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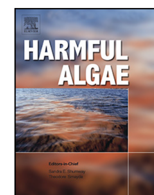

# Characterization of an algicidal bacterium *Brevundimonas* J4 and chemical defense of *Synechococcus* sp. BN60 against bacterium J4

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ABSTRACT

As part of efforts to enhance the strategies explored to eliminate the adverse impacts of cyanobacterial blooms, we isolated an algicidal bacterium, J4, from Lake Taihu. Analysis of 16S rDNA sequence revealed that strain J4 belonged to the genus *Brevundimonas*. Bacterium J4 exhibited algicidal activity mainly through excretion of extracellular algicidal compounds that were further extracted with methanol and purified by silica gel chromatography and high performance liquid chromatography (HPLC). The compounds showed thermal stability, strong polarity and water solubility in J4 cultures. Study on the algicidal activity of J4 against two dominant cyanobacterial bloom-forming species in Lake Taihu showed that J4 exhibited lower algicidal rate against *Synechococcus* sp. BN60 (48.6%, $t = 6$ days) than against *Microcystis aeruginosa* 9110 (91.8%, $t = 6$ days). Additionally, rapid reduction in cell density of J4 was observed in co-cultures of *Synechococcus* sp. BN60 and bacterium J4 but not observed in co-cultures of *M. aeruginosa* 9110 and bacterium J4 during algicidal process, which was the main reason why the algicidal rate of J4 against BN60 was lower than against 9110. The reduction in cell density of J4 resulted from inducible production of antimicrobial-like compound secreted by *Synechococcus* sp. BN60 in co-cultures of *Synechococcus* sp. BN60 and bacterium J4, which reflected a kind of chemical defense from cyanobacteria (BN60) against algicidal bacteria (J4). However, *M. aeruginosa* 9110 had no chemical defense against J4, suggesting that whether cyanobacterial chemical defense occurs or not between cyanobacteria and algicidal bacteria depends on specific cyanobacteria–algicidal bacteria pairs. These results show that not only one-sided algicidal effect but also two-sided reciprocal inhibition interactions exist between algicidal bacteria and cyanobacteria, indicating the complexity of cyanobacteria–algicidal bacteria interactions in Lake Taihu and the need to take the cyanobacterial defensive responses into consideration when assessing potential use of algicidal bacteria.

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## 1. Introduction[1]

Increases in human population and economic development have brought heavy nitrogen/phosphorus loads into waterways, consequently causing many freshwater bodies become eutrophic. Cyanobacterial blooms can be one consequence of eutrophication and frequently occur around the world, often causing large economic losses, e.g. by killing massive fish and reduction of water resources, and seriously threatening humans and livestock health by the accumulation of toxic compounds in food or drinking water (Guo, 2007; Huisman et al., 2005; Wang et al., 2012; Xie, 2008). Accordingly, many researchers have proposed and investigated various strategies for bloom control, including best options for management, such as controlling nutrient input to prevent blooms (Conley et al., 2009; Sengco, 2009), and supplementary methods, such as physical, chemical and biological efforts at stemming blooms (Sengco, 2009; Sigee et al., 1999). However, physical and chemical means are challenging because of their costs and unknown potential environmental damages (Churro et al., 2009; Sengco, 2009). Recently, algicidal bacteria have been associated with termination of cyanobacterial blooms (Manage et al., 2001; Rashidar and Bird, 2001; Zhang et al., 2012) and consequently considered as potential bio-agents for bloom control. Because of their potential effectiveness and species specificity, several algicidal bacteria have been isolated and further investigated their potential use in controlling cyanobacterial blooms (Feng et al., 2013; Hee-jin et al., 2005; Manage et al., 2000; Ren et al., 2010).

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[1] HPLC: high performance liquid chromatography.

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Lake Taihu, the third largest lake in China and a typical shallow freshwater body (surface area: 2338 $km^2$; mean depth: 1.9 m (Wu et al., 2007)), has gradually become hypereutrophic during the last two decades and consequently undergone annual cyanobacterial blooms since 1987 (Ma et al., 2008), in which *Microcystis* and *Synechococcus* are the dominant genera (Chen et al., 2003; Ye et al., 2011). These cyanobacterial blooms cause huge economic losses. For example, a cyanobacterial bloom in Lake Taihu from May 29 to June 4, 2007 caused an estimated US$ 400 million loss in the reduction of drinking water sources and tourism income (Liu et al., 2011). In this study, as part of efforts to enhance the strategies explored to eliminate the adverse impacts of cyanobacterial blooms, a bacterial strain J4 with strong algicidal activity against *Microcystis aeruginosa* was isolated from Lake Taihu. Surprisingly, during comparison of the algicidal effect of J4 against *M. aeruginosa* 9110 and *Synechococcus* species BN60, we had also observed a cyanobacterial defensive response from *Synechococcus* sp. BN60 against the algicidal bacterium J4, resulting in a cell density reduction of J4 in co-cultures of J4 and BN60.

