Original Article

Isolation and Characterization of an Algicidal Bacterium Indigenous to Lake Taihu with a Red Pigment able to Lyse *Microcystis aeruginosa*[*]

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Abstract

Objective To isolate and characterize indigenous algicidal bacteria and their algae-lysing compounds active against *Microcystis aeruginosa,* strains TH1, TH2, and FACHB 905.

Methods The bacteria were identified using the Biolog automated microbial identification system and 16S rDNA sequence analysis. The algae-lysing compounds were isolated and purified by silica gel column chromatography and reverse-phase high performance liquid chromatography. Their structures were confirmed by Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared (FT-IR) spectroscopy. Algae-lysing activity was observed using microscopy.

Results The algae-lysing bacterium LTH-2 isolated from Lake Taihu was identified as *Serratia marcescens*. Strain LTH-2 secreted a red pigment identified as prodigiosin ($C_{20}H_{25}N_3O$), which showed strong lytic activity with algal strains *M. aeruginosa* TH1, TH2, and FACHB 905 in a concentration-dependent manner. The 50% inhibitory concentration (IC_{50}) of prodigiosin with the algal strains was 4.8 (±0.4)×10^{-2} μg/mL, 8.9 (±1.1)×10^{-2} μg/mL, and 1.7 (±0.1)×10^{-1} μg/mL in 24 h, respectively.

Conclusion The bacterium LTH-2 and its pigment had strong *Microcystis*-lysing activity probably related to damage of cell membranes. The bacterium LTH-2 and its red pigment are potentially useful for regulating blooms of harmful *M. aeruginosa.*

Key words: Algicidal bacteria; *Microcystis aeruginosa*; Harmful algal blooms (HABs); *Serratia marcescens*; Algae-lysing

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INTRODUCTION

Harmful algal blooms (HABs) cause severe damage to aquatic ecosystems and human health and have become a serious global problem[1-3]. Techniques to reduce the impact of HABs are urgently needed. Existing techniques such as the application of clay and yellow loess have undesirable secondary effects on bottom-dwelling organisms. Chemical agents often induce chemical toxicity and trigger the release of microcystins into water[2]. Some bacteria also have algae-lysing effects. Algicidal bacteria may play an important role in the decline of HABs and are considered to be a potential solution in regulating HABs[4].

Microcystis is the most commonly found

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bloom-forming cyanobacterium in eutrophic and hyper-eutrophic waters. Many strains of *Microcystis* are known to produce hepatotoxins called microcystins that have been responsible for animal and human poisonings in almost every country where the algae have been found[5-7]. In recent years, harmful *Microcystis* blooms have appeared more frequently and at larger scales in Lake Taihu, the third largest freshwater lake in China[8]. Lake Taihu has a total water surface area of about 2338 km^2 and a water volume of approximately 4.43×10^{12} L. It is essential to local residents for drinking water, recreation, aquaculture, and industrial activity.

In this study, we report the isolation, identification, algae-lysing effects, and mechanism of an indigenous algicidal bacterium LTH-2 from Lake Taihu. Additionally, the paper characterizes a *Microcystis*-lysing pigment obtained from an ethyl acetate extract of the bacterial culture filtrate. The chemical structure of the pigment was determined from a three-dimensional graph, nuclear magnetic resonance (NMR) spectra, gas chromatography/mass spectrometry (GC/MS), and Fourier transform infrared (FT-IR) spectra data, and the mechanism of *Microcystis*-lysis was examined.

MATERIALS AND METHODS

M. aeruginosa Culture

Two strains of *M. aeruginosa* (TH1 and TH2) indigenous to Lake Taihu were obtained during blooms using single-colony isolation[9]. The standard *M. aeruginosa* strain FACHB 905 was purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences. All three strains of algae used in the study contained a phycocyanin intergenic spacer (PC-IGS). TH1 and FACHB 905 produced microcystin-LR, whereas TH2 did not produce microcystin-LR. The *M. aeruginosa* cells were incubated in sterile BG-11 medium[9]. The *M. aeruginosa* strains were maintained as unialgal axenic cultures at 28 °C, pH 7.2, with illumination at 150 µmol photons $m^{-2}s^{-1}$ under a 12-h light/dark regimen.

