

Algicidal activity of *Bacillus* sp. Lzh-5 and its algicidal compounds against *Microcystis aeruginosa*

Zhenghua Li · Mengxin Geng · Hong Yang

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Abstract A freshwater algicidal bacterial strain, Lzh-5, isolated from Lake Taihu, with strong algicidal activity against *Microcystis aeruginosa*, was identified as *Bacillus* sp. based on its phenotypic characteristics and 16S ribosomal RNA (rRNA) gene sequence. The algicidal mode of *Bacillus* sp. Lzh-5 was indirect, attacking *M. aeruginosa* cells by releasing algicidal compounds. Two algicidal compounds (S-5A and S-5B) produced by *Bacillus* sp. Lzh-5 were purified with ethyl acetate extraction, column chromatography, and high-performance liquid chromatography and identified as hexahydropyrrolo[1,2-a]pyrazine-1,4-dione and 3-isopropyl-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione based on liquid chromatography–mass spectrometry, gas chromatography–mass spectrometry, and nuclear magnetic resonance analyses. The active algicidal compounds S-5A (hexahydropyrrolo[1,2-a]pyrazine-1,4-dione) and S-5B (3-isopropyl-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione) displayed high levels of algicidal activity against *M. aeruginosa* 9110, with LD₅₀ values of 5.7 and 19.4 µg/ml, respectively. This is the first report of 3-isopropyl-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione as an algicidal compound. Compounds S-5A and S-5B also induced obvious morphological changes in *M. aeruginosa* 9110. In cocultures of *M. aeruginosa* 9110 and *Bacillus* sp. Lzh-5, the cell density of *Bacillus* sp. Lzh-5 and the concentrations of S-5A and S-5B correlated positively with the algicidal activity. Our results

indicate that strain Lzh-5 and its two algicidal compounds are potentially useful for controlling cyanobacterial blooms in Lake Taihu.

Keywords Algicidal bacteria · Cyanobacterial bloom · *Microcystis aeruginosa* · 3-Isopropyl-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione · Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione

Introduction

Cyanobacterial blooms have become a serious problem all over the world in recent years (Kang et al. 2005) with increasing eutrophication and climate change, especially in China. They cause many problems, including severe damage to aquatic ecosystems and human health. *Microcystis* is the most common bloom-forming cyanobacterium in the world's eutrophic and hypereutrophic waters (Carmichael 2001; Ye et al. 2011; Zurawell et al. 2005). Many strains of *Microcystis* are known to affect drinking water supplies and human health by producing a range of toxins, especially microcystins (Carmichael 2001; Ross et al. 2006; Zurawell et al. 2005). Therefore, an efficient method of controlling the *Microcystis* blooms must be found.

Pollution control is the ultimate strategy for controlling cyanobacterial blooms. However, physical techniques (Lee et al. 2009), chemical techniques (Haughey et al. 2000; Nowack et al. 2011; Paerl et al. 2011), and biological techniques (Cai et al. 2011; Li et al. 2014; Sigeo et al. 1999) have been used to manage the blooms. Because physical techniques are usually too costly to apply and chemical techniques are not environmentally friendly, biological control techniques have received particular attention in recent years because of their potential efficacy, species specificity, and ecofriendliness (Zheng et al. 2012). Algicidal bacteria are considered to cause the decline of

Electronic supplementary material contains phenotypic characteristics of strain Lzh-5, some details for purification (Fig. S4–Fig. S9) and identification (Fig. S10–Fig. S15) of the algicidal compounds.

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blooms (Jeong et al. 2005; Mayali and Azam 2004), so they have potential utility for controlling these blooms.

Various bacterial strains with strong algicidal properties directed against these blooms have been isolated (Hare et al. 2005; Jeong et al. 2000; Lovejoy et al. 1998; Paul and Pohnert 2011; Su et al. 2007; Tian et al. 2012; Wang et al. 2005; Zhang et al. 2013), but only a few algicidal compounds have been purified and identified because their purification is difficult. These compounds include biosurfactants (Wang et al. 2005), proteins (Paul and Pohnert 2013), peptides (Park et al. 2011), bacillamide (Jeong et al. 2003), and pigments (Jeong et al. 2005; Kwon et al. 2010; Nakashima et al. 2006a, b), and most of them were isolated from marine bacterial strains. Very few algicidal compounds have been isolated from freshwater bacterial strains, including argimicin A (Imamura et al. 2000), triterpenoid saponin (Luo et al. 2013), hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, and isatin (Li et al. 2014).

