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Isolation and Characterization of a Newly Isolated Algicidal *Aeromonas* sp.

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A new algicidal bacterium strain FM was isolated and identified as *Aeromonas* sp. based on its 16S rRNA gene sequence analysis. Phylogenic analysis revealed the closest species was an *Aeromonas salmonicida*. The cell-free filtrate of FM culture was proved to contribute to the algicidal effect, indicating strain FM exerted algicidal effect on cyanobacteria by its extracellular substances. The algicidal substances were tested to be non-proteinaceous, heat stable, and with a molecular weight of less than 1 kDa. Strain FM could effectively inhibit the growth of cyanobacteria, including species of *Microcystis*, *Planktothrix*, *Pseudanabaena*, *Oscillatoria* and *Anabaena*. As Aeromonads are ubiquitous in aquatic habitats, *Aeromonas* sp. strain FM has application potential in control of harmful cyanobacteria.

Key words: Algicidal bacterium, Extracellular substances, *Microcystis aeruginosa*, *Aeromonas.*

Cyanobacterial blooming, especially *Microcystis*, occurs worldwide in eutrophic lakes, ponds and reservoirs. Such blooms severely impair water quality and pose operational problems to water treatment plants. Many species of cyanobacteria produce cyanotoxins[1], which creates serious threats to animal and human health. Biological methods for algae removal are of increasingly interest. Bacteria are reported to have potential to inhibit algal growth in water systems. To date, algicidal bacteria of different genera have been isolated from different environments[2-12].

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Most algicidal bacteria are confirmed to inhibit the growth of algae by extracellular substances. Diversified extracellular substances produced by algicidal bacteria, especially marine bacteria strains have shown algicidal activity[8,13-17]. Algicidal bacteria are widespread and taxonomically diversified, thus screening for algicidal bacteria has exhibited an abundance of natural bioagent resource.

In the present study, an algicidal bacterium strain FM was isolated from a eutrophic reservoir and identified as an *Aeromonas* sp. The anti-algae mode was examined and the extracellular substances were tested for algicidal activity. The purpose of this study is to characterize the algicidal effect of the newly isolated *Aeromonas* sp.

MATERIALS AND METHODS

Isolation and identification of the cyanobactericidal bacterium

Water samples collected from eutrophic reservoir (Jinan, China) were spread onto the nutrient agar (NA) plates[3] and incubated at 30 °C

for 24 h. By repeated streaking, clonal colonies were obtained and inoculated onto NA plates respectively for lawns growth. After incubation at 30 °C for 2 d, bacterial cells on each plate was washed off with 4 ml of sterilized BG11 medium, then the collected cell-suspensions were added to 20 ml of exponentially growing *M. aeruginosa* (FACHB 927) culture. Mixed cells in BG11 medium were incubated under the culture condition for *M. aeruginosa* described above, and shaking at 40 rpm reciprocally. The cell-concentration of *M. aeruginosa* was monitored by direct count daily and cell-lysis was observed using a light microscopy. The strain capable of inhibiting of *M. aeruginosa* growth most effectively was selected and named as strain FM. Strain FM was identified as an *Aeromonas* sp. based on its 16S rRNA gene sequence analysis, and the sequence was available under GenBank accession number HM560619.

Microorganisms and culture conditions

M. aeruginosa FACHB 927, *Anabaena spiroides* FACHB 498, *Oscillatoria* sp. FACHB 528, *Planktothrix agardhii* FACHB 920, *Oscillatoria tenuis* FACHB 1052, *Pseudanabaena* sp. FACHB 1277, used in this study was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences (FACHB-Collection; Wuhan, China). The cyanobacterium strain was cultured in BG11 medium at 25 °C under cool white fluorescent light at 130 µmol (photons) /m^2 s, with a 12 h: 12 h (light: dark) cycle. The BG11 medium was prepared according to Rippka *et al.*,[18].

