Isolation, identification, and algicidal activity of aerobic denitrifying bacterium R11 and its effect on *Microcystis aeruginosa*

Jun-feng Su, Si-cheng Shao, Ting-lin Huang, Fang Ma, Kai Zhang, Gang Wen and Sheng-chen Zheng

ABSTRACT

Recently, algicidal bacteria have attracted attention as possible agents for the inhibition of algal water blooms. In this study, an aerobic denitrifying bacterium, R11, with high algicidal activity against the toxic *Microcystis aeruginosa* was isolated from lake sediments. Based on its physiological characteristics and 16S rRNA gene sequence, it was identified as *Raoultella*, indicating that the bacterium R11 has a good denitrifying ability at 30°C and can reduce the concentration of nitrate-N completely within 36 h. Additionally, different algicidal characteristics against *Microcystis aeruginosa* were tested. The results showed that the initial bacterial cell density and algal cell densities strongly influence the removal rates of chlorophyll a. Algicidal activity increased with an increase in the bacterial cell density. With densities of bacterial culture at over 2.4 x 10⁵ cell/mL, algicidal activity of up to 80% was obtained in 4 days. We have demonstrated that, with the low initial algal cell density (OD₆₈₀ less than 0.220), the algicidal activity reached was higher than 90% after 6 days.

Key words | algicidal characteristics, denitrification, *Microcystis aeruginosa*, water blooms

INTRODUCTION

Harmful algal blooms (HABs) are serious global problems, and can cause a series of ecological and environmental problems as well as having significant economic impacts (Zheng et al. 2013). In recent years, HABs have frequently occurred as a result of increasing pollution and climate change, have had negative economic impacts (Bertrand 2013) and have caused ecological damage (Hickey et al. 2013). Therefore, there is an urgent need to develop efficient techniques to control and reduce the adverse impacts of HABs.

In order to reduce algae biomass, several control techniques, including physical and chemical technologies, have been used including yellow loess (Sun et al. 2004) and clay (Zou et al. 2006). Although these methods may be effective, yellow loess and clay can have secondary effects on bottom-dwelling organisms (Pierce et al. 2004). Recently, many algicidal bacteria have been isolated from the environment and identified (Volk & Mundt 2007; Li et al. 2013), and research into the relationship between algae and bacteria has resulted in the isolation of several algicidal bacteria which belong mainly to the *Cytophaga/Flavobacterium/Bacteroidetes* group and the genera *Saprospira* and *Micrococcus*. These bacteria show algicidal activities by either direct or indirect attack on the target algal cells (Mayali & Azam 2004).

In this study, an aerobic denitrifying bacterium was isolated from the eutrophic lake, and identified as having high algicidal activity against the toxic *Microcystis aeruginosa* cyanobacterium. We investigated different algicidal characteristics of *Raoultella* sp. R11 against *Microcystis aeruginosa*, and its denitrifying characteristics in heterotrophic medium (HM).

MATERIALS AND METHODS

Algal cultures and denitrification media

*Microcystis aeruginosa* was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Wuhan, China. An inoculum of the species was grown in
batch culture in 500 mL Erlenmeyer flasks containing 200 mL BG11 medium (sterilized by autoclaving at 121°C for 30 min). The experiments were conducted at 28°C and illuminated with cool white fluorescent light at about 90 μE/m²s with a 12 h/12 h light/dark cycle. In order to reduce any effect caused by minor differences in photon irradiation, the flasks were shaken manually three times each day. *Microcystis aeruginosa*, at an exponential phase, was used unless mentioned otherwise. Cultures were maintained under a sterile condition and at a similar temperature and light conditions to those described previously.

