JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2013. Vol. 7(4), p. 2773-2781

An Oligotrophic Bacteria Isolated from Lake Taihu, Capable of Exhibiting an Effective Algicidal Effect with a Low Cell Density

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(Received: 25 July 2013; accepted: 29 September 2013)

Bacterial isolation is crucial for elucidating the algal-lytic effects of algicidal bacteria and their practical application in controlling cyanobacterial blooms. In this study, algicidal bacterium *Acinetobacter* sp.R14 was isolated from surface water of Lake Taihu by oligotrophic method. It was found that without extra nutrition addition, the inoculation of *Acinetobacter* sp.R14 at a final cell density as low as 7.5×10^3 CFU mL^{-1} could still cause *Microcystis aeruginosa* 9110 cells to decline by 97.95% within 7 days. This is the first report that a bacteria with algicidal activity was identified as genus *Acinetobacter*, and the characteristics of high algicidal efficiency at a low cell density and organic carbon concentration may suggest that the oligotrophic method is a useful strategy to isolate high effeciency algicidal bacteria. It was demonstrated that the cyanobacterial cells were killed by the combination of direct and indirect attack of *Acinetobacter* sp.R14, and the extracellular compounds secreted had a relative stability towards the change of temperature and pH. With strong algicidal activity in conditions close to natural water environment, *Acinetobacter* sp.R14 can be used as a potential agent for controlling dominant cyanobacteria species in Lake Taihu.

Keywords: Algicidal bacteria, Cyanobacterial bloom, *Microcystis aeruginosa*, *Acinetobacter* sp, Oligotrophy.

In last decades, harmful algal blooms (HABs) have been occurred much more frequently which were caused by serious water pollution and global warming[1, 2, 3]. HABs always accompanied by massive fish and shellfish killings, tourism destruction and potential threat to humans[4,5,6]

Various methods have been investigated to control algal blooms such as physical, chemical and biological means[7,8]. Among these methods, biological control agents, including bacteria, viruses, fungi and protozoa[9, 10, 11] were supposed to be potential suppressors in controlling HABs. Studies suggested that algicidal bacteria decline algal blooms by killing the organisms that comprise blooms[12, 13, 14] and they exhibited algicidal activities by direct[15, 16, 17] or indirect attack[18]. Direct attack needs cell to cell contact with alga, and indirect attack is mediated by extracellular compounds secreted by algicidal microorganisms. Interactions between algicidal bacteria and HAB species have been proved to be an important factor in regulating the population of these algae.

Microcystis is reported to be the dominant species in many lakes during cyanobacterial blooms[19, 20, 21]. Some reports have concluded that *Microcystis*, together with *Synechococcus*, are two dominant species during cyanobacteria blooms in Lake Taihu[22, 23]. As a consequence, it is essential to isolate algicidal bacteria with high efficiency on *Microcystis* and *Synechococcus* to control cyanobacteria blooms in Lake Taihu. The relationship between HABs and

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bacteria has always been the focus of bloom control studies, but most of them were conducted with high nutritional organic medium in bacterial initial screening course[24-29]. However, nutrition in most aquatic and terrestrial environments are limited and a large portion of biosphere exists as "oligotrophs" in contrast to "copiotrophs", which are common in environment with greater nutritional opportunities[30, 31]. Although the water contain cyanobacteria blooms always called eutrophic water, it was still oligotrophic for the cell growth of bacteria, as the organic carbon concentration in it was much lower than the organic media, so when the organic carbon media were used in initial screening of algicidal bacteria, those oligotrophic algicidal bacteria may be omitted, which supposed to be one of the dominant functional algicidal bacteria species in termination of cyanobacterial blooms in eutrophic Lakes.

The concentrations of algicidal bacteria isolated by organic medium above which they could exert efficient algicidal effects varied in studies, but mostly based on a high level, ranging from 10^5 to 10^9 cells/mL[26-28, 32-35], which was not in accordance with the real water environment. In addition, extra organic nutrient was always added during algicidal effect examination[15, 27, 29, 34, 36-38], so when these isolated algicidal bacteria being positioned in real water body, they may not exert the same effects as in the laboratory, then their potential usage in controlling algal blooms were impeded.