Van Donk et al. (2011) found that some cyanobacteria and algae have defense responses such as migration, morphological change, cyst formation, and production of bioactive compounds against adverse conditions such as nutrient limitation, competition, or herbivorous zooplanktons. Certain algae could potentially produce cysts as defense responses to algicidal bacteria (Mayali et al., 2007; Nagasaki et al., 2000). These defensive responses enhance their survival and partially shape the structure of their populations in nature (Anderson et al., 2012; Van Donk et al., 2011). Unquestionably, algicidal bacteria also represent a source of mortality for cyanobacteria. In the present study, we focused on the cyanobacterial defensive responses against algicidal bacteria.

## 2. Materials and methods

### 2.1. Cyanobacterial cultures

*Microcystis aeruginosa* 9110 and *Synechococcus* sp. BN60 were isolated from Lake Taihu. Both cyanobacterial strains were axenic. All cyanobacterial cultures used in this study were incubated in 250 mL Erlenmeyer flasks with 100 mL BG11 medium (Stanier et al., 1971) at 25 °C, under 40 μmol photons $m^{-2}\ s^{-1}$, a 12-h light:12-h-dark cycle and amended with fresh medium every month.

### 2.2. Isolation and identification of algicidal bacteria

Algicidal bacteria were isolated during a cyanobacterial bloom in Meiliang Bay of Lake Taihu. Water samples were collected at the Taihu Ecosystem Research Station (31°24′N, 120°13′E) of Meiliang Bay from 0.5 m below the water surface during October 2009. They were collected with a sterile sampler and transported to the laboratory on ice within 4 h.

An aliquot (10 mL) of water samples was inoculated into 100 mL log-phase *Microcystis aeruginosa* 9110 cultures. When the cyanobacterial cell density was below 20% of the control (10 mL sterile water instead of water sample added) during the cultivation, 10 mL co-cultures (*M. aeruginosa* 9110 cultures inoculated with water samples from Lake Taihu) were inoculated into another fresh log-phase *M. aeruginosa* 9110 culture. To promote the possibility of isolating algicidal bacteria, this process was repeated until the consistent reduction of *M. aeruginosa* 9110 density to below 20% of the control within 6 days after inoculation.

An aliquot of co-cultures on day 6 in the last incubation cycle (fifth cycle) was tenfold serially diluted with sterile water and 0.1 mL aliquots of each dilution were spread onto beef extract-peptone agar plates (10 g $L^{-1}$ peptone, 3 g $L^{-1}$ beef extract, 5 g $L^{-1}$ sodium chloride, 1.5% (w/v) agar). The plates were incubated at 30 °C until colonies appeared. Individual colonies of distinct morphology were selected, purified using the method described by Yamamoto and Suzuki (1990) and then cryopreserved at −70 °C in 30% (v/v) glycerol. For screening of algicidal bacteria, bacterial isolates were grown in beef extract-peptone medium (30 °C, 200 rpm) for 24 h and then an aliquot (10 mL) of each bacterial culture was inoculated into 100 mL log-phase *Microcystis aeruginosa* 9110 cultures respectively. Log-phase *M. aeruginosa* 9110 culture (100 mL) inoculated with 10 mL bacterial medium served as a control. The growth of *M. aeruginosa* 9110 was monitored daily by measuring the biomass. Algicidal activity was calculated using the equation described in Section 2.6. The bacterial strains with strong algicidal activity (algicidal rate $A > 80\%$, $t = 6$ days, see Section 2.6) were further analyzed.

Identification of algicidal bacterial strains was accomplished by analysis of their 16S rDNA sequences as previously described (Tian et al., 2012).

### 2.3. Determination of algicidal mode

Bacterium J4 was incubated in beef extract-peptone medium at 30 °C, 200 rpm for 24 h. Bacterial cultures were centrifuged at 12, 000 × *g* for 20 min and the supernatants were passed through 0.22-μm polycarbonate filters to obtain cell-free filtrates. Heat-treated cell-free filtrates were obtained by autoclaving at 121 °C for 20 min. Bacterial cells were collected by centrifugation (5000 × *g*, 20 min), washed twice with sterile water and re-suspended in an equal amount of water. An aliquot (10 mL) of bacterial cultures, cell-free filtrates, heat-treated cell-free filtrates and re-suspended bacterial cells in water was inoculated into 100 mL log-phase *Microcystis aeruginosa* 9110 cultures respectively and cultivated at 25 °C, under 40 μmol photons $m^{-2}\ s^{-1}$ and a 12-h light:12-h dark cycle. *M. aeruginosa* 9110 culture (100 mL) inoculated with 10 mL bacterial medium acted as a control. The algicidal rates ($t = 6$ days) of differently treated J4 cultures were calculated according to the change of cell density of *M. aeruginosa* 9110 on day 6 after inoculation of J4. Significant differences in algicidal rate were determined using one-way ANOVA with SPSS version 19.0 (IBM, USA).

