Interactive effects of algicidal efficiency of *Bacillus* sp. B50 and bacterial community on susceptibility of *Microcystis aeruginosa* with different growth rates

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**ABSTRACT**

Application of algicidal bacteria is a promising way in the control of harmful algal blooms. However, many environmental factors can strongly influence the algicidal efficiency of algicidal bacteria against harmful algae. Till now, the relationship between algicidal efficiency and environmental factors is still elusive. In order to explore the key factors which play important role in determining the algicidal efficiency of an algicidal bacterium *Bacillus* sp. B50 against *Microcystis aeruginosa*, the susceptibilities of thirteen *M. aeruginosa* strains to algicidal bacterium *Bacillus* sp. B50 were tested. The results indicated that these thirteen *M. aeruginosa* strains could be classified into three groups: high susceptible strains, moderate susceptible strains and, resistant ones. The susceptibility of these *Microcystis* strains to *Bacillus* sp. B50 did not correspond with microcystins-producing characteristics or cellular phenotype of *M. aeruginosa*. However, the susceptibility has a linear relationship with growth rate of these *M. aeruginosa* strains. Bacterial community in the culture of the resistant strains could modify the susceptibility of susceptible one to the stress from algicidal bacterium, but this modification could be diminished by enhancing inoculation density of algicidal bacterium.

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**Introduction**

*Microcystis* spp., the major microcystins-producing cyanobacteria, have been commonly found worldwide, and they frequently form severe algal blooms in many eutrophic water bodies. Taking an example from China, two large lakes, Lake Dianchi and Lake Taihu, are all suffered severe *Microcystis* dominated water blooms. Several approaches, such as chemical algicides and physical manipulations, have been proposed to control harmful algal blooms, but most of them are impracticable because of high cost or subsequent secondary pollution (Shao et al., 2013). An alternative approach for the removal of cyanobacterial blooms was proposed to the application of algicidal bacteria (Nakamura et al., 2003). However, the field application of algicidal bacteria is very limited due to the influence of environmental factors. Many biotic and abiotic factors, e.g. bacterial community in algal culture (Mayali and Doucette, 2002), dissolved organic matter (Su et al., 2007), pH (Shi et al., 2004), can affect the susceptibility of algae to algicidal bacteria.

Different cyanobacterial strains, even belonging to the same species, show many differences in intrinsic characteristics and cellular phenotypes. For example, the *Microcystis aeruginosa* can be divided into toxic group and non-toxic ones based on the ability of microcystin production. *Microcystis* strains, with two types of cellular phenotype as single cell and colonial form, usually co-exist in natural waters (Kessel and Eloff, 1975; Reynolds et al., 1981). Recently, some studies reported that intrinsic characteristics and phenotypes of algae could affect the responses of algae to environmental stress (Liu et al., 2007; Wu et al., 2007).

As for the algae used in assessment of algicidal ability and algicidal efficiency of algicidal bacteria, most studies tested only one or two algae strains in the assessment, and then concluded that such genus was (which the tested alga(e) belonged to) susceptible or unsusceptive to certain algicidal bacterium. In this way, inaccurate conclusions are inevitable since those assessments ignored the differences of phenotypes and intrinsic physiological characteristics among different strains even belonging to one species.

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In order to explore the factors which affect the susceptibility of algae to algicidal bacterium, the susceptible characteristics of thirteen *M. aeruginosa* strains, including toxic and non-toxic ones, unicellular and colonial strains, to an algicidal bacterium were studied. Correlation analysis was conducted to link the susceptible characteristics with cellular phenotype and intrinsic physiological characteristics of these *Microcystis* strains.

**Materials and methods**

**Algal strains and culture conditions**

Thirteen *M. aeruginosa* strains were used in this study. Among them, *M. aeruginosa* FACHB905 and *M. aeruginosa* FACHB1023 were kindly provided by the Freshwater Algae Culture Collection of Chinese Academy of Sciences; *M. aeruginosa* PCC 7806 was originally obtained from the Pasteur Culture Collection of Cyanobacteria in France and also kindly provided by the Freshwater Algae Culture Collection of Chinese Academy of Sciences; *M. aeruginosa* NIES-843 was kindly provided by National Institute for Environmental Studies in Japan; *M. aeruginosa* CHAB440, *M. aeruginosa* CHAB109, *M. aeruginosa* CHAB456, *M. aeruginosa* CHAB587, *M. aeruginosa* CHAB439, *M. aeruginosa* CHAB2162, *M. aeruginosa* CHAB2170, *M. aeruginosa* CHAB724 and *M. aeruginosa* CHAB4370 were isolated from China by us. All *M. aeruginosa* strains were grown in CT liquid medium (pH 8.2) (Ichimura, 1979) at 25 ± 1 °C, under a 12:12 light/dark cycle with a light intensity of 30 μmol photons/s/m² provided by cool white fluorescent tubes.