The aims of this study were (I) to isolate an oligotrophic bacterium possessing algicidal effect against *Microcystis aeruginosa* by use of oligotrophic screening method, (II) to determine the algicidal effects of the isolated bacterial strain at low bacteria concentrations, (III) to determine the algicidal mode, (IV) to examine algicidal effects on other cyanobacteria and alga species.

MATERIALS AND METHODS

Cyanobacteria culture

The cyanobacterial strain *Microcystis aeruginosa* 9110 (*M.aeruginosa* 9110) was isolated from Lake Taihu and cultivated in BG11 medium[39] at 25 °C under an illumination of 100 umol photon/m^2/s on a 12:12 h light–dark cycle. *M.aeruginosa* 9110 culture at log-phase, approximately 5×10^6 cells/mL was used for the screening of algicidal bacterial isolates and examination of their algicidal activity.

Water sample collection

Water samples were collected at a depth of 0.5 m below the surface in Meiliang Bay (31°242 N, 120°132 E) of Lake Taihu in September and December 2011. Immediately after collection, the water was injected into sampling bottles (with prior hot humid sterilization) and transported to the laboratory as soon as possible, and the total organic carbon (TOC) concentration of water sample was tested by total organic carbon analyzer (Shimadzu company, Japan).

Isolation of algicidal bacteria by oligotrophic method

Algicidal bacteria were isolated by the plaque method. The cyanobacteria cells were collected by centrifuging exponential phase *M. aeruginosa* 9110 culture at 4000 rpm for 15 min, then washed and mixed with water sample collected from Lake Taihu. The mixture was spread onto BG11 agar plates followed by incubation under the conditions described above.

Bacteria were picked out from apparent plaque, where *M. aeruginosa* 9110 had died out, and purified by streaking onto nutrient broth (NB) agar plates (3 gL^{-1} beaf extract, 10 gL^{-1} peptone, 5 gL^{-1} Nacl and 15 gL^{-1} agar) several times. Isolated bacteria were cultivated at 30 °C for 12 h and cryopreserved at -20 °C in NB medium containing 40% glycerol until use.

Algicidal effects of the oligotrophic-algicidal bacteria

For secondary screening of algicidal strains, isolates were grown in 5 mL NB medium at 30 °C, 180 rpm for 12 h and each bacterial culture (5 mL) was inoculated into 100 mL *M.aeruginosa* 9110 culture, an equal volume of NB medium was added into *M.aeruginosa* 9110 cultures instead of bacterial inoculation as control. The cells of *M.aeruginosa* 9110 were quantified by direct counting under a light microscope (Olympus company, Japan) with the aid of a haemocytometer (Shanghai qiujing instrument company, China) after 4 days (t=4 d) of inoculation.

The algicidal activity was measured by the following equation[40]: $A(\%) = (1 - T_t / C_t) \times 100$, where A means algicidal activity, T (treatment) and C (control) represent cell or chlorophyll-a densities of *M.aeruginosa* 9110 with and without bacterium

inocula respectively, and t is incubation time.

PCR amplification of 16S rDNA and sequencing

Since in the secondary screening, strain R14 exhibited the most efficient algicidal activity on *M.aeruginosa* 9110, it was picked up to continue further study. The bacterial strain R14 was identified by PCR amplification of 16S rDNA gene sequence[41], BLAST analysis[42], and comparison with sequences in GenBank database.

16S rDNA genes were amplified by a polymerase chain reaction (PCR) thermocycler (Eppendorf company, Germany), using universal primers 27F, 1492R and genomic DNA as template. PCR was conducted for thirty-five thermal cycles, which consisted of denaturing for 1 min at 94 °C, then annealing for 1 min at 55 °C, followed by extension for 1 min at 72 °C, and finally extending for 5 min at 72 °C. The PCR products were tested by agarose gel electrophoresis and then sequenced on an ABI-Prism 3730 automated sequencer, finally comparing with the sequences in the Genbank database (http://www.ncbi.nlm.nih.gov/blast) to determine the closest phylogenetic types of strain R14.

Algicidal activity of R14 at low cell concentrations

To examine the algicidal effects of R14 at different cell concentrations, strain R14 was inoculated into 5 mL NB medium and cultivated at 30 °C, 180 rpm for 12 h. After being centrifugated (4000 rpm, 15 min), washed and diluted with sterilized BG11 medium in series, 5 mL bacterial cells were inoculated into 100 mL *M. aeruginosa* 9110 culture at final concentrations of 7.5×10^2, 7.5×10^3 and 7.5×10^4 CFU mL^{-1} respectively. After inoculation, cyanobacterial cells were counted everyday for 7 days. An equal volume of sterilized BG11 medium instead of R14 was added to *M.aeruginosa* 9110 as control.