### 2.4. Interactions between bacterium J4 and each of two cyanobacterial species (M. aeruginosa *9110 and* Synechococcus *sp. BN60)*

To compare the interactions between bacterium J4 and each of the two cyanobacterial species, an aliquot (10 mL) of stationary-phase J4 cultures (cell density $1.3–1.5 \times 10^{10}$ CFU $mL^{-1}$) was inoculated into 100 mL log-phase cultures of *Microcystis aeruginosa* 9110 and 100 mL log-phase cultures of *Synechococcus* sp. BN60 respectively. The controls were 100 mL log-phase cyanobacterial cultures inoculated with an equal volume of beef extract-peptone medium instead of J4 cultures. The additional control was 100 mL BG11 medium inoculated with 10 mL J4 cultures. All tests and controls were incubated at 25 °C, under 40 μmol photons $m^{-2}\ s^{-1}$ and a 12-h light:12-h dark cycle. Cell density of *M. aeruginosa* 9110 and chlorophyll-*a* concentration of *Synechococcus* sp. BN60 were evaluated daily and then algicidal rate was calculated according to Section 2.6. In the meantime, cell density of algicidal bacterium J4 was also monitored daily by the CFU method performed on beef extract-peptone agar plates (Su et al., 2007).

### 2.5. *Antimicrobial effect of cell-free filtrates of co-cultures of Synechococcus sp. BN60 and algicidal bacterium J4*

The co-cultures of *Synechococcus* sp. BN60 and bacterium J4 (designated as co-cultures (BN60 and J4)), co-cultures of *Microcystis aeruginosa* 9110 and bacterium J4 (designated as co-cultures (9110 and J4)), and log-phase *Synechococcus* sp. BN60 cultures on day 6 were collected and then treated by centrifugation and filtration as described in Section 2.3 to obtain cell-free filtrates. The heat-treated cell-free filtrates of co-cultures (BN60 and J4) were also obtained by autoclaving at 121 °C for 20 min. After being supplemented with 10% (v/v) fresh beef extract-peptone medium, the above mentioned cell-free filtrates were inoculated with 1% (v/v) log-phase cultures of J4 (cell density $7.4 \times 10^7$ CFU $mL^{-1}$ after inoculation) respectively and incubated at 30 °C, 200 rpm for 24 h. Then, the cell density of J4 was determined by the CFU method performed on beef extract-peptone agar plates (Su et al., 2007). Instead of the cell-free filtrates, sterile BG11 medium and sterile water were also supplemented with 10% (v/v) fresh bacterial medium and subjected to the same protocol as controls. All experiments were repeated in triplicate and results are given as mean ± standard deviation of raw data.

### 2.6. *Determination of algicidal activity*

The algicidal activities of algicidal bacteria were determined by evaluating changes of the biomass of cyanobacteria in the presence of the algicidal bacteria and calculated using the following equation

$$A = \left(1 - \frac{D_{t\text{-test}}}{D_{t\text{-control}}}\right) \times 100\%$$

where $A$ is algicidal rate; $D_{t\text{-test}}$ and $D_{t\text{-control}}$ are cell densities (cells $mL^{-1}$) of *Microcystis aeruginosa* 9110 or chlorophyll-*a* concentrations ($\mu g\, L^{-1}$) of *Synechococcus* sp. BN60 in tested and control cultures respectively; $t$ (day) is co-cultivation time of the cyanobacteria and algicidal bacteria. Cell density of *M. aeruginosa* 9110 was determined using a hemocytometer and an optical microscope (BH – 2, Olympus, Japan). Since *Synechococcus* sp. BN60 is not countable due to its small size and tendency to form aggregations or colonies, chlorophyll-*a* concentration was used as an index of biomass according to previous protocol (Huang and Cong, 2007; Pápista et al., 2002). All experiments were repeated in triplicate and results are given as mean ± standard deviation of raw data.

### 2.7. *Extraction and purification of algicidal compounds*

Cell-free filtrates of J4 cultures, obtained as described in Section 2.3, were concentrated and dried at 50 °C under reduced pressure in an evaporator (RE52-3, Shanghai Huxi Analysis Instrument Factory Co., China). Dried filtrates were soaked with methanol to extract algicidal compounds. After concentration, tenfold-concentrated methanol extracts were subjected to silica gel column chromatography (commercial silica gel, Qingdao Haiyang Chemical Group Co., China; 200–300 mesh; 1 cm i.d. × 50 cm) using an eluent of methanol/chloroform (5:5, v/v) at 1 mL $min^{-1}$ with monitoring at 254 nm. Fractions containing algicidal compounds were collected and further purified by high performance liquid chromatography (HPLC) with reverse-phase column (Surpsil C18-EP, 5 μm, 4.6 mm i.d. × 250 mm, Dikma, China), which was eluted with ultrapure water at 1 mL $min^{-1}$ and monitored at 210 nm.

Algicidal effects of all fractions from gel column chromatography and HPLC were monitored by a cyanobacterial-lawn protocol (Tian et al., 2012).

## 3. Results

### 3.1. *Isolation and identification of algicidal bacteria*

A total of 86 bacterial strains were isolated from Lake Taihu and screened for algicidal activity against *Microcystis aeruginosa* 9110. The screen yielded 13 strains with strong algicidal activity (algicidal rate $A > 80\%$, $t = 6$ days). Strain J4 had the strongest algicidal activity ($A = 93.0\%$, $t = 6$ days). Based on 16S rDNA sequence analysis, J4 belonged to the genus *Brevundimonas* (α-proteobacteria). Its 16S rRNA gene sequence was deposited in GenBank under accession number KC429672.