Lake Taihu (119° 54′–120° 36′ N, 30° 56′–31° 33′ E) is the third largest freshwater lake in China (surface area, 2,338 km²; mean depth, 1.9 m) (Tian et al. 2009; Wu et al. 2007) and is a vital economic resource and natural treasure for 30 million people living in the basin. With water eutrophication, cyanobacterial blooms have appeared more frequently and on a larger scale in recent years, especially in Meiliang Bay of Lake Taihu (Qin et al. 2010; Rinta-Kanto et al. 2005; Tian et al. 2009; Ye et al. 2011), and *Microcystis* is one of the dominant species in these cyanobacterial blooms (Ye et al. 2011).

In this study, an algicidal bacterium, *Bacillus* sp. Lzh-5, with strong algicidal activity against *Microcystis aeruginosa*, was isolated from Lake Taihu, and two algicidal compounds (hexahydropyrrolo[1,2-a]pyrazine-1,4-dione and 3-isopropyl-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione) secreted by *Bacillus* sp. Lzh-5 were purified and identified.

Materials and methods

Cyanobacterial culture

M. aeruginosa 9110 isolated from Lake Taihu was cultivated in BG11 medium at 25 °C under 40 μmol photons/(m² s) and a 12 h:12 h (light: dark) cycle (Tian et al. 2012). Cell densities of *M. aeruginosa* 9110 were quantified using a hemocytometer under a light microscope (magnification ×400). *M. aeruginosa* 9110 has been deposited in the China General Microbiological Collection Center (CGMCC) under accession number CGMCC-9118.

Isolation and screening of algicidal bacteria

Water samples in cyanobacterial blooms were collected at the Taihu Ecosystem Research Station (31° 24′ N, 120° 13′ E) in Meiliang Bay using a Ruttner standard water sampler in

October 2010. The collected water samples were transferred into sterile bottles immediately and transported to the laboratory in a mini-icebox. Using the screening approach described by Li et al. (2014), strain Lzh-5, which showed strong algicidal activity against *M. aeruginosa* 9110, was isolated from the water samples and selected for further study. After being re-streaked three times onto beef extract peptone medium agar plates to obtain purified isolate (Yamamoto and Suzuki 1990), strain Lzh-5 was cryopreserved at –70 °C in beef extract peptone medium containing 20 % glycerol. Cell densities of strain Lzh-5 in cultures were determined by the colony-forming unit (CFU) method performed on beef extract peptone medium agar plates (Yamamoto and Suzuki 1990).

Characterization and identification of strain Lzh-5

For identification of the algicidal bacteria, strain Lzh-5 was subjected to taxonomic analysis using conventional physiological and biochemical tests (XiuZhu and Cai 2001). Extraction of bacterial chromosomal DNA from strain Lzh-5 and PCR amplification using primer pair 27F/1492R (27F: 5′-AGAGTTTGATCATGGCTCAG-3′; 1492R: 5′-GGTTACCTTGTTACFACTT-3′) for the 16S ribosomal RNA (rRNA) gene were performed as described by Wu et al. (2007). The PCR products were then sequenced on an ABI-Prism 3730 automated sequencer (PE Applied Biosystems, USA). The sequence of strain Lzh-5 (GenBank accession number HQ896845) was analyzed using RDP classifier software (version 2.2) and compared with sequences in the GenBank database (<http://www.ncbi.nlm.nih.gov/blast>) using the BLAST program.