Algicidal tests

For the algicidal test, cell suspensions were added into BG11 cultures of *M. aeruginosa* during the exponential phase (10^6 cells /ml). The mixed systems were then incubated at 30 °C under 130 µmol (photons) /m^2 s with a 12 h: 12 h (light: dark) cycle and shaking at 40 rpm. In the control, an equal volume of sterile BG11 medium was added into the algal culture. The cell concentration of cyanobacteria was monitored by direct count under light microscopy. The algicidal activity of strain FM was evaluated by the following equation: Algicidal activity (%) = $(1-T_t/C_t) \times 100$, where T and C are the algae cell densities of treatment group and control, respectively, and t is the testing time. All tests were carried out in triplicate.

Determination of mode of algicidal action

To prepare a cell suspension of strain FM, cells of about 7×10^8 cells/ml were collected from the NA plates and suspended in sterile BG11 medium, after which the cell suspension was centrifuged. The cells were resuspended with BG11 medium and used as washed cells. And the supernatant was then filtered through a membrane filter (Millipore, 0.22 µm pore size). Equal volumes of washed cell suspension and cell-free filtrate prepared as described were added into the *M. aeruginosa* culture for algicidal tests seperately. The control was prepared as described above.

Characterization of the algicidal extracellular substance

The cell-free filtrate as described above was also subjected to algicidal test after the following treatment similar to Kim *et al.*,[9]. (1) Heat treatment: the cell-free filtrate was autoclaved at 121 °C for 30 min. (2) Proteinase-K treatment: the cell-free filtrate was digested with 200 µg/ml of proteinase-K (Sigma), keeping in a 55 °C water bath for 3 h. All tests were carried out in triplicate. Then equal volumes of treated cell-free filtrate were used for algicidal tests.

To estimate the molecular weight of the algicidal substances, cell-free filtrate of strain FM was dialyzed against distilled water using dialysis membrane (Spectra/Por CE, Spectrum) with 1 kDa, 3.5 kDa, 7 kDa molecular weight cutoff (MWCO) over 24 h. The algicidal activity of the treated dialysate (2.5 ml) was tested in 6-well cell culture plates by mixing with equal volume of *M. aeruginosa* (FACHB 927) culture (cell density of 5.3×10^6 cells/ml after mixing). Equal volume of sterile water instead of, the cell-free filtrate of strain FM was added to the alga culture to serve as control. Algicidal activity was detected after 7 d. The co-culture condition and algicidal activity determination was the same as described above.

Algicidal effect of extracellular substances of strain FM on harmful algae

To examine algicidal activity of extracellular substances of strain FM, cell-free filtrate of 1.5×10^8 cells/ml and 1.5×10^9 cells/ml strain FM prepared as described above was added into Erlenmeyer flasks containing cultures of *Anabaena spiroides* FACHB 498, *Oscillatoria* sp. FACHB 528, *Planktothrix agardhii* FACHB 920, *Oscillatoria*

tenuis FACHB 1052 and *Pseudanabaena* sp. FACHB 1277 respectively. The algae density was 3.5×10^6 cells/ml. The mixed systems were then incubated at 30 °C under 20 µmol (photons) /m^2 s with a 12 h: 12 h (light: dark) cycle and shaking at 40 rpm, following with BG11 cultures of each alga as control. All tests were carried out in triplicate.

RESULTS AND DISCUSSIONS

Identification and phylogenic analysis of strain FM

Strain FM is a Gram negative rod. The 16S rRNA gene sequence of FM exhibited 99% identity with that of *Aeromonas* available in GenBank nucleotide sequence database. Phylogenic analysis revealed the closest species was *Aeromonas salmonicida* (GQ869651) (Fig. 1).

Aeromonads are ubiquitous in aquatic habitats worldwide and have strongly adapted to a variety of environments. As a frequent-blooming species, *M. aeruginosa* has shown strong viability in fresh water. In a natural ecosystem, it is advantageous to use a widespread microorganism as a biocontrol agent against the harmful algal bloom. Therefore, indigenous strains of *Aeromonas* could be expected as potential anti-cyanobacterial agents against *M. aeruginosa* in aquatic ecosystems.