### 3.2. *Algicidal mode of bacterium J4*

Different treatments of bacterial cultures exhibited different algicidal activity against *Microcystis aeruginosa* 9110 (Fig. 1). The algicidal rate of bacterial cultures (93.0%, $t = 6$ days) was approximately 9% higher than that of cell-free filtrates of bacterial cultures (84.2%, $t = 6$ days), as well as 64.2% higher than that of re-suspended bacterial cells in water (28.8%, $t = 6$ days) ($P < 0.01$). No significant difference was observed in the algicidal rate between cell-free filtrates and heat-treated cell-free filtrates (84.3%, $t = 6$ days) ($P > 0.920$). These results suggested that strain J4 exhibited algicidal activity mainly by excreting heat-stable extracellular algicidal compounds.

### 3.3. *Extraction and purification of algicidal compounds*

Fractions with a retention time of 2.5–3.5 h on silica gel column chromatography had algicidal effects on *Microcystis aeruginosa* 9110 and *Synechococcus* sp. BN60 (data not shown). These fractions were further purified by HPLC, resulting in one fraction (retention time of 5–6 min on HPLC (data not shown)) that had algicidal effect shown as forming a clear inhibition zone on cyanobacterial lawns of *M. aeruginosa* 9110 or *Synechococcus* sp. BN60 (Fig. 2). These results suggested at least one kind of algicidal compound existed in the J4 cultures.

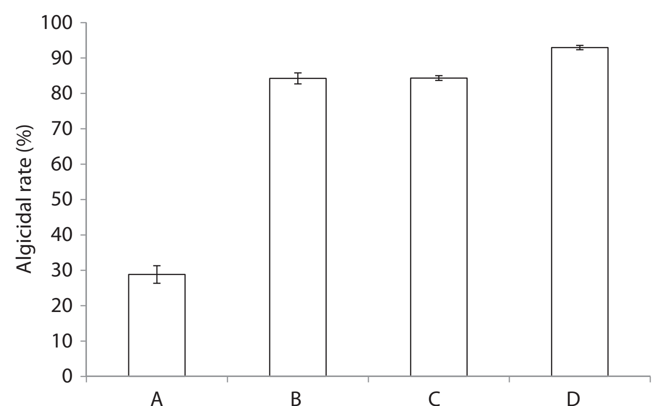


**Fig. 1.** Algicidal rates of different J4 cultures against *M. aeruginosa* 9110. Aliquots (10 mL) of differently treated bacterial cultures were inoculated into 100 mL log-phase *M. aeruginosa* 9110 cultures respectively. Differently treated bacterial cultures included re-suspended bacterial cells in water (A), cell-free filtrates (B), heat-treated cell-free filtrates (C) and bacterial cultures (D). Beef extract-peptone medium was inoculated into 100 mL log-phase *M. aeruginosa* 9110 cultures as the control. Algicidal rate (A, %) was calculated by the following equation: $A = (1 - D_{t\text{-test}}/D_{t\text{-control}}) \times 100\%$, $D_{t\text{-test}}$ and $D_{t\text{-control}}$ were cell density of *M. aeruginosa* 9110 in tests and control after 6 days cultivation respectively. Bars are triplicate means of algicidal rate. Significant differences in algicidal rate between different tests are: A and B, A–D $P < 0.01$; B and C $P \geq 0.920$. Data are mean ± S.D. from at least three independent assays.