Isolation and Screening of Algae-lysing Bacteria

Water samples were collected from Lake Taihu, China, a freshwater, eutrophic lake, during a *Microcystis* bloom in 2009. Samples were serially diluted with sterile water and 0.1 mL aliquots of each dilution were inoculated onto nutrient agar (NA, 2% agar) plates. Isolated bacteria were inoculated into 100 mL of liquid nutrient broth (NB) medium at 30 °C at 130 rpm/min for 48 h. For each bacterial culture, one mL of culture was added into 20 mL of *M. aeruginosa* FACHB 905 culture. The mixed algal-bacterial cultures were incubated under the algal culture conditions described above. Daily cell number counts of *M. aeruginosa* were obtained under a microscope using a hemocytometer. The following formula was used to calculate the algae-lysing activity: algae-lysing activity (%)= (1-*Tt*/*Ct*)×100%, where *T* (treatment) and *C* (control) are the cell concentrations of treatments and the control. Bacteria exhibiting algae-lysing activities against *M. aeruginosa* were selected for further study.

Identification of Bacteria

The algae-lysing isolates were identified by the Biolog automated microbial identification system (Biolog Inc, Hayward, CA, USA) using the MicroLog™ System, Release 4.2, which analyzes the utilization of 95 different carbon-sources[10]. The isolates were also identified by phylogenetic analysis. The bacterial 16S rRNA gene was amplified by PCR using two universal primers, 27F (5'-AGAGTTTGATCMTGGCTC AG-3') and 1492R (5'-TACGGYTACCTTGTTACGACT T-3') in 50 µL reactions containing 100 ng template DNA, 1 × PCR Buffer (Mg^{2+} Free), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP mixture, 400 nmol/L of each primer, and 1.25 units of Taq DNA polymerase. The PCR was run with 30 cycles of denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 63 °C, and extension for 90 seconds at 72 °C, with a final elongation step of 10 min at 72 °C[3]. The PCR products were sequenced by the Genscript Biotechnological Company in Nanjing. Comparisons of nucleotide sequences were performed using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST) and the Ribosomal Database Project (RDP) database (http://rdp.cme.msu.edu/seqmatch). Sequences were aligned using the program ClustalW, and a phylogenetic tree was constructed using the neighbor-joining method based on the software MEGA 3.1. The trees were tested with the bootstrap procedure using 1000 random samples[11].

Lysis of M. aeruginosa by Bacterium LTH-2

The bacterial culture was prepared as above, diluted with sterile BG11 (1 mL) to initial densities of 6×10^8 CFU/ mL, and inoculated into 20 mL of

exponentially growing cultures of the three strains of *M. aeruginosa* (3×10^6 cells/mL each). To investigate algae-lysis by secreted bacterial compounds, bacterial cultures were centrifuged at 5000× g for 10 min, and the supernatants were filtered through a 0.22 μm Millipore-Express PES membrane. Red filtrates were used directly or heat-treated in a water bath at 100 °C for 20 min. The filtrates were added to *M. aeruginosa* FACHB 905 cultures at a 1:20 volume ratio, as described above for bacterial cultures. As a control, sterile NB medium was added to the algal cultures instead of bacterial culture. Cells numbers of *M. aeruginosa* were counted with a hemocytometer under a microscope (BX41TF, Olympus, Tokyo, Japan) daily for three days. The following formula was used to calculate the algae-lysing activity: algae-lysing activity (%)=(1-*Tt*/*Ct*)×100%, where *T* (treatment) and *C* (control) are the cell concentrations of treatments and the control, respectively, and *t* is the incubation time. All experiments were repeated in triplicate.