Determination of algicidal mode

To examine the algicidal mode of strain R14, the cell-free filtrate was harvested by centrifuging the exponential phase bacteria culture at 4000 rpm for 15 min and the supernatants were filtrated through 0.22 um millipore membrane filters. Cell-free filtrate and bacterial culture (5 mL) were separately added, in triplicate, to 100 mL exponential growing *M.aeruginosa* 9110 cultures to test algicidal activity. In the case of control, an equal volume of NB medium was added instead of cell-free filtrate or bacterial culture. The algicidal activity was evaluated after 4 and 7 days by examining the cyanobacteria cell density.

Influence of temperature and pH on algicidal compounds

To test the heat stability of algicidal compounds excreted by R14, the cell free filtrates were treated at -20, 4, 50 and 80 °C for 30 min. To test the effect of pH on algicidal compounds, the pH of the cell free filtrates were adjusted to 2.0, 5.0, 9.0 and 12.0 and maintained for 30 min, then readjusted to 7.0. The algicidal activity was monitored after inoculating 5 mL treated filtrates to 100 mL *M.aeruginosa* 9110 culture and 5 mL sterilized NB medium was added as control. The algicidal activity of untreated cell free filtrate (30 °C, pH7.0) was also tested to be compared.

Algicidal effects on other cyanobacteria and alga species

To investigate the algicidal effects of strain R14 towards other cyanobacteria and alga species in the blooms, 5 mL logarithmic phase bacterial culture of R14 was inoculated into 100 mL exponential-phase cultures of *Microcystis aeruginosa* PCC7806, *Microcystis viridis* FACHB-979, *Oscillatoria* sp.BN35, *Chlorophyta* sp.B1, *Chlamydomonas* sp.BS3 and *Synechococcus* sp.BN60 respectively. Among these strains, the first two were purchased from Freshwater Algae Culture Bank (FACHB), and the other four were isolated from Meiliang Bay of Lake Taihu, among which *Synechococcus* sp.BN60 is another dominant species in cyanobacterial blooms of Lake Taihu. All of these bacteria-cyanobacteria/algal cultures were cultivated in illumination incubator under the conditions described above. In the case of control, an equal volume of sterilized NB medium was added to each cyanobacteria/alga culture instead of R14 culture. The concentration of chlorophyll-a (chl-a) was evaluated by acetone digestion method[43] after 4 days of inoculation, and the algicidal activity was computed by the equation described above.

Statistical analysis

All experiments were repeated in triplicate and all data were represented as triplicate means± standard deviation. The significant difference of repeated results was evaluated by t-test and a p value < 0.05 was considered significantly different.

RESULTS

Water Sample collection

Water samples were collected in September and December of 2011. In September, the cyanobacterial aggregates in lake were small and tightly packed, colored in dark green; while in December, the cyanobacteria filaments were large and incompact, lighter in color, which showed evident signs of decay. The temperature of the lake water was 28 and 12°C respectively, and TOC concentration was 21.6 and 8.0 mg/L separately.

Isolation of algicidal bacteria by using oligotrophic method

A total of seventy-four strains were isolated by the appearance of blank zones on BG11 agar plates on which mixture of *M. aeruginosa* 9110 cells and water samples from lake Taihu was spread (Fig.1). These oligotrophic bacteria grew slowly on the oligotrophic medium, and it would take 4 days before apparent colony occurred around the blank zones on BG11 agar plates.

Algicidal effects of the oligotrophic-algicidal bacteria

Among seventy-four bacterial strains isolated above, eleven strains exhibited algicidal activities against *M.aeruginosa* 9110 which were designated as strain R14, R15, R18, R19, R20, R24, R25, R31, R32, R33, R39, and their algicidal activities (t=4 d) on *M.aeruginosa* 9110 were shown in figure.2. From the results, we can see that strain R14, isolated from the water sample collected in October 2011 (termination period of cyanobacterial bloom in Lake Taihu), appeared to exert the strongest algicidal efficiency ($p < 0.05$) and was selected for further study.