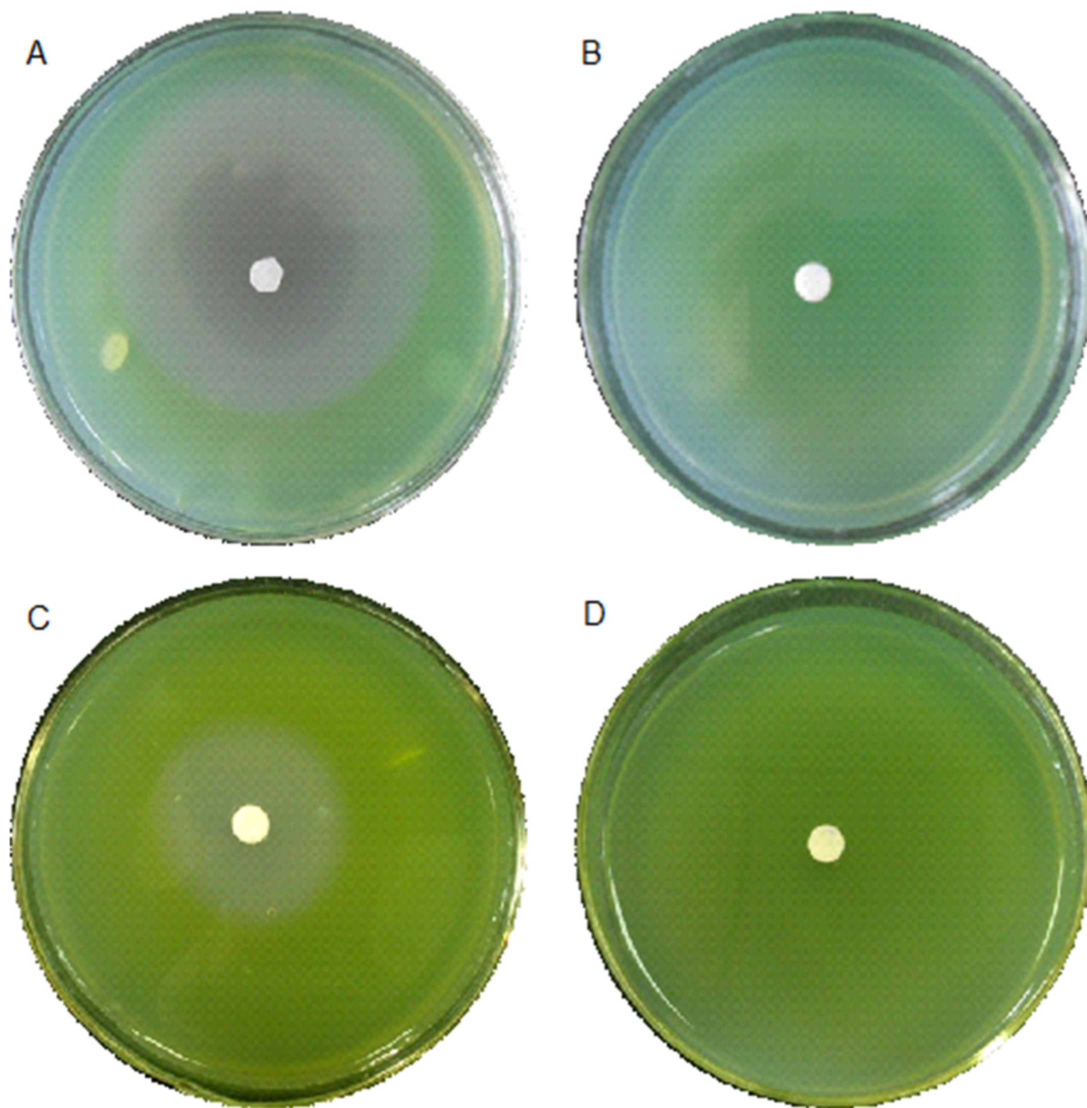


**Fig. 2.** Algicidal effects of HPLC fractions on cyanobacterial-lawns. A/C: algicidal effect of the fraction (retention time: 5–6 min) against *M. aeruginosa* 9110 and Synechococcus sp. BN60; B/D: algicidal effects of all the other fractions (no algicidal effects) against *M. aeruginosa* 9110 and Synechococcus sp. BN60.

### *3.4. Reduction of J4 density during co-culturing with* Synechococcus *sp. BN60*

*Synechococcus* sp. BN60 and *Microcystis aeruginosa* 9110 cultures were inoculated with equal volume of J4 cultures respectively, and then chlorophyll-*a* concentrations of BN60 and cell densities of 9110 in co-cultures and controls were monitored (Fig. 3A). In controls, chlorophyll-*a* concentrations in *Synechococcus* sp. BN60 cultures increased from 67.3 $\mu g\,L^{-1}$ on day 0 to 444 $\mu g\,L^{-1}$ on day 6 while cell densities of *M. aeruginosa* 9110 increased from $5.2 \times 10^6$ cells $mL^{-1}$ on day 0 to $3.0 \times 10^7$ cells $mL^{-1}$ on day 6. In experiments that J4 cultures were inoculated, chlorophyll-*a* concentrations of *Synechococcus* sp. BN60 continued to increase from 67.3 $\mu g\,L^{-1}$ on day 0 to 228 $\mu g\,L^{-1}$ on day 6. However, cell densities of *M. aeruginosa* 9110 decreased gradually from $5.2 \times 10^6$ cells $mL^{-1}$ on day 0 to $2.5 \times 10^6$ cells $mL^{-1}$ on day 6. These results showed that bacterium J4 exhibited stronger algicidal activity against *M. aeruginosa* 9110 ($A$ = 91.8%, $t$ = 6 days) than against *Synechococcus* sp. BN60 ($A$ = 48.6%, $t$ = 6 days).

In the meantime, the cell density of J4 in co-cultures (BN60 and J4) decreased from $1.7 \times 10^9$ CFU $mL^{-1}$ on day 2 to $7.0 \times 10^5$ CFU $mL^{-1}$ on day 6 (Fig. 3B). However, the cell density of J4 in co-cultures (9110 and J4) or cultivated alone in BG11 medium did not change from 1.0 to $1.4 \times 10^9$ CFU $mL^{-1}$ after 6 days. These results suggested that the reduction in cell density of J4 in co-cultures (BN60 and J4) might result from inhibitory factors presented in the co-cultures.