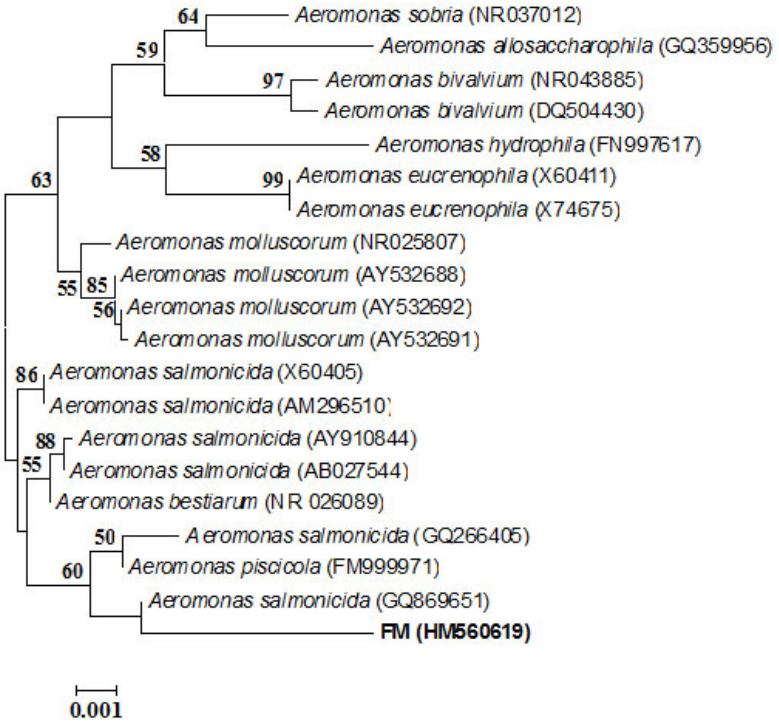

Fig. 1. Phylogenetic relations of strain FM and related strains inferred from 16S rRNA gene sequence. The tree was constructed using the neighbor-joining method. The reliability of the inferred trees was tested by bootstrap analysis using 1,000 resamplings. Bootstrap values beyond 50% are denoted at the branching points. The scale bar indicates 0.001 nucleotide substitution per position. GenBank accession numbers used for phylogenetic analysis are shown after the bacterial strain name. Strain FM was marked in boldface.

Algicidal mode of *Aeromonas* sp. strain FM

To clarify whether strain FM exerted an inhibitory effect against *M. aeruginosa* via direct attack or an indirect mode, the anticyanobacterial activities of the washed cells of strain FM and the cell-free filtrate were tested. As shown in Fig. 2, the effects of washed FM cells and their cell-free filtrate on the growth of *M. aeruginosa* differed from each other significantly. The cell-free filtrate inhibited the growth of *M. aeruginosa*, whereas

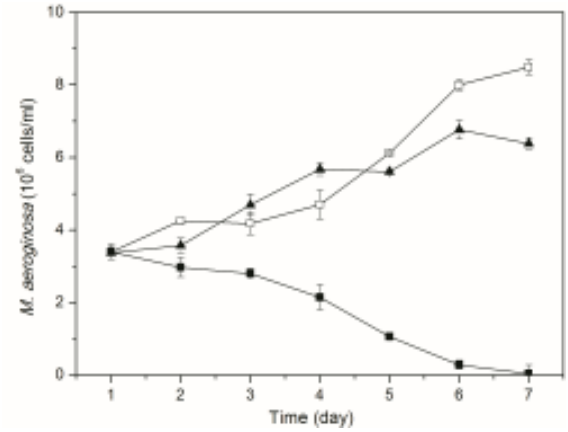

Fig. 2. Growth of *M. aeruginosa* with the addition of cell-free filtrate (■) and washed cells (▲) of *Aeromonas* sp. strain FM in contrast to untreated *M. aeruginosa* control (□). Data are the mean ± standard deviation of three independent "assays".

the washed FM cells did not. These results indicated that the effects of FM against *M. aeruginosa* occured via its extracellular substances; thus, strain FM inhibits the growth of *M. aeruginosa* via indirect attack.