16S rDNA sequencing and analysis

Comparison of 16S rDNA of R14 with sequences in Genbank database (http://www.ncbi.nlm.nih.gov/blast) indicated that strain R14 was most closely related to *Acinetobacter haemolyticus* strain DSM 6962 (99% homology, GenBank database accession number NR_026207.1), so strain R14 could be identified as *Acinetobacter* sp.R14. The 16S rDNA sequence of *Acinetobacter* sp.R14 had been deposited in GenBank database with the accession number JX845720, and R14 bacterial culture was preserved in China General Microbiological Collection Center (CGMCC) with the number CGMCC No.6550.

Algicidal activity of R14 at low cell concentrations

As shown in fig.3, significant difference of algicidal effect occurred at different initial R14 cell densities in co-cultures of *M.aeruginosa* 9110 and R14 ($p < 0.05$), according which two groups were classified: the initial R14 cell densities of 7.5×10^4, 7.5×10^3 CFU mL^{-1} (group A) and 7.5×10^2 CFU mL^{-1} (group B).

Table 1. Algicidal effect of strain R14 against several cyanobacterial and algal strains

Harmful algal bloom-forming species	Algicidal effect (%)
Microcystis aeruginosa PCC7806	-6.9±8.16
Microcystis viridis FACHB-979	37.3±2.06
Oscillatoria sp. BN35	-7.2±5.24
Synechococcus sp. BN60	55.1±5.23
Chlorophyta sp. B1	-40.0±8.23
Chlamydomonas sp. BS3	-13.9±3.23

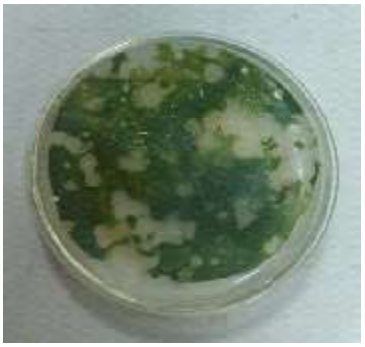

Fig. 1. Blank zones on BG11 agar plate formed by algicidal bacteria

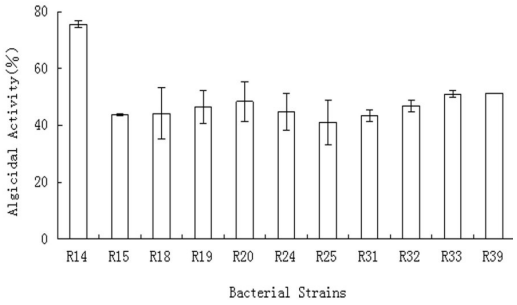

Fig. 2. The algicidal activity of bacteria isolated by oligotrophic medium after 4 days of inoculation

In group A, the cell growth of *M.aeruginosa* 9110 was restrained. The algicidal effect was evident after 2 days of R14 inoculation (average 44.33%-24.00%), but no algicidal activity was observed in group B. After 7 days of inoculation, the cell densities of *M.aeruginosa* 9110 in group A were only 1.7% and 2.05% of the control. In contrast, the cell growth of *M.aeruginosa* 9110 in group B was not affected much, and it's cell density was 73.77% of the control.

The results suggested that without extra nutrient addition, strain R14 could have a significant algicidal effect against *M.aeruginosa* 9110 with an initial cell density as low as 7.5×10^3 CFU mL^{-1}.

Algicidal mode of strain R14

To determine whether strain R14 exert algicidal activity by producing bioactive compounds, cell-free filtrate and bacterial culture of R14 were added to *M.aeruginosa* 9110 cultures respectively. The results were shown in Fig. 4. The cell growth of *M.aeruginosa* 9110 inoculated with cell-free filtrate was decreased by 30.6% (t=4 d) and 61.6% (t=7 d), compared to 72.6% (t=4 d) and 94.6% (t=7 d) inoculated with bacterial culture. These results indicated that strain R14 displayed algicidal effect on *M.aeruginosa* 9110 through the combination of direct attack and excretion of algicidal substances.

