacterial cell density was heavily and rapidly decreased, leading to the continuous decrease in the cell density of the LAB. According to Fraleigh and Burnham (1988), the densities of host cyanobacterial cells and the levels of their derived organic matters (DOM) influence bacterial growth. Therefore, it seems that the rapid exhaustion of the main food source (the cells of *A. flos-aquae* and their DOM concentration) by cyanobacteriacidal bacteria could cause rapid decrease of the bacterial densities as reported by Kang et al. (2012b). It is thought that if the LAB is present in natural ecosystem containing no or little target cyanobacterial cells, its population could not survive and increase to a threshold density.

However, our host range revealed that the LAB can also utilize several algae and cyanobacteria, including *A. flos-aquae*, *A. crassa*, and *A. circinalis* as a generalist, without regard for the morphological characteristics. This may indicate that the LAB would not necessarily have to lyse *Anabaena* cells for its growth or survival if other host algae such as *Scenedesmus*, *Pediastrum*, *Cyclotella*, *Stephanodiscus*, and *Peridinium* exist abundantly in the natural environment. Generally, a generalist has the ability to survive, due to a broad range of alternative prey, given a low abundance of their primary prey (Koss and Snyder 2004). Moreover, microcosm testing using natural water indicated that a reduction in cell density was clearly apparent not only

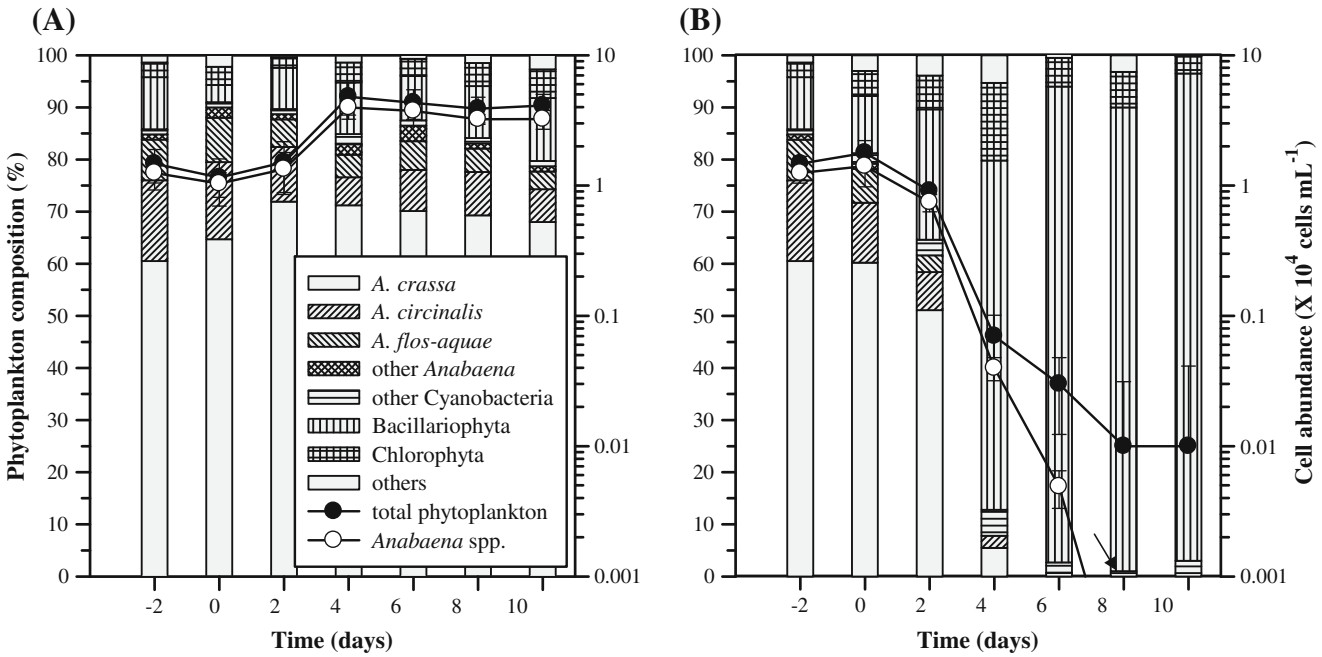

Fig. 7 Time course of cell abundances of total phytoplankton and *Lactobacillus paraplantarum* and composition (%) of major phytoplankton (genus *Anabaena*, the other Cyanobacteria, Bacillariophyta, Chlorophyta, and others) in **a** negative control without LAB and **b** LAB treatment with pellet of *L. paraplantarum* during the microcosm experiment. The *arrow* indicates the time for complete lysis of natural *Anabaena* cells. Data are the mean±SD from at least three independent assays