Isolation of the Algae-lysing Red Pigment from a Cell-free Supernatant of LTH-2 Culture

The algicidal bacterium was cultured in a 1-L Erlenmeyer flask containing 600 mL of NB medium. The cells were removed by centrifugation, and the supernatant was washed three times with an equal volume of ethyl acetate. The ethyl acetate fraction was concentrated by evaporation and stored at -20 °C. The ethyl acetate extract was separated by silica-gel column chromatography (2.5×30 cm, kieselgel 60, Merck, Germany) with a gradient elution consisting of petroleum ether, petroleum ether-ethyl acetate (from 50:1 to 5:1, v/v). The petroleum ether-ethyl acetate (5:1, v/v) fraction contained a red pigment and showed algae-lysing activity. The red pigment was re-purified by reversed-phase high performance liquid chromatography (HPLC) with a Zorbax Extend C_{18} column (4.6×150 mm, 5 μm, Agilent, USA). It was eluted with a mixture of methanol and 0.1% glacial acetic acid (6.5: 3.5, v/v) at a flow rate of 1 mL/min. Subsequently, the purified pigment was lyophilized and stored at -20 °C.

Structural Determination of the Red Pigment

The purity, absorbance (measured from 200 nm to 900 nm), and concentration of the pigment were determined with an Agilent 1100 HPLC. The pigment was dissolved in acidic methanol. The molecular mass of the pigment was determined using a GC/MS (7890A/5975C, Agilent, USA). Helium was used as the carrier gas at a flow rate of 1 mL/min in split mode (1:20). The oven temperature program was as follows: 1 min at 70 °C, increasing to 280 °C at a rate of 15 °C/min, and holding at 280 °C for 2 min. Other instrumental parameters were as follows: the electron energy was 70 eV, the mass range scanned was 12-450 amu, the ion source temperature was 230 °C, the injector temperature was 280 °C and the quadrupole temperature was 150 °C. After the red pigment was dissolved in $CDCl_3$, the ^1H- and ^{13}C-NMR spectra were recorded on a Bruker DRX spectrometer. The chemical shifts were referenced to an internal trimethylsilyl (TMS) signal. An FT-IR spectrum of the compound was recorded with a Nicolet iS10 FT-IR spectrometer.

Algae-lysing Effects of the Pigment Tested with M. aeruginosa

Purified pigment was dissolved in methanol, and 20 μL aliquots were added to 980 μL of exponentially growing cell suspensions of each of the three algal strains[12]. The concentrations of red pigment in *M. aeruginosa* cultures were from 2.3×10^{-2} μg/mL to 3.0 μg/mL. Algae were counted every 12 h. All experiments were repeated in triplicate. The IC_{50} values were calculated using SPSS 13.0. Lysis of *Microcystis* cells was observed using a photomicroscope (Axio Imager 2, Carl Zeiss, Jena, Germany).

Membrane Integrity of M. aeruginosa Cells Exposed to Purified Pigment

Exponentially growing *M. aeruginosa* FACHB 905 cultures (3×10^6 cells/mL) were exposed to purified pigment at concentrations of 9.4×10^{-2} μg/mL, 18.8×10^{-2} μg/mL, 37.5×10^{-2} μg/mL, and 7.5×10^{-1} μg/mL. After staining with PI, the membrane integrity of *M. aeruginosa* cells was determined using a FACS Calibur flow cytometer equipped with a xenon-ion excitation lamp (488 nm). Fluorescence was collected at 560-590 nm. The change in cell membrane integrity was observed after a 2 h exposure. All experiments were repeated in triplicate.

Nucleotide Sequence Accession Number

The nucleotide sequence of a 16S rRNA gene from bacterium TH-2 was deposited in the NCBI database with accession number HM640277.