### *3.5. Inhibition of J4 by cell-free filtrates of co-cultures (BN60 and J4)*

After 24 h cultivation, the cell density of J4 in cell-free filtrates of co-cultures (BN60 and J4) with and without heat-treatment decreased to $6.0–7.0 \times 10^6$ CFU $mL^{-1}$ (Fig. 4A and B), which was much lower than that in the other cell-free filtrates and controls ($4.0–6.0 \times 10^8$ CFU $mL^{-1}$) (Fig. 4C–F). These results indicated that there existed antimicrobial-like compounds with heat stability in co-cultures (BN60 and J4), which were produced by *Synechococcus* sp. BN60 during co-culturing. Lack of inhibition on J4 by cell-free filtrates of log-phase *Synechococcus* sp. BN60 cultures (Fig. 4E) suggested that the production of antimicrobial compounds by BN60 was triggered by the presence of J4 during the algicidal process. Furthermore, lack of inhibition on J4 by cell-free filtrates of co-cultures (9110 and J4) (Fig. 4F) implied that only specific

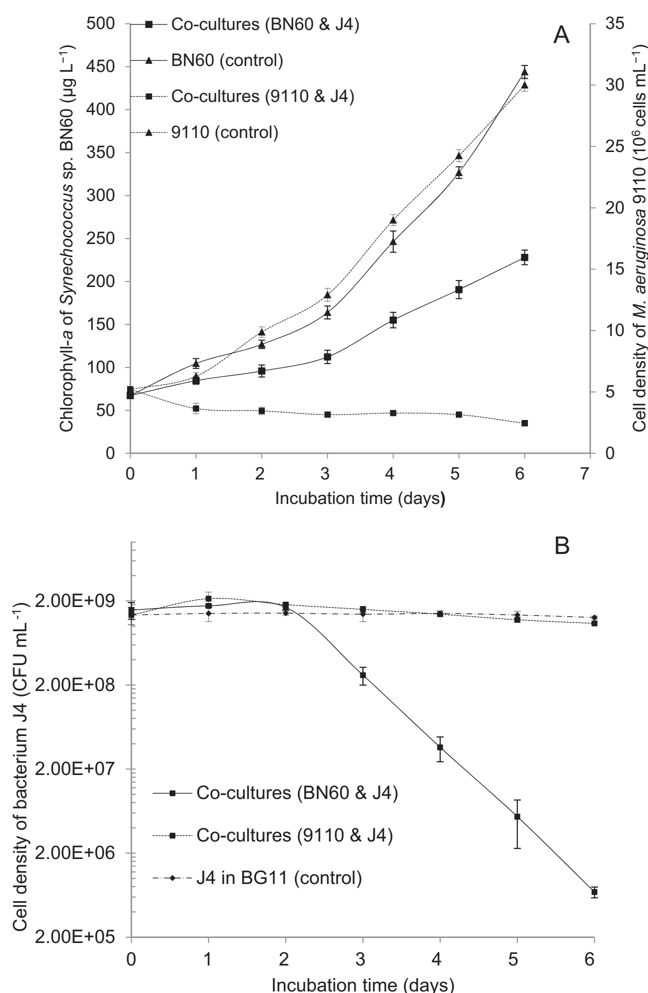


**Fig. 3.** Interaction between bacterium J4 and each of two cyanobacterial species. (A) Dynamics of chlorophyll-*a* concentration of Synechococcus sp. BN60 and cell density of *M. aeruginosa* 9110 in co-cultures and controls; (B) dynamics of cell density of J4 in co-cultures and controls. Co-cultures (9110 and J4): 10 mL J4 cultures were inoculated into 100 mL log-phase *M. aeruginosa* 9110 cultures; co-cultures (BN60 and J4): 10 mL J4 cultures were inoculated into 100 mL log-phase Synechococcus sp. BN60 cultures. Beef extract-peptone medium (10 ml) was added to cyanobacterial cultures (100 mL) as controls (9110 (control), BN60 (control)), and J4 cultures (10 mL) were inoculated into BG11 medium (100 mL) as another control (J4 in BG11 (control)). The inoculation took place on day 0. Cell density of *M. aeruginosa* 9110 and J4 and chlorophyll-*a* concentration of Synechococcus sp. BN60 in the cultures were evaluated daily. Data are mean ± S.D. from at least three independent assays.

cyanobacteria could be triggered by the presence of specific algicidal bacteria to produce antimicrobial-like compound.

## 4. Discussion

The algicidal activity of isolated strains of bacteria in this study was determined by evaluating changes in the biomass of cyanobacteria (or algae), as described by other researchers (Kang et al., 2005; Kim et al., 2007, 2008; Pokrzywinski et al., 2012; Tian et al., 2012). As cyanobacteria are autotrophic, whereas algicidal bacteria are heterotrophic, it suggests that there is no carbon source competition between them. The competition of algicidal bacteria with cyanobacteria for other inorganic nutrients (e.g., nitrogen/phosphorus) could be also negligible because these inorganic nutrients are sufficient in their co-cultivation process. Besides, the biomass of each axenic cyanobacterial species incubated alone in BG11 medium could continuously increase for more than 10 days and then maintain at maximum-level for

