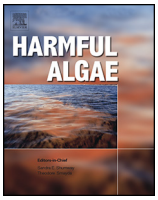

Characterization of an algicidal bacterium *Brevundimonas* J4 and chemical defense of *Synechococcus* sp. BN60 against bacterium J4

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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

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 × 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 7.4×10^7 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

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

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 Tm()Tj/8.21

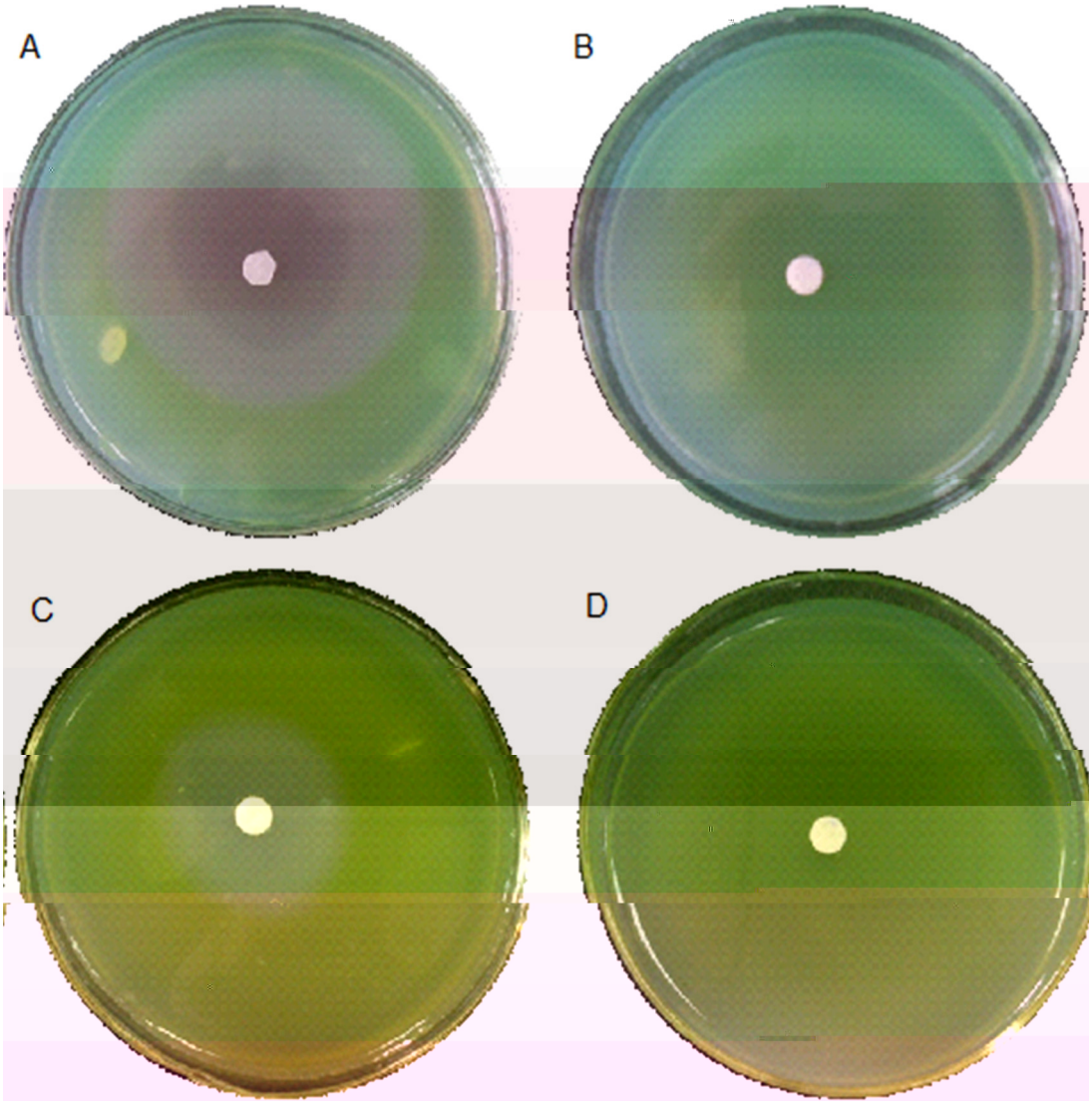

Fig. 2. Algicidal effects of HPLC fractions on cyanobacterial-lawns. A/C: algicidal effect of the fraction (retention time: 5–6 min) against *M. aeruginosa* 9110 and Synechococcus sp. BN60; B/D: algicidal effects of all the other fractions (no algicidal effects) against *M. aeruginosa* 9110 and Synechococcus 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 5.2×10^6 cells mL^{-1} on day 0 to 3.0×10^7 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 5.2×10^6 cells mL^{-1} on day 0 to 2.5×10^6 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 1.7×10^9 CFU mL^{-1} on day 2 to 7.0×10^5 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 1.4×10^9 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

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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. 3CFig.00-.5669123.1239511.8235Tm()Tj/F1Tf02Tm(lower)Tj/F21Tf.5669008u5To58140TD(CFig.08er)wer

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