Algicidal activity of strain Lzh-5

Strain Lzh-5 was incubated at 28 °C under 200rpm for 24 h; after which, 10 ml of bacterial suspensions were inoculated into a test flask containing 90 ml of exponential-phase cultures of *M. aeruginosa* 9110 (1.0×10⁷ cells/ml) and cultivated at 25 °C under 40 μmol photons/(m² s) and a 12 h:12 h (light: dark) cycle. A control was tested using sterile beef extract peptone medium inoculation instead of the bacterial culture inoculation. The algicidal activity (*A*, %) of strain Lzh-5 against *M. aeruginosa* 9110 was calculated by the following equation (Tian et al. 2012):

$$A = \left(1 - \frac{D_{t-\text{treatment}}}{D_{t-\text{control}}} \right) \times 100$$

where $D_{t-\text{treatment}}$ (cells/ml) and $D_{t-\text{control}}$ (cells/ml) are the cell densities of *M. aeruginosa* 9110 in cultures with and without strain Lzh-5 inoculation, respectively; t (day) stands for the inoculation time.

Algicidal mode of strain Lzh-5

To determine the algicidal mode of strain Lzh-5 against *M. aeruginosa* 9110, the strain Lzh-5's bacterial suspension was centrifuged at 4,000×g for 20 min; after which, the supernatant was passed through a 0.22-μm membrane filter to obtain cell-free supernatant. The cell-free supernatant was concentrated tenfold using a centrifugal dryer; the sediment of the culture was washed twice with sterile water in the mean time and re-suspended in sterile water, and after which, 200 μL of the concentrated cell-free supernatant and suspension of the washed bacterial cells were added to the cyanobacterial lawn of *M. aeruginosa* 9110 and incubated under the cyanobacterial growth conditions for 2 days, respectively. As a control, 200 μL of tenfold concentrated beef extract peptone medium was added to the cyanobacterial lawn of *M. aeruginosa* 9110. Cyanobacterial lawn was developed using the method described by Li et al. (2014).

Extraction and purification of algicidal compounds

Strain Lzh-5 was incubated at 28 °C under 200 rpm in beef extract peptone medium for 48 h. Crude extracts were obtained from the bacterial suspension by extraction with an equal volume of ethyl acetate, followed by collecting and evaporating the ethyl acetate layer. The crude extracts dissolved in water were filtered through a 0.22-μm membrane filter to get filtrate; after which, the filtrate was subjected to column chromatography (commercial silica gel, Qingdao Haiyang Chemical Group Co., China, 200–300 mesh; 1×50 cm, flow rate 1 ml/min; UV detection at 254 nm). A fraction from the silica gel column that exhibited algicidal effect based on the cyanobacterial lawn test was applied to a semi-preparative high-performance liquid chromatography (HPLC) column (Supersil™ C18-EP, 5 μm, 10.0×250 mm, Dikma, China, flow rate 4 ml/min; mobile phase 20 % methanol aqueous solution; UV detection at 210 nm) on a liquid chromatography system (1260 Infinity, Agilent, USA). Fractions A and B from semi-preparative HPLC were proved to have algicidal effect and further purified by a reversed-phase HPLC column (Supersil™ C18-EP, 5 μm, 4.6 mm×250 mm, Dikma, China; flow rate 1 ml/min; UV detection at 210 nm) and eluted with 100 % ultrapure water and 10 % methanol aqueous solution as mobile phase, respectively. The purified two compounds S-5A and S-5B were verified to have algicidal effect based on the cyanobacterial lawn test.

Identification of the algicidal compounds S-5A and S-5B

LC–MS, GC–MS, and NMR were applied to determine the chemical structure of the algicidal compounds S-5A and S-5B. LC/MS spectrometers (Agilent Technologies HPLC 1290-MS 6230, USA, for ESI-MS) were used to record

ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-TOF MS) of S-5A and S-5B under the condition described previously (Li et al. 2014). The separation was performed on a ZORBAX Extend-C18 column (1.8 μm, 2.1×50 mm) using an eluent composed of 5 % methanol aqueous solution. The injection volume was 2 μL and the flow rate was 0.2 ml/min. The total effluent from the detector was transferred directly to the hybrid IT/TOF mass spectrometer without splitting. The mass spectrometer was equipped with an electrospray ionization source and operated in positive mode for S-5A and S-5B. Data acquisition and processing were carried out using the LC/MS Qualitative Analysis B.04.00 software supplied with the instrument.