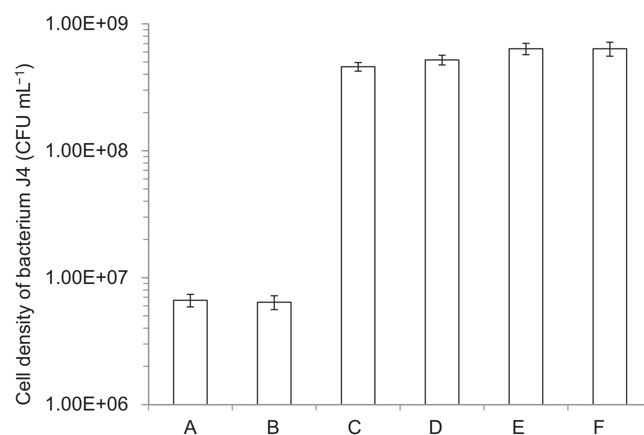


**Fig. 4.** Cell density of J4 in different mediums after 24 h cultivation. Cell-free filtrates of co-cultures (BN60 and J4) without heat-treatment (A) and with heat-treatment (B), cell-free filtrates of Synechococcus sp. BN60 cultures (E), cell-free filtrates of co-cultures (9110 and J4) (F), sterile water (C, as control), and sterile BG11 medium (D, as control) were supplemented with beef extract-peptone medium (10%, v/v) and then inoculated with log-phase J4 cultures (1% v/v) respectively. Cell density of J4 was determined after incubation at 30 °C for 24 h. Bars represent triplicate means of bacterial cell density. Data are mean ± S.D. from at least three independent assays.

more than 2 weeks. Thus, the significant differences in cyanobacterial biomass between the tests (inoculated with algicidal bacterial cultures) and controls (inoculated with beef extract-peptone medium) under the same incubated conditions could be solely attributed to algicidal bacteria but not resources competition or nutrient limitation, indicating that the determination method for algicidal activity based on changes in the biomass of cyanobacteria is reliable.

To date, most known algicidal bacteria are classified within either the cytophaga-Flavobacterium-Bacteroidetes group or the γ-Proteobacteria group (Mayali and Azam, 2004; Roth et al., 2008). However, many algicidal bacteria belonging to other genera have been identified and characterized, e.g. AMA-03 (*Ruegeria atlantica*) (Amaro et al., 2005), and LE17 (*Roseobacter* clade-affiliated) (Mayali et al., 2008). In this study, molecular identification showed that algicidal bacterium J4 belonged to the α-proteobacteria subclass, genus *Brevundimonas*. To the best of our knowledge, this is the first report of algicidal bacteria belonging to the genus *Brevundimonas*.

In general, algicidal mode of algicidal bacteria could be summarized as either direct or indirect attacks (Mayali and Azam, 2004; Pokrzywinski et al., 2012). Direct algicidal attacks mean that algicidal bacteria physically come in to contact with target cells and kill them directly, while indirect algicidal attacks are generally considered to be chemically mediated aggressions by algicidal compounds secreted from algicidal bacteria (Mayali and Azam, 2004). The reported algicidal compounds secreted by algicidal bacteria comprise peptides or enzymes (Chen et al., 2011; Imamura et al., 2000; Lee et al., 2000; Paul and Pohnert, 2011; Wang et al., 2012), biosurfactants (Wang et al., 2005), pigments (Nakashima et al., 2006; Sakata et al., 2011) and antibiotic-like substances (Dakhama et al., 1993). Compared to the number of isolated algicidal bacteria strains, fewer algicidal compounds have been successfully extracted, purified and identified. This is likely due to the difficulties in purification of algicidal compounds with various characteristics from different algicidal bacteria (Doucette and Powell, 1998; Skerratt et al., 2002). In the present study, significant differences in algicidal activity of differently treated J4 cultures (Fig. 1) indicated that J4 exhibited algicidal activity mainly through indirect attacks. The results of subsequent purification experiments further suggested at least one kind of algicidal

compound produced by bacterium J4. Based on thermal stability and polarity, these compounds are unlikely to be enzymes or surfactants (Pokrzywinski et al., 2012). The results of HPLC purification of bacterial compounds demonstrated that several other compounds with similar chemical properties existed in the bacterial culture, obstructing purification.

Manage et al. (2000) found that the algicidal bacterium *Alcaligenes denitrificans* increased to certain density before it exhibited strong algicidal activity against three axenic *Microcystis* species. A report by Mayali and Doucette (2002) also showed that the cell density of the algicidal bacterium 41-DBG2 increased before it had strong algicidal effects on xenic *Karenia brevis* C2. Consequently, the cell density of algicidal bacteria has been widely recognized as an important factor of influencing the algicidal activity. In this study, we observed the reduction in cell density of J4 in co-culture with *Synechococcus* sp. BN60 during the algicidal process. After 6 days of co-cultivation, the cell density of J4 in co-cultures (BN60 and J4) was four orders of magnitude lower than that in co-cultures (9110 and J4) (Fig. 3B). Meanwhile, bacterium J4 exhibited lower algicidal activity against *Synechococcus* sp. BN60 ($A$ = 48.6%, day 6) than against *Microcystis aeruginosa* 9110 ($A$ = 91.8%, day 6) (Fig. 3A). Therefore, the significant reduction in the cell density of J4 could at least partially explain why bacterium J4 exhibited lower algicidal activity against *Synechococcus* sp. BN60 than against *M. aeruginosa* 9110.