A BG11 growth medium was used (Rippka et al. 1979). The BG11 included the following reagents per litre: NaNO₃ 0.02 g; K₂HPO₄ 0.04 g; MgSO₄·7H₂O 0.075 g; CaCl₂·2H₂O 0.036 g; citric acid 0.006 g; ferric ammonium citrate 0.006 g; EDTA·Na₂ 0.001 g; Na₂CO₃ 0.02 g; A5 (trace element) solution 1 mL. The contents of the A5 solution were as follows per litre: H₃BO₃ 2.86 g; ZnSO₄·7H₂O 0.22 g; Na₂MoO₄·2H₂O 0.039 g; MnCl₂·4H₂O 1.81 g; CuSO₄·5H₂O 0.079 g; Co(NO₃)₂·6H₂O. The enrichment medium (EM) included the following reagents per litre (pH 7.0–7.2): NaHCO₃ 1.0 g; NaNO₃ 0.02 g; K₂HPO₄ 0.1 g; FeSO₄ 0.5 g; Na₂S 0.5 g; MgSO₄·7H₂O 0.05 g; CaCl₂ 0.05 g; TE (trace element) solution 2 mL. The HM included the following reagents per litre (pH 7.0): CH₃COONa 0.1 g; NaNO₃ 0.02 g; K₂HPO₄ 0.02 g; MgCl₂ 0.01 g; CaCl₂ 0.01 g, and TE (trace element) solution 2 mL. The contents of TE solution were as follows per litre: MgSO₄·7H₂O 0.5 g; EDTA 1.0 g; ZnSO₄ 0.2 g; MnCl₂·4H₂O 0.1 g; FeSO₄·7H₂O 0.5 g; CuSO₄·5H₂O 0.5 g; CoCl₂·6H₂O 0.2 g. The Luria Bertani (LB, pH 7.0–7.2) medium, containing 10 g/L of peptone, 5 g/L of yeast extract and 10 g/L of NaCl, was used for culture preservation. The final pH of the medium was adjusted by 1 mol/L NaOH or HCl solution.

**Isolation of algicidal bacteria**

The sediment samples were collected during March 2014 from the eutrophic Qu Jiang lake (Xi’an, China). For algicidal bacteria isolation, 100 mL sediment samples and 100 mL EM were transferred to a sterilized 1,000 mL flask with 50 mL log phase *Microcystis aeruginosa* and were co-cultured in an incubator for a week to check the lytic activity. The temperature of the incubator was set at 28°C, with fluorescent illumination at 90 μE/m²s with a 12 h/12 h light/dark cycle. Decolorized groups (compared with axenic control culture) were chosen for algicidal bacteria isolation. Bacteria–cyanobacteria mixed cultures were then diluted, streaked on LB agar plates and incubated for 48–72 h at 30°C. Colonies with different colony color and morphological shape were chosen for isolation and purified three times by repeated streaking and were co-cultured with *Microcystis aeruginosa* to confirm the lysing effect. To test the algicidal activity of strains, a small sample of each colony was inoculated on plates of BG11 medium containing a tested algal species. As a result, the most promising one, named bacterium R11, was stored at −20°C in tubes containing 40% glycerol.

**Identification and sequence analysis of 16S rRNA gene**

Morphological observations were carried out using a field emission scanning electron microscope (FESEM). For FESEM (Quanta 600FEG) observation, the samples were processed (Zhang et al. 2011). Morphological changes of *Microcystis aeruginosa* with treatment or without treatment with R11 were observed by FESEM.

After isolation, the bacterium was identified by polymerase chain reaction (PCR) amplification of the 16S rDNA gene and BLAST analysis, followed by comparison with sequences in the GenBank nucleotide database. The 16S rRNA of the R11 was amplified by PCR according to Su et al. (2015). The sequence was compared with available 16S rRNA gene sequences in GenBank by BLAST. A phylogenetic tree was constructed using the MEGA 5.1 program. The PCR products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide for visualization.

**Estimation of heterotrophic denitrification capability**

The bacterium R11 was tested in HM. The pure culture was enriched in an aerobic chamber at 30°C for about 72 h. When the culture had become obviously turbid, 75 mL of cell suspension was transferred into 1,000 mL flasks with 750 mL HM. The flasks were incubated aerobically at 30°C with shaking at 120 rpm. The samples were taken from flasks periodically to determine the optical density at 600 nm (OD₆₀₀), pH, and the concentration of NO₃⁻-N and NO₂⁻-N.

**Determination of bacterial cell density on algicidal activity**

The bacterium R11 was incubated in sterilized LB medium and reached a logarithmic growth phase at 30°C overnight. Sterilized 50 mL tubes with 25 mL *Microcystis aeruginosa* (OD₆₈₀ equal to 0.314) were inoculated with 1.2 × 10⁵,
1.8×10^5, 2.4×10^5, 3.0×10^5 and 3.6×10^5 cell/L of bacterial cultures (OD_{600} equal to 1.007), respectively. Controls consisted of Microcystis aeruginosa from the same volume of the sterilized LB medium. The chlorophyll a concentrations of Microcystis aeruginosa were measured every 4 days.

**Statistical analysis**

The denitrification rate formula is \((C_0 - C_n)/h\). \(C_0\) is the initial concentration of NO_3^-N; \(C_n\) is the final concentration of NO_3^-N at \(n\) hour, and \(h\) is the time of R11 treatment. Nitrate and nitrite removal ratio formula is \((C_0 - C_n)/C_0 \times 100\%\). \(C_0\) is the initial concentration and \(C_n\) is the final concentration of NO_3^-N and NO_2^-N. The algicidal efficiency was determined according to reduction of chlorophyll a content (Shao et al. 2015). Data were analysed by Microsoft Excel and Origin 9.0 software.