GC/MS spectrometers with HP-5 ms columns (0.25 μm, 0.25 μm×30 m) (Agilent Technologies 6850/5975C, USA, for EI-MS) were applied to record gas mass spectra (EI-MS) of S-5A and S-5B using the method described by Li et al. (2014). Helium was used as the carrier gas and the flow rate was maintained at 1 ml/min. Column temperatures were increased from 100 to 300 °C at 10 °C/min. The mass spectra of S-5A and S-5B were compared with those in the NIST/EPA/NIH Mass Spec. Library (Version 2.0), respectively.

The H-NMR spectra of the two compounds were recorded using a NMR spectrometer (400 MHz, Avance III, Bruker, Switzerland). Chemical shifts were expressed as *d* values (ppm) with trimethylsilyl (TMS) as the internal standard.

LD₅₀ of S-5A and S-5B against *M. aeruginosa* 9110

To elucidate the algicidal activity of S-5A and S-5B, different amounts of S-5A (or S-5B) were added to exponential-phase cultures of *M. aeruginosa* 9110, with the serial concentrations ranging from 5 to 100 μg ml⁻¹, respectively. A control was tested using sterile water inoculation instead of the algicidal compound inoculation. The cell densities of *M. aeruginosa* 9110 were measured after 24 h of incubation under the cyanobacterial growth condition. The viability of *M. aeruginosa* 9110 was calculated by the following equation:

$$V (\%) = D_{\text{treatment}}/D_{\text{control}} \times 100$$

where *V* indicates viability and *D*_{treatment} and *D*_{control} are cell densities of *M. aeruginosa* 9110 in cultures with and without S-5A (or S-5B) inoculation, respectively. The LD₅₀ of S-5A (or S-5B) calculated from the dose–response curve is the concentration of S-5A (or S-5B) that causes the death of 50 % *M. aeruginosa* cells in the culture after 24-h co-incubation.

Observation of morphological change in *M. aeruginosa* 9110

S-5A (or S-5B) was added to the cell suspensions of *M. aeruginosa* 9110 at an initial concentration of $10 \mu\text{g ml}^{-1}$, respectively, and the suspensions were incubated at 25°C for 24 h under the cyanobacterial growth condition. After incubation, the *M. aeruginosa* 9110 cells were observed at a magnification of $\times 1,000$ using an Eclipse 50i Microscope (Nikon) (Nakashima et al. 2006a).

Quantification of S-5A and S-5B in cocultures by LC–MS

Ten-milliliter bacterial suspensions of strain Lzh-5 were inoculated into test flasks containing 90 ml *M. aeruginosa* 9110 cultures, and 10 ml of sterile beef extract peptone medium was inoculated into 90 ml *M. aeruginosa* 9110 cultures as a control. The mixtures were cultured under the cyanobacterial growth condition for 6 days. From the 1st day to the 6th day, cell densities of strain Lzh-5 and *M. aeruginosa* 9110 in the cocultures were determined using the methods described above; after which, aliquot portion of the cocultures was mixed with an equal volume of ethyl acetate for the extraction of S-5A and S-5B. After keeping the mixture in a separation funnel for 24 h, the ethyl acetate layer was collected and evaporated to obtain crude extracts. The crude extracts dissolved in 1 ml water were filtered through a $0.22\text{-}\mu\text{m}$ membrane filter to get filtrate; after which, the filtrate and standard solutions of S-5A and S-5B were subjected to LC–MS analysis in positive mode, respectively. Using the LC–MS Qualitative Analysis B.04.00 software supplied with the instrument, MS Data was acquired and processed; after which, mass chromatograms corresponding to S-5A and S-5B were extracted and integrated from the total ion chromatogram. S-5A and S-5B's concentrations in the filtrate were determined by comparing the peak areas of S-5A (or S-5B) with those of the standards (Armando et al. 2012). Ethyl acetate extraction recoveries were evaluated by the method described by Ran et al. (2013). The concentrations of S-5A (or S-5B) in the cocultures were calculated according to S-5A (or S-5B)'s concentrations in the filtrates, the volume of co-cultures to be extracted, and the extraction recoveries.