in *Anabaena*-like structure, but also in the Bacillariophyta composed overwhelmingly of its another preys, *Cyclotella* and *Stephanodiscus*. Although two diatoms held a dominant position among phytoplankton assemblage at the latter half of experimental period, another bloom of new dominant species including two diatoms did not fortunately occur under the application of the LAB. Kang et al. (2011) reported that a field application of bacteria as a biological control may cause problems such as marked regeneration of nutrients and subsequent blooms of other phytoplankton. Thus, our results suggest that a LAB, *L. paraplantarum* may be a potential bio-agent for use in controlling freshwater *Anabaena* in a field application if there is no secondary bloom by a resistant species to this bacterium.

In the present study, the significant decrease in *A. flos-aquae* cell density was observed when the LAB was added to the cyanobacterial culture, and also when only the co-culture filtrate was added. Moreover, our microscopic observations verified that the cyanobacteriacidal bacterium could lyse cells of *A. flos-aquae* indirectly without cell-to-cell contact during the whole experimental period. The cyanobacteriacidal modes of bacteria generally proceed in one of three ways (Sigee et al. 1999): (1) direct attack (Imai et al. 1993); (2) release of extracellular compounds (Lovejoy et al. 1998; Kang et al. 2007); or (3) bacterial entrapment, which may involve lysis through the release of lysozyme-like enzymes on the bacterial surface (Kang et al. 2005). Of these, the indirect contact type of cyanobacteriacidal bacteria can be economically more advantageous for the development of cyanobacteriacidal chemicals originating from bacteria, owing to the maintenance of high algaecidal activity irrespective of surrounding environmental constraints and to less toxicity to the environment when compared with existing algicides such as copper based algicides (Kang et al. 2012a), although any chemically mediated substances may easily be diluted by mixing in running water.

Generally, cyanobacteriacidal compounds originating from bacteria are either proteins, peptides, amino acids, antibiotics, biosurfactants, bacillamide, hydroxylamine, lipid peroxidants or others (Ren et al. 2010). In our study, the extracellular substance(s) in the culture filtrates related to algaecidal activity were chemical compound(s) that are heat-resistant and not lysed by proteinase, indicating that they may not be proteins and large size molecular chemicals. Thus, it is possible that these cyanobacteriacidal substance(s) could be LAB-derived antimicrobial substances such as organic acids, lactic acid, hydrogen peroxide, diacetyl, fatty acids or phenylacetic acid, or bacteriocin (Kim et al. 2009). It is, moreover, thought that the LAB should excrete two or more cyanobacteriacidal substances capable of lyse *Anabaena* cells into surrounding water because diethyl ether and ethyl acetate extract can dissolve nonpolar and medium-polar substance, respectively when compared with other organic solvent and water fractions. Therefore, further work is required to isolate and identify extracellular cyanobacteriacidal compounds originating from the LAB strain tested using chromatographic technology.

Considering that cyanobacteriacidal bacteria may serve as a latent agent of disease for various organisms (including humans) in a water ecosystem by replacing the host alga as the main prey with other organisms (Ren et al. 2010), LABs may be better candidates compared with existing cyanobacteriacidal bacteria. The reason is due to the fact that they are (1) metabolically versatile bacteria known as biocontrol and bioremediation agents against various pathogens (Bringel et al. 2005), (2) are generally recognized as safe for human and water ecosystem use (Torriani et al. 2001), and (3) are accessible from bacteria culture collection centers (Haarman and Jan 2006). To the best of our knowledge, no LABs have been described as cyanobacteriacidal bacteria. Thus, this is the first report to demonstrate that LABs have cyanobacteriacidal activity against bloom-forming cyanobacteria.

In conclusion, this study suggests that LAB may be an attractive bio-agent for controlling natural HABs if there is no recurring bloom by another algal and cyanobacterial species after the field application of the bacterium. We showed that inoculation of 10^5 cells mL^{-1} of LAB into the initial or developing (10^4 to 10^5 cells mL^{-1}) blooms can effectively prevent blooms and induce early curtailment of excess cyanobacterial propagation of *A. flos-aquae*. Also, we showed the presence of two or more (heat-resistant small size molecular) cyanobacteriacidal substances outside of the bacterial cell, which most likely promotes lysis of *A. flos-aquae*. The isolation and identification of algaecidal compounds are currently underway in our laboratory.