RESULTS

Isolation and Identification of Algae-lysing Bacteria

Among the 160 bacterial strains isolated, six strains showed algicidal activity against *M. aeruginosa*. The algicidal activities of strains LTH-1, 2, 3, 4, 5, and 6 were 83.99%, 84.3%, 74.23%, 67.76%, 72.59%, and 75.00% in 48 h, respectively. Among these six isolates, LTH-2 exhibited the highest algicidal activity with *M. aeruginosa*. Strain LTH-2 was identified as *Serratia marcescens* by the Biolog automated microbial identification system after 17 h of incubation (Species ID: *Serratia marcescens*, Probability: 98%, Similarity: 0.5, Distance: 7.80). The result was confirmed by 16S rDNA sequence analysis. The LTH-2 sequence exhibited the greatest similarity (99%) to that of *S. marcescens* (accession no. AB061685). A phylogenetic tree shows the relationship between the LTH-2 strain and other closely related members (Figure 1). Based on this information, strain LTH-2 belongs in the *S. marcescens* grouping.

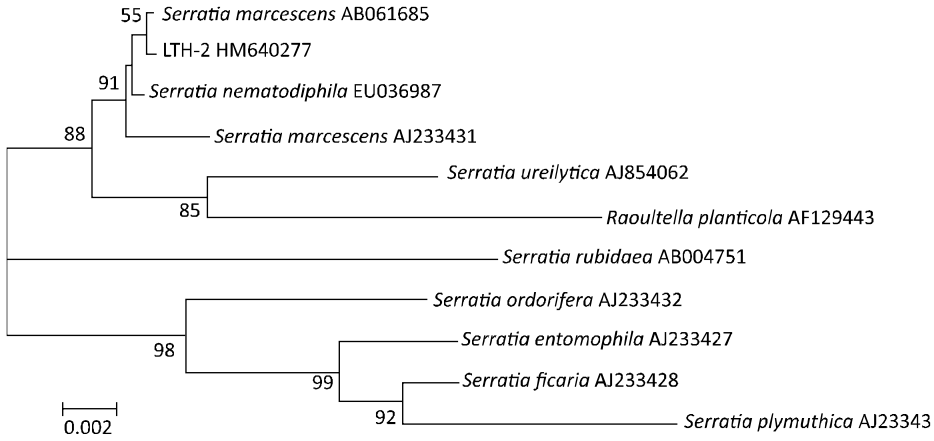

Figure 1. Phylogenetic tree based on bacterial 16S rDNA gene sequence of the isolate LTH-2 strain and closely related members. Numbers at nodes are levels of bootstrap support (%). The codes after the names are Genebank Accession numbers.

Algae-lysing Mechanism of the Bacteria LTH-2 Tested with M. aeruginosa

Rapid lysis of *M. aeruginosa* TH1, TH2, and FACHB 905 occurred in the presence of the LTH-2 strain (Figure 2). The average lysis activities of *M. aeruginosa* TH1, TH2, and FACHB 905 cells were 62.6%, 51.0%, and 75.7% in 24 h, 72.4%, 70.0%, and 84.3% in 48 h and 79.0%, 74.6%, and 87.7% in 72 h. In addition, the average lysis activities of FACHB 905 cells caused by cell filtrates and heat-treated cell filtrates were 80.7% and 79.3%, respectively, in 72 h. These results showed that the bacterium LHT-2 causes lysis of *Microcystis* cells mainly through an indirect attack.

Characterization of the Red Pigment

The purity of the pigment produced by LTH-2 was 99.0% as determined by HPLC (Figure 3A). The pigment showed a strong absorption maximum at about 535 μm (Figure 3B). The molecular mass of the pigment determined by GC-MS was 323.2 Da (m/z

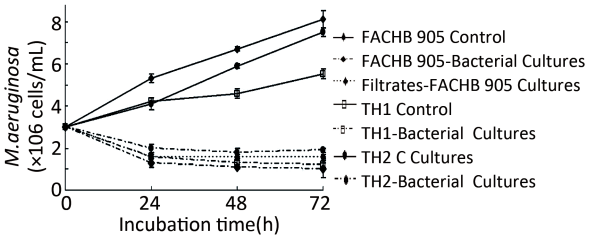

Figure 2. Concentration of *M. aeruginosa* in mixed culture at various incubation times. Data are expressed as mean±SD.