Influence of temperature and pH on algicidal compounds

Results showed that the algicidal activities of treated cell-free filtrate were average 42.90% (t=4 d) and 59.87% (t=7 d), and the algicidal activities of the untreated cell-free filtrate were 38.4% (t=4 d) and 68.1% (t=7 d) at 30 °C (Fig.5.a) which demonstrated that the algicidal compounds secreted by R14 were heat stable. On the other hand, when the supernatant being treated in extreme pH condition such as pH=2 and 12, the algicidal activity was reduced to average 23.9% (t=4 d) and 50.55% (t=7 d) compared to average 45.75% (t=4 d) and 65.6% (t=7 d) when treated at

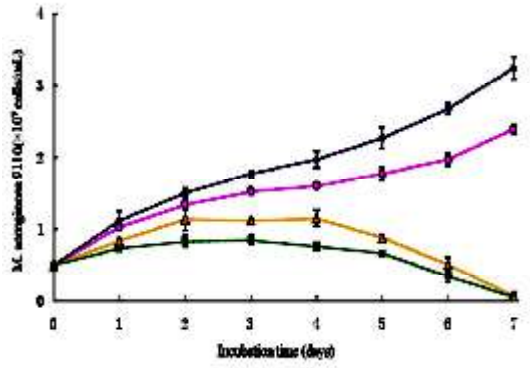

Fig. 3 . Cell growth of *M.aeruginosa* 9110 inoculated with various initial concentrations of algicidal bacterium R14

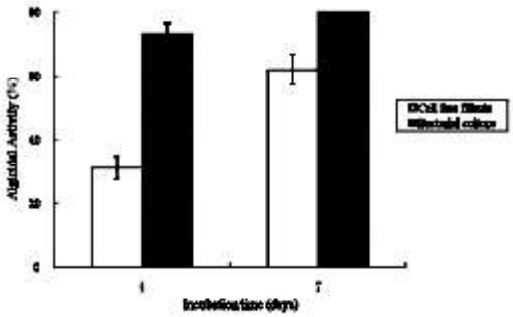

Fig. 4. Algicidal effects of the cell free filtrate and bacterial culture of R14

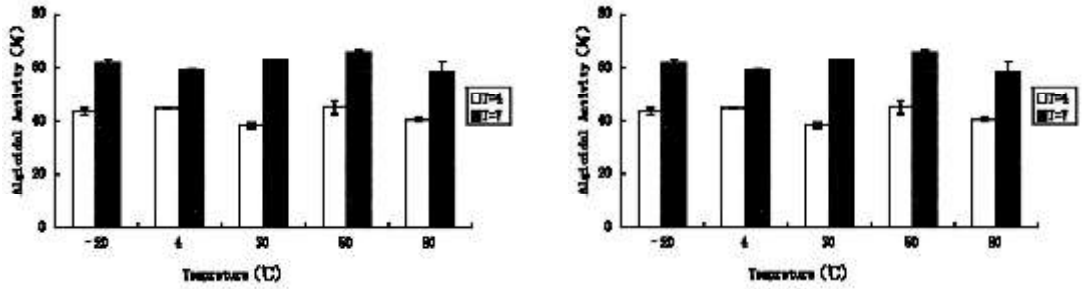

Fig. 5(a) Temperature steady of the algicidal compounds excreted by strain R14.
(b) pH steady of algicidal compounds excreted by strain R14

pH=5 and 9 (Fig. 5.b), which indicated that the algicidal compounds excreted by R14 were susceptible to extreme pH condition, but not totally inactivated.

Algicidal effects on other cyanobacteria and alga species

The algicidal efficiency of strain R14 was tested against other algal species, and the algicidal activities after 4 days of inoculation were shown in Table1.

Compared to the remarkable algicidal activity on *M.aeruginosa* 9110, strain R14 showed lower algicidal effect on *Synechococcus* sp. BN60 (average chl-a reduction of 55.1%) and *M.viridis* FACHB-979 (37.3%). Interestingly, the cell growth of *Chlorophyta* sp.B1, *Chlamydomonas* sp.BS3, *M.aeruginosa* PCC7806 and *Oscillatoria* sp. BN35 were slightly promoted by bacterial strain R14 (Table 1).

DISCUSSION

In this study, we reported an oligotrophic bacteria R14 which could efficiently inhibit the growth of *M.aeruginosa* 9110. Oligotrophic bacteria were those that could be cultured on the medium contained 1-15 mg/L carbon for the first time[44]. Semenov[45] suggested that oligotrophs may also grow in rich environments, but poor media were more suitable for bacterial isolation as the competitive advantage of their oligotrophy could be easily exploited in nutrient limited environment.