**Algicidal bacterium and algicidal efficiency assessment**

An algicidal bacterium, *Bacillus* sp. B50 was isolated from Lake Donghu, China. This strain was found to kill *M. aeruginosa* through releasing dissolved algicidal agent(s) (Shao et al., 2014). *Bacillus* sp. B50 was inoculated and incubated at 28 °C for 24 h with shaking at 150 rpm (revolution per minute) in modified CT medium (tryptone, yeast extract and sodium citrate were added into CT medium at a final concentration of 0.2 g/L, 0.1 g/L and 2 g/L, respectively). The culture of *Bacillus* sp. B50 was harvested by centrifugation at 10,000 × g for 10 min, and adjusted to 1 × 10⁸ CFU/mL with sterilized CT medium. Appropriate volume of bacterial suspensions were inoculated into 250 mL Erlenmeyer flask containing 90 mL exponential-phase *M. aeruginosa* cultures. The filtrates were added into 250 mL Erlenmeyer flask containing 90 mL exponential-phase *M. aeruginosa* cultures, and the filtrates were substituted with 10 mL sterilized modified CT medium in control treatment (Prior to the present experiment, we already observed that 10% modified CT medium had no obvious effect on the growth of *M. aeruginosa* in CT medium after a following 5 days incubation). *M. aeruginosa* strains were then cultured under a 12:12 light/dark cycle with a light intensity of 30 μmol photons/s/m² at 20 °C and 30 °C, respectively. Chl α content of all treatments were determined according to the method mentioned above.

**Bacterial community in *M. aeruginosa* culture**

Total DNAs of *M. aeruginosa* cultures were extracted using CTAB method as described by Lin et al. (2010). The V3 region of 16S rRNA gene was amplified by touchdown PCR in 50 μL reactions using primers 341F with a GC clamp (5'-CGCCCGGCGCGCCGGCCGGGGCGGCCGGGGGGCCCCGACGGGCTACGGAGGCAGCAG-3') and 517R (5'-ATTACCGGGCTGTGAGG-3') as described by Riemann et al. (1999). Touchdown PCR reactions were conducted as described by Mayall and Doucette (2002). PCR products were loaded on 8% polyacrylamide gels (acrylamide: bisacrylamide = 37:1). These gels contained denaturant concentrations of 45–65% (100% is 7M urea and 40% v/v deionized formamide). Electrophoresis was performed in a D-code system (BIORAD Inc., Hercules, CA, USA) at 60 °C for 12 h at 70 V in 1 × TAE buffer. Gels were stained for 40 min with SYBR® Gold (Molecular Probes, Eugene, OR, USA) in 1 × TAE buffer and photographed with a standard UV transilluminator using a SYBR Green/Gold photographic filter (UVP Biochemi HR, CA, USA).

**Relationship between unattached bacteria associated with *M. aeruginosa* culture and algicidal efficiency of algicidal bacterium**

The effect of unattached bacteria associated with *M. aeruginosa* CHAB4370 culture on the algicidal efficiency of *Bacillus* sp. B50 against *M. aeruginosa* NIES-843 was investigated. The culture of *M. aeruginosa* CHAB4370 was filtered using 0.22 μm (bacterium free) or 0.8 μm (*M. aeruginosa* free) cellulose acetate membrane. The filtrates were added into *M. aeruginosa* NIES-843 axenic culture with a ratio of 10%. CT re-suspended *Bacillus* sp. B50 was added at a final density of 1 × 10⁶ CFU/mL or 1 × 10⁷ CFU/mL. Table 1 showed the detail information about the design of this experiment. Chl α content was determined every 24 h.