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References

Beakes G, Canter HM, Jaworski GHM (1988) Zoospores ultrastructure of *Zygorhizidium affluens* Canter and *Z. planktonicum* Canter, two hytrids parasitizing the diatom *Asterionella Formosa*. Hassall. Can J Bot 66:1054–1067

Berg KA, Lyra C, Sivonen K, Paulin L, Suomalainen S, Tuomi P, Rapala J (2009) High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms. ISME J 3:314–325

Billkova A, Kinova Sepova H, Bukovsky M, Bezakova L (2011) Antibacterial potential of lactobacilli isolated from a lamb. Vet Med 56:319–324

Bradford MM (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bringel F, Castioni A, Olukoya DK, Fekis GE, Torriani S, Dellaglio F (2005) *Lactobacillus plantarum* subsp. *argentoratensis* subsp. nov., isolated from vegetable matrices. Int J Syst Evol Microbiol 55: 1629–1634

Carmichael WW (1992) A status report of planktonic cyanobacteria (blue-green algae and their toxins). #EPA/600/R-92/079. United States Environmental Protection Agency, Washington, DC, USA

Choi H-J, Kim B-H, Kim J-D, Han M-S (2005) *Streptomyces neyagawaensis* as a control for the hazardous biomass of *Microcystis aeruginosa* (Cyanobacteria) in eutrophic freshwater. Biol Control 33:335–343

Curk M-C, Hubert J-C, Bringel F (1996) *Lactobacillus paraplantarum* sp. nov., a new species related to *Lactobacillus platarum*. Int J Syst Bacteriol 46:595–598

de Man JD, Rogosa M, Sharpe ME (1960) A medium for the cultivation of *Lactobacilli*. J Appl Bacteriol 23:130–135

de Vuyst L, Vandamme EJ (1994) Antimicrobial potential of lactic acid bacteria. In: DeVuyst L, Vandamme EJ (eds) Bacteriocins of lactic acid bacteria: microbiology, genetics and applications, 1st edn. Blackie Academic, London, pp 1–8

Fraleigh PC, Burnham JC (1988) Myxococcal predation on cyanobacterial populations: nutrient effects. Limnol Oceanogr 33: 476–483

Giraud E, Gosselin L, Raimbault M (1992) Degradation of cassava linamarin by lactic acid bacteria. Biotechnol Lett 14:593–598

Gueguen Y, Chemardin P, Labrot P, Arnaud A, Galzy P (1997) Purification and characterization of an intracellular β-glucosidase from a new strain of *Leuconostoc mesenteroides* isolated from cassava. J Appl Microbiol 82:469–476

Gumbo JR, Ross G, Cloete TE (2010) The isolation and identification of predatory bacteria from a *Microcystis* algal bloom. Afr J Biotechnol 9:663–671

Haarman M, Jan K (2006) Quantitative real-time PCR analysis of fecal *Lactobacillus* species in infants receiving a prebiotic infant formula. Appl Environ Microbiol 72:2359–2365

Imai I, Ishida Y, Hata Y (1993) Killing of marine phytoplankton by a gliding bacterium *Cytophaga* sp., isolated from the coastal sea of Japan. Mar Biol 116:527–532

Kang Y-H, Kim J-D, Kim B-H, Kong DS, Han M-S (2005) Isolation and characterization of a bio-agent antagonistic to diatom, *Stephanodiscus hantzschii*. J Appl Microbiol 98:1030–1038

Kang Y-H, Kim B-R, Choi H-J, Seo JG, Kim B-H, Han M-S (2007) Enhancement of algicidal activity by immobilization of algicidal bacteria antagonistic to *Stephanodiscus hantzschii* (Bacillariophyceae). J Appl Microbiol 103:1983–1994

Kang Y-H, Jung SW, Jo S-H, Han M-S (2011) Field assessment of the potential of algicidal bacteria against diatom blooms. Biocontrol Sci Tech 21:969–984

Kang Y-H, Jung SW, Joo J-H, Han M-S (2012a) Use of immobilized algicidal bacteria to control natural freshwater diatom blooms. Hydrobiologia 683:151–162

Kang Y-H, Park C-S, Han M-S (2012b) *Pseudomonas aeruginosa* UCBPP-PA14 as a useful bacterium capable of lysing *M. aeruginosa* cells and degrading microcystins. J Appl Phycol 24:1517–1525