Algicidal bacteria have been shown to inhibit algal growth effectively through direct or indirect attack. Direct attacks were conducted via cell-to-cell contact initiated by predatory bacteria, while indirect attacks are considered to be chemically mediated though production of extracellular substances. Most algicidal bacteria that have been identified to date have been reported to exert effects via the indirect mode[19-21].

Characterization of the algicidal extracellular substance of strain FM

To characterize the extracellular substances of strain FM associated with

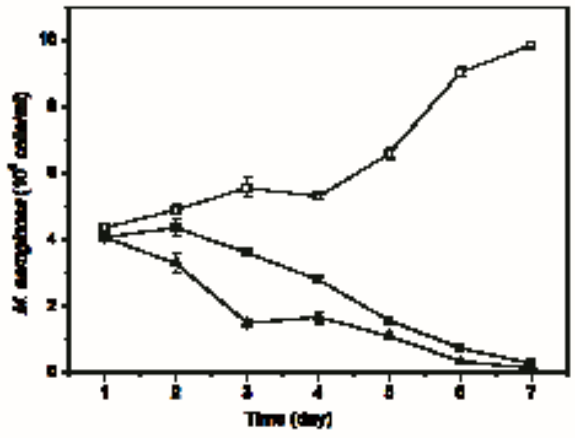

Fig. 3. Growth of M. aeroginosa with the addition of cell-free extracellular substances treated by proteinase-K (■) and heat (▲) in contrast to untreated *M. aeruginosa* control (□). Data are the mean ± standard deviation of three independent "assays".

anticyanobacterial effect, the cell-free filtrate was further treated and subjected to algal removal test. As shown in Fig. 3, growth of *M. aeruginosa* was significantly inhibited after treatment with the cell-free filtrate digested by proteinase-K.

The result indicated that the extracellular substance associated with anticyanobacterial effect was not protein-like compound. Moreover,

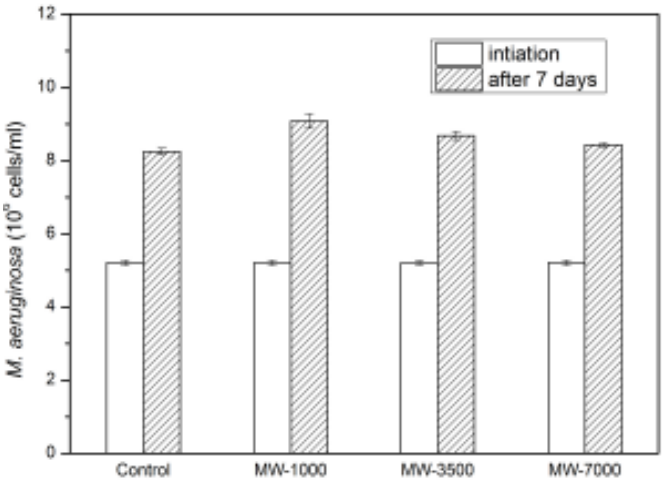

Fig. 4. The cell density change of *M. aeruginosa* (FACHB 927) at initial concentration of 5.3×10^6 cell/ml without the cell-free filtrate of strain FM (control), the treated retentate from 1 kDa (MW-1000), 3.5 kDa (MW-3500), 7 kDa (MW-7000) molecular dialysis membrane were added. Pairs of bars showed the algal density before (left bar) and after (right bar) treatment for 7days. Data are expressed as the mean of triplicate "assays"

the heat stability of the extracellular substances associated with anticyanobacterial effect was examined after autoclave treatment. Similarly, the cyanobacterial density decreased significantly with the addition of heat-treated cell-free filtrate.