**Statistics**

Significant differences were determined using statistical software SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by LSD post-hoc test was used to calculate the significant difference among different treatments (≥3 groups); T-

**Table 1**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Filtrate of <em>M. aeruginosa</em> CHAB4370 cultures</th>
<th><em>Bacillus</em> sp. B50 inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.22 μm filtrate</td>
<td>0.22 μm</td>
<td>Not inoculation</td>
</tr>
<tr>
<td>0.8 μm filtrate</td>
<td>0.8 μm</td>
<td>Not inoculation</td>
</tr>
<tr>
<td>0.22 μm filtrate + low inoculum</td>
<td>0.22 μm</td>
<td>1 × 10⁶ CFU/mL</td>
</tr>
<tr>
<td>0.22 μm filtrate + high inoculum</td>
<td>0.22 μm</td>
<td>1 × 10⁷ CFU/mL</td>
</tr>
<tr>
<td>0.8 μm filtrate + high inoculum</td>
<td>0.8 μm</td>
<td>1 × 10⁷ CFU/mL</td>
</tr>
</tbody>
</table>
test was used to calculate the significant difference between two samples; Pearson bivariate correlation was used in the correlation analysis. Differences were considered to be significant at \( p < 0.05 \).

**Results**

**Algicidal efficiency of Bacillus sp. B50 on different *M. aeruginosa* strains**

The susceptibilities of thirteen *M. aeruginosa* strains to *Bacillus* sp. B50 showed great differences. As shown in Fig. 1, cells of *M. aeruginosa* FACHB905 and *M. aeruginosa* NIES-843 were completely dead on day 5 after inoculation of *Bacillus* sp. B50. The growths of *M. aeruginosa* CHAB439, *M. aeruginosa* FACHB1023, *M. aeruginosa* CHAB456 and *M. aeruginosa* PCC 7806 were strongly suppressed by *Bacillus* sp. B50, and the algicidal efficiency was 71.08%, 62.52%, 60.33% and 66.90% respectively during a five day incubation. *Bacillus* sp. B50 had a moderate algicidal effect on *M. aeruginosa* CHAB440, *M. aeruginosa* CHAB109, *M. aeruginosa* CHAB2162, *M. aeruginosa* CHAB2170 and *M. aeruginosa* CHAB724, and the algicidal efficiency was 37.8%, 26.17%, 34.10%, 15.27% and 22.56%, respectively. *M. aeruginosa* CHAB4370 and *M. aeruginosa* CHAB587 were resistant to *Bacillus* sp. B50.

**Effect of temperature on algicidal efficiency**

As for these thirteen *M. aeruginosa* strains, *M. aeruginosa* CHAB4370 and *M. aeruginosa* CHAB587 were still resistant to the filtrate of *Bacillus* sp. B50 both at 20 and 30 °C (Fig. 2). Among the other eleven *M. aeruginosa* strains, there were eight strains showed higher susceptive to the filtrate at 30 °C than it at 20 °C. Though the mean value of algicidal efficiency of *Bacillus* sp. B50 filtrate on *M. aeruginosa* CHAB440 and *M. aeruginosa* CHAB2170 at 30 °C was higher than it at 20 °C respectively, statistical analysis (T-test) indicated that those differences did not reach significant level (\( p > 0.05 \)).

**Bacterial community in *M. aeruginosa* culture**

In order to determine the relationship between algal susceptibility and bacterial community in algal culture, the diversities of bacterial community in four low susceptible *M. aeruginosa* cultures (*M. aeruginosa* CHAB4370, *M. aeruginosa* CHAB587, *M. aeruginosa* CHAB724, and *M. aeruginosa* CHAB2170) and four high susceptible *M. aeruginosa* cultures (*M. aeruginosa* NIES-843, *M. aeruginosa* PCC 7806, *M. aeruginosa* FACHB905, *M. aeruginosa* FACHB1023) were determined using denaturing gradient gel electrophoresis (DGGE).

Fig. 1. Algicidal activities of *Bacillus* sp. B50 against different *M. aeruginosa* strains. Data presented as average value ± standard deviation (\( n = 3 \)). TS: toxic single cell strain; NTS: non-toxic single cell strain; TC: toxic colonial strain; NTC: non-toxic colonial strain.