323.2), which corresponds to that of the antibiotic prodigiosin (C20H25N3O) (Figure 4). The NMR chemical shifts of the compound were as follows: 1H-NMR (CDCl3, 500 MHz, ppm) δ 0.90 (t, 3H, H-19), 1.26 (m, 2H, H-18), 1.31 (m, 2H, H-17), 1.53 (m, 2H, H-16), 2.39 (t, 2H, H-15), 2.56 (s, 3H, H-14), 3.97 (s, 3H, H-20), 6.05 (s, 1H, H-6), 6.29 (m, 1H, H-2), 6.56 (d, 1H, H-11), 6.89 (d, 1H, H-3), 7.07 (brs, 1H, H-9), 7.26 (m, 1H, H-1), 12.58 (brs, 1H, H-21), and 12.76 (brs, 1H, H-22); 13C-NMR (CDCl3, 125MHz, ppm) δ 11.85

(C-14), 14.01 (C-19), 22.48 (C-18), 25.40 (C-15), 29.96 (C-16), 31.49 (C-17), 58.54 (C-20), 93.68 (C-6), 111.13 (C-2), 115.87 (C-9), 117.47 (C-3), 119.56 (C-8), 122.46 (C-4), 125.77 (C-10), 127.61 (C-1), 128.96 (C-11), 129.53 (C-12), 147.02 (C-13), 147.53 (C-5), and 166.56 (C-7). FT-IR absorption in KBr was at 3445, 2956, 2924, 2854, 1625, 1544, 1461, 1376, 1260, 1134, 1060, 995, and 726 cm^{-1}.

Influence of the Pigment on M. aeruginosa

M. aeruginosa responded to the pigment in a concentration-dependent manner. The IC_{50} values of the pigment with TH1, TH2, and FACHB 905 cultures were 4.8 (±0.4)×10^{-2} μg/mL, 8.9 (±1.1)×10^{-2} μg/mL, and 1.7 (±0.1)×10^{-1} μg/mL, respectively.

The algae-lysing process was investigated under a microscope (Figure 5). Lysis of *Microcystis* cells most likely began with damage to the cell membranes, followed by penetration of the pigment into the algal cells (Figure 5B). Subsequently, the cells lost their integrity and burst, leading to the appearance of some algal fragments (Figure 5C and D).

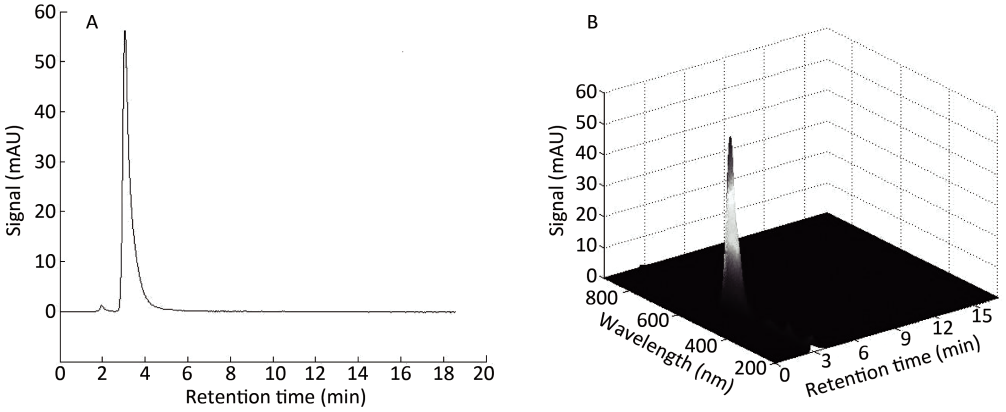

Figure 3. The purity and three-dimension graph of the pigment determined by HPLC (A) HPLC profile of prodigiosin from LTH-2 (99.0% purity). (B) Three-dimensional graph of the red pigment dissolved in acidic methanol.