**RESULTS AND DISCUSSION**

**Bacterial identification using phylogenetic analysis**

Morphological and genetic analyses were performed in order to identify the bacterium R11. This bacterium was shown to be Gram-negative, non-motile and non-pigmented in an LB agar plate. The FESEM (Figure 1(a)) image demonstrates that it is a short rod-shaped bacterium (<1.5 μm).

16S rRNA was amplified with PCR, in order to genetically characterize the bacterium R11, and approximately 1,500 bp of 16S rRNA was obtained and sequenced. A phylogenetic tree was reconstructed based on this sequence and the sequences of other phylogenetically related strains (Figure 1(b)). The results indicated that the bacterium R11 is most closely related to Raoultella ornithinolytica strain CIP 103364 (99% similarity). These results, combined with the morphological identification, identified the bacterium R11 (GenBank No. KT005386) as Raoultella.

**Denitrification with the bacterium R11**

As the data in Figure 2(a) show, R11 is able to utilize organic carbon as a carbon source to sustain growth under aerobic conditions, and therefore it is a chemoheterotrophic bacterium. In order to investigate further the existence of aerobic denitrification, periplasmic nitrate reductase (NAP) was successfully amplified. It has been suggested that NAP may be involved in the aerobic conversion of nitrate to nitrite. Biomass growth (determined by OD_{600}) increased between 0 and 36 h, and the stationary phase began after 36 h. However, it reached an exponential phase at 40 h in HM, and the cell growth was not significant after 40 h, probably due to the decrease of nutrients in media. It reached a stationary phase at 40 h, and the OD_{600} increased from 0.214 to 0.214. The maximum growth rate was calculated to be 0.005 h^-1, which is consistent with a previous report (Su et al. 2015).

During the incubation of bacterium R11 in HM at 30 °C, nitrate-N concentration decreased and organism biomass (indicated by OD_{600}) increased at the same time. Nitrate-N concentration decreased significantly within 12 h and decreased slowed between hours 12 and 44. Nitrite began to accumulate in the first 56 h, due to nitrate reduction, and its content did not decrease until 44 h. The bacterium
R11 was able to reduce NO$_3$-N concentration from 5.59 mg/L to 0 mg/L in 36 h, and the denitrifying efficiency was 100%, with a denitrification rate of 0.155 mg/(L·h).

As nitrate reduction increased during the experimental process, the pH gradually decreased at first, and increased by the end. For batch experiments at the initial pH 6.73, pH became 6.58 in the end, but pH maintained a certain range. These results indicate that the best denitrification rate was at pH above 6.5, which is in accordance with the findings of Koenig & Liu (2001).

**Morphological changes of Microcystis aeruginosa**

Figure 2 shows the FESEM images of normal *Microcystis aeruginosa* cells, and these cells treated with bacterial culture ($3.0 \times 10^5$ cell/L) for 7 days. Normal *Microcystis aeruginosa* cells are round, plump, and have a spherical shape with a rough exterior (Figure 2(b)). Compared with normal cells, the cells in the presence of bacterial culture are different, and in Figure 2(c), these cells, which are distorted, compared with normal spherical shape, and flattened, are represented. This
indicates that R11 possibly invades Microcystis aeruginosa cells, or adheres to the surface of Microcystis aeruginosa cells. Most algal cells may be cracked and inclusion may have leaked out, which is also consistent with a previous report (Zhang et al. 2011).

Figure 2 | Changes in nitrogen compound, OD_600 and pH by Raoultella sp. R11 in HM (a). FESEM of Microcystis aeruginosa treated with 3.0 × 10^5 cell/L bacterial culture of bacterium R11: normal cells (b); treated cells (c). Spectrum of Microcystis aeruginosa cells (d).
Figure 3 | Chlorophyll a concentrations with different initial densities of bacterium R11 (a). Chlorophyll a concentrations with different initial algal cell density (b) and experimental picture (c) at 6 days.
Spectral scanning of Microcystis aeruginosa

As shown in Figure 2(d), cyanobacterial cells (Microcystis aeruginosa) were used for spectral scanning. In the range of visible light (400–800 nm), the wavelength of 443 nm and 680 nm had strong absorption peaks for Microcystis aeruginosa (OD680 = 0.6943). As seen for the corresponding substances, the wavelength of 680 nm was a pigment absorption peak. Therefore, the optical density absorption at 680 nm was monitored, and it indirectly determined the cell densities of the cyanobacterial samples.