To the best of our knowledge, this is the first observation of rapid reduction in bacterial cell density (J4) during co-culturing process of algicidal bacteria (J4) and axenic cyanobacteria (BN60). Generally, reduction of bacterial cell density could be attributed to nutrient limitation or certain inhibitory factors in the cultures. Preliminary analysis in our study (data not shown), as well as in previous studies (Manage et al., 2000; Mayali and Doucette, 2002; Su et al., 2007), demonstrated that algicidal bacteria can use the compounds released from cyanobacterial (or algal) cell lysis. Moreover, in the present study, the cell density of J4 in co-cultures (9110 and J4) or incubated alone in BG11 medium did not significantly change over 6 days (Fig. 3B). It strongly indicates that nutrient limitation could not play major role in the reduction of cell density of J4 in co-cultures (BN60 and J4). Hence, it must be certain inhibitory factors in co-cultures (BN60 and J4) resulting in the reduction of J4 density in the co-cultures.

The decrease in cell density of J4 in co-cultures (BN60 and J4) resulted from the antimicrobial-like compounds that existed in the co-cultures (Fig. 4). Furthermore, the antimicrobial-like compounds were induced products released by *Synechococcus* sp. BN60 in co-cultures (BN60 and J4) when J4 exerted algicidal effects on *Synechococcus* sp. BN60. In previous studies, cyanobacteria and algae have been demonstrated possessing various strategies to protect themselves from adverse surrounding conditions (Anderson et al., 2012; Van Donk et al., 2011). The strategies are proposed to be of three types of defense for their survivals: morphological change (formation of spines, colonies and thick cell walls), life cycle change (formation of cysts, reduced recruitment rate) and production of bioactive compounds (Van Donk et al., 2011). As a chemical defense for survival, it was reported that some cyanobacteria could produce bioactive compounds to respond to the attacks from herbivorous zooplanktons and consequently reduce the density of these grazers (Jang et al., 2003; Pajdak-Stos et al., 2001). Beyond doubt, algicidal bacterium J4 was adverse surrounding effects of *Synechococcus* sp. BN60 in the co-cultures. Thus, the inducible production of antimicrobial-like compounds by *Synechococcus* sp. BN60 could be a chemical defense that mitigates the algicidal effects from J4. It was the chemical defense of *Synechococcus* sp. BN60 against J4 that resulted in a cell density decrease of J4 in co-cultures (BN60 and J4), consequently leading to the decrease of algicidal effects of J4 against BN60.

Interactions between bacteria and cyanobacterial species are an important factor in regulating the cyanobacterial population (Manage et al., 2001; Paerl et al., 2001; Su et al., 2007; Zhang et al., 2012). The interactions include one-sided (only one organism affected by another) and two-sided (both bacteria and caynobacterial species affected by each other) effects that can be harmful or beneficial (Kodama et al., 2006). To date, previous studies on algicidal bacteria have mainly focused on their one-sided algicidal effects on cyanobacteria, but not on the defensive responses from cyanobacteria against algicidal bacteria. In the present study, the cyanobacterial chemical defense against algicidal bacteria indicates that not only one-sided algicidal action (e.g. J4 vs. 9110) but also two-sided reciprocal inhibition interactions (e.g. J4 vs. BN60) between algicidal bacteria and cyanobacteria exist in co-occurring algicidal bacteria-cyanobacteria populations. Occurrence of chemical defenses in *Synechococcus* sp. BN60 but not in *Microcystis aeruginosa* 9110 against bacterium J4 implies that what type of interaction occurs between them depends on specific algicidal bacteria–cyanobacteria pairs. Furthermore, the fact that the chemical defense in *Synechococcus* sp. BN60 resulted in lower algicidal activity of bacterium J4 against BN60 than against *M. aeruginosa* 9110 suggests that the type of interactions between algicidal bacteria and cyanobacteria could influence the algicidal activity of algicidal bacteria. Nevertheless, continued exploration of how bacterium J4 interacts with other cyanobacterial species is still necessary to determine what role bacterium J4 plays in regulating bloom dynamics, as well as to provide more information for field-based studies (e.g. mesocosms).

In summary, in the present study, algicidal bacterium J4 exhibited strong algicidal activity against dominant cyanobacterial bloom-forming species *Microcystis aeruginosa*, indicating its potential use in bio-controlling cyanobacterial blooms in Lake Taihu. The finding of chemical defense in *Synechococcus* sp. BN60 against bacterium J4 promotes our understanding about the interactions between algicidal bacteria and cyanobacteria in Lake Taihu, e.g. the complexity and species-specificity. Although a difficult task, it will be essential to investigate bacterium J4 under natural conditions in lake Taihu in future when assessing the potential role of this bacterium not only as an natural regulator of bloom dynamics, but also as part of a blooms management and control strategy.

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