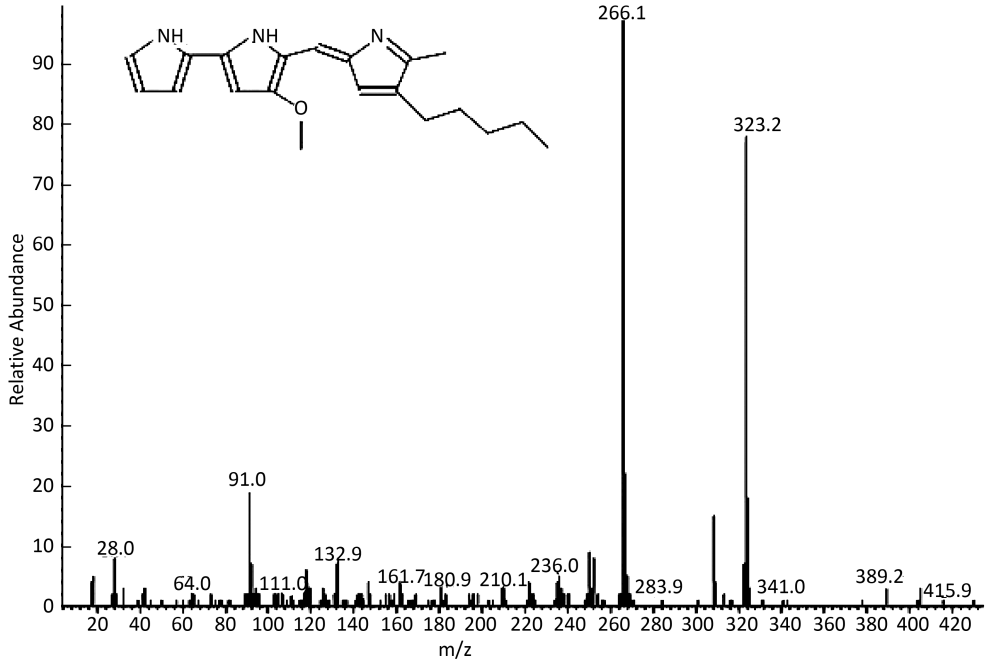

Figure 4. GC/MS mass spectrum and the structural formula of the red pigment produced by *S. marcescens* LTH-2.

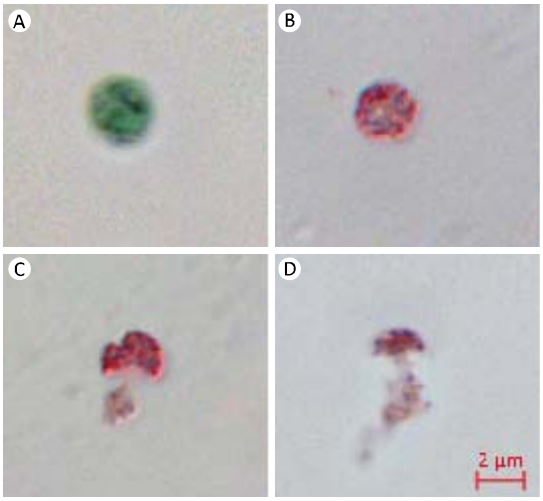

Figure 5. Light micrographs of the algae-lysing process of *M. aeruginosa* FACHB 905 treated with the red pigment (A) control cells, (B) cells penetrated with purified prodigiosin, (C) and (D) severely damaged and burst cells. The concentration of purified prodigiosin was 7.5×10^{-1} µg/mL. Scale bar = 2 µm.