To investigate the molecular weight range of the algicidal substances, the cell-free filtrate of strain FM was dialyzed against distilled water using different MWCO dialysis membrane and the algicidal activity of the retentate were tested. No significant algicidal activity was shown in all three fractions of 7 kDa retentate (MW-7000), 3.5 kDa retentate (MW-3500), and 1 kDa retentate (MW-1000) (Fig. 4).

Substances of bacterial origin have also been reported to have algicidal activity, such as L-amino acid oxidase[22], peptides[23], indole, 3-oxo-α-ionone[24], rhamnolipid[25], and quinolone derivative[26]. In the present study, the extracellular substances associated with anticyanobacterial effect were heat-stable and non-protein. Moreover, the algicidal substances were assumed to be low molecular weight molecules (< 1 kDa). Thus, further studies need to be conducted for the isolation and

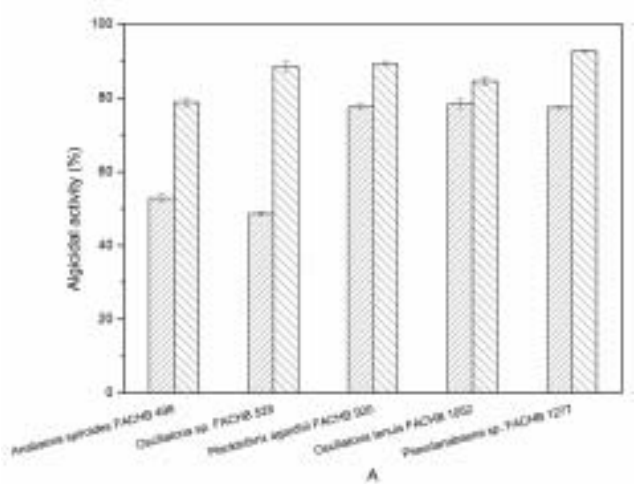

Fig. 5. Effects of extracellular substances prepared from *Aeromonas* sp. strain FM on different algae. Columns showed each alga cultured in BG11 with extracellular substances from 1.5×10^8 cells/ml of *Aeromonas* sp. strain FM (left bar) and from 1.5×10^9 cells/ml of strain FM (right bar). Data are expressed as the mean of triplicate "assays"

identification of the specific algicidal substances.

Algicidal effect of extracellular substance of strain FM on harmful algae

The algicidal range of strain FM against some harmful algae species is presented in Fig. 5. After incubated for 6 d, strain FM showed strong inhibition against *Pseudanabaena* sp. FACHB 1277 (92.7%). A relatively weak algicidal effect was shown against *Oscillatoria* sp. FACHB 498 (78.8%).

Genera such as *Anabaena* and *Microcystis* can secrete toxins[1], which caused damage to the fresh water. Meanwhile, *Oscillatoria* sp. FACHB 528 and *Pseudanabaena* sp. FACHB 1277 were found to release geosmin and 2-methylisoborneol to water. These off-flavor compounds affected the taste and odor of the water were detrimental in many aquaculture facilities[27]. Therefore, toxic cyanobacteria elimination has attracted much attention. It should be more applicable that an algicidal bacterium could target more than one toxic species.

The biological control of harmful algae has attracted much attention due to its ecological benefits in nature. In the present study, the newly isolated algicidal bacterium *Aeromonas* sp. FM was confirmed to remove algae through its heat-stable extracellular substances. Use of such substances for algae removal avoided the invasion of alien species, thus lowered the damage to environment. Moreover, extracellular substance of strain FM could inhibit the growth of cyanobacteria strains of *Anabaena*, *Oscillatoria*, *Planktothrix*, and *Pseudanabaena*. Therefore, strain FM has application potential for algal bloom control.

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