The influence of bacterial culture density on algicidal activity

The influence of bacterial cell density on algicidal activity is shown in Figure 3(a). The bacterium R11 showed good algicidal activity, and the removal rates of chlorophyll a increased with the bacterial culture density. Initially, the concentrations of chlorophyll a decreased quickly. When the initial bacterial culture density of R11 was over 2.4 × 10^5 cell/mL, the algicidal activity reached 80% within 4 days. Bacterial cultures at concentrations of 1.2 × 10^5, 1.8 × 10^5, 2.4 × 10^5, 3.0 × 10^5, and 3.6 × 10^5 cell/mL were incubated with the samples containing Microcystis aeruginosa cells, and after 8 days the maximum algicidal activity of 40.00, 40.82, 90.38, 90.70 and 94.00% was obtained, respectively. These results were in accordance with the results of Yoon et al. (2008), which showed that HYY0512-PK05 degraded more than 90% of Peridinium bipes cells within 8 days after the inoculation of bacteria at a density of ≥10^7 cells·mL^{-1} to the lag or logarithmic growth phase of the algal culture.

Previous studies identified the bacterial inoculum levels (Manage et al. 2000; Lin et al. 2014) as primary determinants of algicidal activity. Larger bacterial concentrations can exert more effective algicidal activity. Nakashima et al. (2006) and Mu et al. (2007) reported that algicidal activity increased with the bacterial cell density.

The influence of algal cell density on bacterial algicidal activity

The influence of algal cell density on algicidal activity is shown in Figure 3(b) and 3(c). These results show that the reduction of chlorophyll a was considerable, and the removal rates decreased as algal cell densities increased. When the initial algal cell density (OD_680) was more than 0.220, algicidal activity was shown to be about 80%, for the initial densities below 0.220, algicidal activity was over 80%. Finally, when the bacterial culture at the concentration of 3.0 × 10^5 cell/L was introduced to Microcystis aeruginosa culture at the densities of 0.120, 0.220, 0.301, 0.417, 0.517 and 0.615, after 6 days, the maximum algicidal activity of 95.24, 93.55, 86.67, 84.21, 81.97, and 90.36%, respectively, was obtained.

This suggests that the removal rates of chlorophyll a were higher. The chlorophyll a concentration in natural water blooms is usually higher than 100 µg/L, and therefore the bacterium R11 could be of great practical significance in water bloom treatments. A similar result was obtained in a previous study (Mu et al. 2007), in which it was demonstrated that Bacillus fusiformis removes 70% of the chlorophyll a content of the cultures with less than 550 µg/L chlorophyll a, within 7 days. Yoon et al. (2012) reported that the density of M. aeruginosa also affects the algicidal activity of Pseudomonas aeruginosa PA14. In summary, bacterium R11 may have potential applications in water treatment due to the relationship between algae and bacteria. The potential implications of these findings to open pond microalgae culturing systems based on use of waste-waters as nutrient may provide new insight into microbial degradation of nitrate and Microcystis aeruginosa.

CONCLUSIONS

In this study, an aerobic denitrifying bacterium, R11, exhibited high algicidal activity against a toxic Microcystis aeruginosa, which was isolated from the sediments. On the basis of analysis of the 16S rRNA sequence and physiological identification, the isolated strain was identified as Raoultella. The bacterium R11 had good denitrification ability and the removal ratio of nitrate reached 100% in 36 h. Meanwhile, it appears that the bacterial and algal cell densities had strong influences on the removal ratios of chlorophyll a. Bacterial cultures at concentrations of 1.2 × 10^5, 1.8 × 10^5, 2.4 × 10^5, 3.0 × 10^5 and 3.6 × 10^5 cell/mL were incubated with the samples containing Microcystis aeruginosa cells, and after 8 days, the maximum algicidal activity of 40.00, 40.82, 90.38, 90.70 and 94.00% was obtained, respectively. When the bacterial culture, at a concentration of 3.0 × 10^5 cell/L, was introduced to the Microcystis aeruginosa culture at densities of 0.120, 0.220, 0.301, 0.417, 0.517 and 0.615, after 6 days, the maximum algicidal activity of 95.24, 93.55, 86.67, 84.21, 81.97 and 90.36%, respectively, was obtained.
ACKNOWLEDGEMENTS

This research work was partly supported by the National Key Technology Research and Development Program of the Ministry of Science and Technology of the People’s Republic of China (No. 2012BAC04B02), supported by Open Project of State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology (No. QA201518) and the Key Laboratory of the Education Department of Shan Xi Province (No. 12JS051).

REFERENCES


First received 9 July 2015; accepted in revised form 27 January 2016. Available online 12 February 2016