After a two hour exposure to red pigment concentrations of 9.4×10^{-2} µg/mL, 18.8×10^{-2} µg/mL, 37.5×10^{-2} µg/mL, or 7.5×10^{-1} µg/mL, the fraction of cells exhibiting membrane damage was 5.1%±0.6%, 8.0%±0.6%, 10.3%±1.3%, and 14.4%±0.6%, respectively. As PI is a nucleotide-binding stain, the fluorescence of cells stained with PI indicated cell membrane damage. The results demonstrated the existence of a dose dependent relationship for the pigment causing cell membrane damage.

DISCUSSION

Most of the known algicidal bacteria belong to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group or the *γ-Proteobacteria*. In general, the bacteria lyse algae through production of extracellular algae-lysing products or through cell-to-cell contact[4]. The available data suggest that the lytic activity of the CFB group can involve either direct or indirect interactions with target cells, whereas the *γ-Proteobacteria* employ primarily an indirect mode of attack[3-4]. The cell-free supernatant of the *γ-Proteobacteria* strain LTH-2 showed strong algae-lysing activity that was only slightly weaker than the activity of the bacterial culture. Therefore, the results revealed that LTH-2 lysed *M. aeruginosa* mainly through extracellular substances released as an indirect attack. These results indicated that *γ-Proteobacteria* primarily employ an indirect mode. This is the first report that an indigenous *S. marcescens* strain from Lake Taihu is able to lyse local or exogenous strains of the alga, *M. aeruginosa*.

Algicidal bacteria are known to produce a wide variety of algae-lysing compounds, including proteases[13], harmane[14], surfactin[15], and rhamnolipid biosurfactants[16]. The isolation, purification, and characterization of algae-lysing compounds is always difficult because of their varied characteristics. Because the cell filtrate treated at 100 °C for 20 min exhibited similar algae-lysing activity compared to unheated filtrate, the algae-lysing compound secreted by the bacterium LTH-2 was heat-tolerant and unlikely to be an enzyme[17]. A *Microcystis*-lysing red pigment was isolated from the supernatant fluid and was identified as prodigiosin. Its purity was very high according to HPLC, GC-MS, NMR, and FT-IR spectra.

Prodigiosin is a cytotoxic compound showing a broad range of activities including apoptotic activity against human cancer cells and antimalarial activity[8,18]. Algae-lysing activity against the red tide phytoplankton, *Heterosigma akashiwo*, *Heterocapsa circularisquama*, *Cochlodinium polykrikoides*, *Gyrodinium impudicum*, and *Alexandrium tamarense* was reported after 2005[19-20]. The LD_{50} values of the prodigiosin-like pigment against some red tide phytoplanktons were estimated to be 5.0-12.5 µg/mL. Completely purified prodigiosin showed a significant algae-lysing activity against *C. polykrikoides* at a concentration of 10^{-3} mg/L within 60 min of co-incubation[12,19-20]. However, there have been few reports of algae-lysing activity against freshwater phytoplankton. In this study, the algae-lysing activity of prodigiosin against the freshwater phytoplankton, *M. aeruginosa,* was documented. Activity against indigenous strains of *M. aeruginosa* from Lake Taihu, and features of the *Microcystis*-lysing process were reported and discussed for the first time. The results showed that prodigiosin from LTH-2 may be a useful agent for removing *Microcystis* blooms because of the algae-lysing activity demonstrated with *M. aeruginosa*. This work expands the range of harmful algae known to be lysed by prodigiosin.

In conclusion, the indigenous bacterium *S. marcescens* LTH-2 from Lake Taihu and its algae-lysing red pigment, prodigiosin, have significant lytic activity against *M. aeruginosa* strains including the local strains TH1 (toxic), and TH2

(nontoxic), and the reference strain FACHB 905. The lytic activity is probably related to damage of algal cell membranes.

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