Fig. 2. Algicidal activities of *Bacillus* sp. B50 filtrate on *M. aeruginosa* at different temperatures. * is \( p < 0.05 \) (T-test).
Relationship between unattached bacteria in *M. aeruginosa* culture and the algicidal efficiency of algicidal bacterium

As indicated above (Section 3.1 in Results), *M. aeruginosa* NIES-843 was susceptible to the attack of *Bacillus* sp. B50 while *M. aeruginosa* CHAB4370 was resistant to this bacterium. In order to further explore the effect of bacterial community in algal culture on algicidal activity of *Bacillus* sp. B50 against *M. aeruginosa*, the effect of *M. aeruginosa* CHAB4370 filtrate on algicidal efficiency of *Bacillus* sp. B50 against *M. aeruginosa* NIES-843 was studied. As shown in Fig. 3, the unattached bacteria in *M. aeruginosa* CHAB4370 culture did not show to have significant effect on the growth of axenic *M. aeruginosa* NIES-843, since the biomass of *M. aeruginosa* NIES-843 treated with bacterium free filtered cultures (passed through 0.22 μm micropore membrane) had no significant difference with the one’s treated with *M. aeruginosa* CHAB4370 free filtered cultures (passed through 0.8 μm micropore membrane) during a 3 days culture. Among the thirteen *M. aeruginosa* strains tested in this study, *M. aeruginosa* NIES-843 is the only strain susceptible to the attack of *Bacillus* sp. B50 at an algicidal bacterium inoculation density of 1 × 10^6 CFU/mL. The growth of *M. aeruginosa* NIES-843, treated with *M. aeruginosa* CHAB4370 filtrate (0.22 μm), was significantly inhibited by *Bacillus* sp. B50 at a B50 inoculation density of 1 × 10^6 CFU/mL, but the algicidal activity was inhibited by the filtrate which passed through 0.8 μm cellulose acetate membrane. However, this inhibitory effect of filtrate on algicidal activity was diminished when the final inoculation density of *Bacillus* sp. B50 reached 1 × 10^7 CFU/mL.

**Fig. 3.** DGGE profiles of 16S rRNA gene of bacterial communities in eight *M. aeruginosa* cultures. Lane A: *M. aeruginosa* NIES-843, lane B: *M. aeruginosa* FACHB905, lane C: *M. aeruginosa* PCC 7806, lane D: *M. aeruginosa* FACHB1023, lane E: *M. aeruginosa* CHAB4370, lane F: *M. aeruginosa* CHAB724, lane G: *M. aeruginosa* CHAB3170, lane H: *M. aeruginosa* CHAB587.

Except *M. aeruginosa* NIES-843, high bacterial diversity was presented in these seven *M. aeruginosa* cultures, including resistant and susceptible ones (Fig. 3). However, no specific band was found to relate the resistant or susceptible characteristics of *M. aeruginosa* to *Bacillus* sp. B50.

**Fig. 4.** Effect of the filtrate of *M. aeruginosa* CHAB4370 culture on the algicidal activity of *Bacillus* sp. B50 against *M. aeruginosa* NIES-843. Different lower case letters within columns indicate statistically significant difference by one-way ANOVA (*p* < 0.05). The cultures of *M. aeruginosa* NIES-843 received the following treatments: 1) 0.8 μm filtrate: added the filtrates of *M. aeruginosa* CHAB4370 which passed through 0.8 μm micropore membrane and not inoculated *Bacillus* sp. B50; 2) 0.22 μm filtrate: added the filtrates of *M. aeruginosa* CHAB4370 which passed through 0.22 μm micropore membrane and not inoculated *Bacillus* sp. B50; 3) 0.8 μm filtrate + low B50: added the filtrates of *M. aeruginosa* CHAB4370 which passed through 0.8 μm micropore membrane and inoculated *Bacillus* sp. B50 at a density of 1 × 10^7 CFU/mL; 4) 0.22 μm filtrate + low B50: added the filtrates of *M. aeruginosa* CHAB4370 which passed through 0.22 μm micropore membrane and inoculated *Bacillus* sp. B50 at a density of 1 × 10^7 CFU/mL; 5) 0.8 μm filtrate + high B50: added the filtrates of *M. aeruginosa* CHAB4370 which passed through 0.8 μm micropore membrane and inoculated *Bacillus* sp. B50 at a density of 1 × 10^7 CFU/mL; 6) 0.22 μm filtrate + high B50: added the filtrates of *M. aeruginosa* CHAB4370 which passed through 0.22 μm micropore membrane and inoculated *Bacillus* sp. B50 at a density of 1 × 10^7 CFU/mL.
Discussion