Kim J-D, Han M-S (2004) Enzyme profiles of alga-lytic bacterial strain AK-13 related with elimination of cyanobacterium *Anabaena cylindrica*. Kor J Environ Biol 22:184–191

Kim M, Lee S-J, Seul K-J, Park Y-M, Ghim S-Y (2009) Characterization of antimicrobial substance produced by *Lactobacillus paraplantarum* HNUC25 isolated from kimchi. Kor J Microbiol Biotechnol 37:24–32

Koss AM, Snyder WE (2004) Alternative prey disrupt biocontrol by guild of generalist predators. Biol Control 10:1–9

Lee J-H, Kim M, Um S (2004) PCR-based detection and identification of *Lactobacillus plantarum*, *Lactobacillus pentosus*, and *Lactobacillus paraplantarum* in kimchi. Food Sci Biotechnol 13:754–757

Leia V, Amoa-Awuab WKA, Brimerc L (1999) Degradation of cyanogenic glycosides by *Lactobacillus plantarum* strains from spontaneous cassava fermentation and other microorganisms. Int J Food Microbiol 53:169–184

Lovejoy C, Bowman JP, Hallegraeff GM (1998) Algicidal effects of a novel marine *Pseudoalteromonas* isolated (class Proteobacteria, Gamma subdivision) on harmful algal bloom species of the genera *Chatonella*, *Gymnodinium*, and *Heterosigma*. Appl Environ Microbiol 64:2806–2813

Manage PM, Kawabata Z, Nakano S (2000) Algicidal effect of the bacterium *Alcaligenes denitrificans* on *Microcystis* spp. Aquat Microb Ecol 22:111–117

NIES (2004) Microalgae and Protozoa. In: Watanabe MM, Hiroki M, Kasai F, Kawachi M, Shimizu A, Erata M, Mori F, Yumoto K (eds) NIES-collection: list of strains, 6th edn. National Institute for Environmental Studies, Tsukuba, Japan, pp 50–51

Park YT, Park JB, Chung SY, Song BC, Lim WA, Kim CH, Lee WJ (1998) Isolation and algicidal properties of *Micrococcus* sp. LG-1 possessing killing activity for harmful Dinoflagellate *Cochlodinium polykrikoides*. J Kor Fish Soc 31:767–773

Park BS, Baek SH, Ki J-S, Cattolico RA, Han M-S (2012) Assessment of EvaGreen-based quantitative real-time PCR assay for enumeration of the microalgae *Heterosigma* and *Chattonella* (Raphidophyceae). J Appl Phycol 24:1555–1567

Reim RL, Shane MS, Cannon RE (1974) The characterization of a *Bacillus* capable of blue-green bactericidal activity. Can J Microbiol 20:981–986

Ren H, Zhang P, Liu C, Xue Y, Lian B (2010) The potential use of bacterium strain R219 for controlling of the bloom-forming cyanobacteria in freshwater lake. World J Microbiol Biotechnol 26:465–472

Rubenchik L I, Bershova O I, Knizhnik ZP (1965) On the interrelationship of *Anabaena* with bacteria and actinomycetes. In: Ecologia i physiologia sinezelenych vodorosleiy. Nauka Moscow: 223–226

Saarisalo E, Skytta E, Haikara A, Jalava T, Jaakkola S (2007) Screening and selection of lactic acid bacteria strains suitable for ensiling grass. J Appl Microbiol 102:327–336

Shilo M (1970) Lysis of blue green algae by myxobacter. J Bacteriol 104: 453–461

Sigee DC, Glenn R, Andrews MJ, Bellinger EG, Butler RD, Epton HAS, Hendry RD (1999) Biological control of cyanobacteria: principles and possibilities. Hydrobiologia 395/396:161–172

Soomro AH, Masud T, Anwaar K (2002) Role of latic acid bacteria (LAB) in food preservation and human health—a review. Pak J Nutr 1(1):20–24

Torriani S, Clementi F, Vancanneyt M, Hoste B, Dellaglio F, Kersters K (2001) Differentiation of *Lactobacillus plantarum*, *L. pentosus* and *L. paraplantarum* species by RAPD-PCR and AFLP. Syst Appl Microbiol 24:554–560

Walker HL, Patrick CL (1998) Method of isolating and propagating microorganisms and viruses. U.S. Patent No. 5,739,019