However, for the initial screening of algicidal bacteria, most studies proceeded with organic carbon media. Salomon et al.[26] spread water sample on agar plates containing 1 gL^{-1} TRIZMA base, 1 gL^{-1} tryptone, 1 gL^{-1} yeast extract and 1.5% (w/v) agar, Nakamura et al.[25] reported that the initial screening of algicidal bacteria was conducted with skim milk agar plates, Hare *et al.* [27] isolated the algicidal bacteria by spreading water sample onto R_2A agar plates, Su *et al.*[28] used 2216E broth agar plates to isolate algicidal bacteria. As these medium contain a high level of organic nutrient, it is more likely to isolate the bacteria favored an eutrophic environment. Besides, there were few reports adopting inorganic medium in initial screening of algicidal bacteria[46,47]. In these studies, cyanobacterial lawns were prepared on agar medium primarily, then water sample was spread, so it can be inferred that before the bacteria inoculation, the cell and extracellular compounds produced by cyanobacteria may have cumulated to a comparative high level, which may be utilized by algicidal bacteria for cell growth. While in this study, the water sample was mixed with cyanobacteria cells, then spread on inorganic agar plates, allowing the bacteria cells grow with limited organic nutrient. As most of fresh water environment are nutrition limited and oligotrophs occupy a large percentage of the whole microbiology, it counts much for this study using BG11 medium to isolate oligotrophic bacteria with algicidal activity.

Using BG11 medium in initial screening of algicidal bacteria, seventy-four algicidal bacterial strains were isolated from Lake Taihu, among which 11 strains showed algicidal activity in secondary screening. Among these isolated strains, strain R14 exhibited the most significant algicidal efficiency which was selected for further study. According to 16S rDNA gene sequencing and phylogenetic analysis, strain R14 was identified as genus *Acinetobacter* and this is the first report that a bacterium belongs to genus *Acinetobacter* possessing algicidal activity. It suggested that strain R14 may proved to be a valuable agent to control cyanobacterial blooms in Lake Taihu.

The concentration above which algicidal bacteria could exert an efficient algicidal effect always based on a high level such as 10^9 cells/mL[27], 10^8 cells/mL[26,28,33,35], 10^7 cells/mL[34], 10^6 cells/mL[32] and 10^4 cells/mL[47]. From the report of Ye *et al.*[23], the total number of bacteria in Lake Taihu was 6.25×10^7 to 1.57×10^{11} copies/mL, so the concentration of a single certain bacteria species can hardly reach that high amount. Doucette *et al.*[24] indicated that the algicidal activity was significantly affected by the initial density of algicidal bacteria, and Fraleigh *et al.*[48] assumed that alga-lytic effects would emerge only when the bacteria density beyond the algicidal threshold. Results in this study indicated that the threshold density above which bacterium R14 exhibited algicidal effects against *M.aeruginosa* 9110 was about 7.5×10^3 CFU mL^{-1}, which is much lower than those have reported. It may be indicated that the oligotrophic screening method is helpful to isolate the algicidal strains with lower algicidal threshold.

Sampling time is very important to the

isolation of efficient algicidal bacteria. In this study, two typical time were chosen to collect water sample in Lake Taihu, the bloom explosion (September) and termination period (December). According to the algicidal effect examination of the isolated bacteria, all of those that could exert an apparent algicidal effect were isolated from the termination period, which may suggest that during the termination period of cyanobacterial blooms, there would be more high efficiency algicidal bacteria playing an important role in the decay of cyanobacterial bloom. Our results were in accordance with Doucette's [24] study, he found that the algicidal bacteria isolated from waters with undetectable harmful alga was much more effecient than the one isolated from alga-containing water.

CONCLUSIONS

Our results showed a high algicidal efficiency strain R14, which was isolated by oligotrophic method and exhibited an effective algicidal activity with a low cell density, providing a possible way to control alga blooms by microbial strategy. For a better understanding of oligotrophic bacteria R14, many studies are needed such as isolation and identification of responsible algicidal compounds, explaination of biochemical process and cooperation with other algicidal bacteria isolated and so on. The interactions between phytoplankton and algicidal bacteria are very complex, so much more attention should be paid to make it an efficient action in controlling cyanobacteria blooms.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No. 21277089), the National Basic Research Program (973) of China (No. 2012CB720802), the National High Technology Research and Development Program (863) of China (No.2011AA100901) , and the National Natural Science Foundation of China (No.J1210047).

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