*M. aeruginosa* can be divided into toxic group and non-toxic group according to production of microcystins or not. Previous studies indicated that these two *M. aeruginosa* types exhibited differences in competition ability (Kardinaal et al., 2007) as well as the responses to environmental stresses. Non-toxic *M. aeruginosa* was more susceptible to pyrogallol stress than toxic *M. aeruginosa* (Liu et al., 2007); however, the toxic strain was more sensitive than non-toxic one to the allelopathic effect from *Stratiotes aloides* (Mulderij et al., 2005). As for these thirteen *M. aeruginosa* strains examined in this study, it includes seven microcystins producing strains and six non-microcystins producing strains (Fig. 1). Pearson correlation analysis was carried out between algicidal efficiencies of the strains and some pathogenic microorganisms to antibiotics stress related to their growth rates (Brown et al., 1988; Evans et al., 1990). Our previous study showed that the algicidal activity of *Bacillus* sp. B50 was mediated through secreting extracellular algicidal substance, and the metabolism inhibition may be involved in the algicidal mechanism of algicidal substances against *M. aeruginosa* (Shao et al., 2014). We hypothesized that the *M. aeruginosa* with high growth rate may had high uptake rate on algicidal substance from outside to cell, which in turn showed high susceptibility to *Bacillus* sp. B50. Far to our knowledge, this is the first report to reveal that growth rate of *M. aeruginosa* correspond to its susceptibility to an algicidal bacterium.

A comparison experiment was carried out to study the algicidal efficiency of the cell free filtrate of *Bacillus* sp. B50 towards these thirteen *M. aeruginosa* strains at 20 °C and 30 °C, respectively. As showed in Fig. 2, for most tested *M. aeruginosa* strains, the strains which incubation at 30 °C showed higher susceptible than that incubation at 20 °C. The growth rate of *M. aeruginosa* at 30 °C is higher than that at 20 °C. Thus, these results also indirectly suggested that susceptibilities of these *M. aeruginosa* strains to *Bacillus* sp. B50 was corresponding to their growth rates.

Our previous work indicated that algicidal bacteria *Bacillus* sp. B50 showed no algicidal effect on *M. aeruginosa* NIES-843 at an inoculation level of 1.9 × 10^5 CFU/ml (Shao et al., 2014). However, the growth of *M. aeruginosa* NIES-843, treated with bacterium free filtrate of *M. aeruginosa* CHAB4370 (passed through 0.22 μm), was significantly inhibited by *Bacillus* sp. B50 at a B50 inoculation density of 1 × 10^6 CFU/ml, but no inhibitory effect was observed when the culture of *M. aeruginosa* CHAB4370 passed through 0.8 μm cellulose acetate membrane. Most of bacteria could be

![Fig. 5](image1.png)  
**Fig. 5.** Correlation analysis between algicidal efficiency (on day 3) and the characteristics of microcystins production (A) and cellular phenotype (B).

![Fig. 6](image2.png)  
**Fig. 6.** Correlation analysis between the growth rate of *M. aeruginosa* strain and the algicidal efficiency (on day 3) at a B50 inoculation level of 1 × 10^7 CFU/ml (A) and 1 × 10^8 CFU/ml (B), respectively.
intercepted by 0.22 μm micropore membrane, but many bacteria could pass through the 0.8 μm micropore membrane. The results presented in Fig. 4 suggested that the bacterial community in the culture of *M. aeruginosa* CHAB4370 could relieve the algicidal activity of *Bacillus* sp. B50 against *M. aeruginosa* NIES-843. Interestingly, this inhibitory effect on the algicidal activity was diminished when the final inoculation density of *Bacillus* sp. B50 reached $1 \times 10^7$ CFU/mL. One possible reason for this phenomenon is that the bacteria in the filtrate of *M. aeruginosa* CHAB4370 inhibited the growth/activity of *Bacillus* sp. B50 and caused this algicidal bacterium not able to secret enough algicidal agent(s) to trigger the lysis of *M. aeruginosa* NIES-843 at a B50 inoculation density of $1 \times 10^6$ CFU/mL. However, *Bacillus* sp. B50 could accumulate high enough algicidal agent(s) to cause the lysis of *M. aeruginosa* NIES-843 when the inoculation density reached $1 \times 10^7$ CFU/mL. These results suggested that the inhibitory effect of bacterial community on algicidal activity could be eliminated by increasing the inoculum of algicidal bacteria.

Conclusions

The susceptibilities of thirteen *M. aeruginosa* strains, including different microcystins-producing characteristics and different cellular phenotype, showed great differences under the stress of algicidal bacterium *Bacillus* sp. B50 inoculation. The susceptibility has a linear relationship with growth rate of these *M. aeruginosa* strains. Bacterial community in the culture of the resistant strains could modify the susceptibility of susceptible one to the stress from algicidal bacterium, but this modification could be diminished by enhancing inoculation density of algicidal bacterium.

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References