In the mean time, strain Lzh-5 was incubated at 28°C under 200 rpm in beef extract peptone medium for 24 h. From 0 to 24 h, cell densities of strain Lzh-5 were determined every 2 h; after which, aliquot portion of the bacterial suspensions was mixed with an equal volume of ethyl acetate for the extraction and quantification of S-5A and S-5B using the methods described above.

Algicidal range and antibacterial effect of S-5A and S-5B

M. aeruginosa 9110 (CGMCC-9118) and *Synechococcus* sp. BN60 (CGMCC-9117) were isolated from Lake Taihu, while

M. aeruginosa PCC7806, *Microcystis viridis* FACHB-979, *Chroococcus* sp. FACHB-193, *Anabaena* sp. FACHB-1140, and *Oscillatoria* sp. FACHB-1083 were purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences, China (FACHB). To determine the algicidal range of S-5A and S-5B, all these seven cyanobacterial strains were used to develop the cyanobacterial lawn, respectively; after which, $200 \mu\text{L}$ of S-5A and S-5B ($10 \mu\text{g ml}^{-1}$) was added to the cyanobacterial lawn, respectively. As a control, $200 \mu\text{L}$ of sterile water was added to the cyanobacterial lawn. The cyanobacterial lawn was incubated under the cyanobacterial growth conditions for 2 days; after which, the diameter of zone of inhibition (millimeter) on the cyanobacterial lawn was measured.

The antibacterial effect of S-5A and S-5B was tested against the test strains of bacteria using standard disc diffusion assay (Nishanth Kumar et al. 2014). The test strains of bacteria were *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Shewanella* sp., *Sphingobacterium* sp., and *Stenotrophomonas* sp., which were purchased from the CGMCC under accession numbers CGMCC-1.8723, CGMCC-1.2620, CGMCC-1.10612, CGMCC-1.8676, CGMCC-6549, CGMCC-8281, and CGMCC-6548, respectively; $200 \mu\text{L}$ of S-5A and S-5B (1, 10, 20, 50, and $100 \mu\text{g ml}^{-1}$, respectively) was added to the bacterial lawn made by the method described previously (CLSI 2006), respectively. As a control, $200 \mu\text{L}$ of sterile water was added to the bacterial lawn. The agar plates were incubated for 24 h at 28°C , and the diameters of inhibition zone (millimeter) on the discs were measured.

Results

Screening algicidal strains

Fifty-six bacterial strains were isolated from surface water samples collected from Meiliang Bay in Lake Taihu in October 2010. Eight strains displayed algicidal effects against *M. aeruginosa* 9110, and strain Lzh-5 was chosen as the algicidal strain for subsequent experiments.

Identification of strain Lzh-5

The colonies of strain Lzh-5 were round, slightly convex, and ivory white, measuring 1–1.5 mm in diameter after incubation for 24 h (SI Fig. S1). The biochemical profile of strain Lzh-5 was determined, as shown in SI Table S2. The isolated bacterium was Gram-positive, rod-shaped, motile, and oxidase- and catalase-positive. Strain Lzh-5 grew at temperatures ranging from 20 to 40°C , but did not grow at temperatures $<4^\circ\text{C}$

>45 °C, or at a pH <5 or pH >10. The Lzh-5 isolate showed phenotypic characteristics similar to those of *Bacillus* sp. The 16S rRNA gene sequence of strain Lzh-5 was determined. Searches of the RDP and BLAST databases revealed that the 16S rRNA sequence of strain Lzh-5 (GenBank accession number HQ896845) was closely related to those of the genus *Bacillus*, with 99 % sequence similarity to *Bacillus* sp. SAP02_1 (99 % homology, GenBank accession number JN872500). Based on its morphological, biochemical, and genetic characteristics, strain Lzh-5 was designated *Bacillus* sp. Lzh-5. Strain Lzh-5 has been deposited in the CGMCC under accession number CGMCC-8282.

Algicidal activity of strain Lzh-5

As shown in SI Fig. S2, strain Lzh-5 (1×10^8 cells/ml [initial concentration]) did not inhibit the growth of *M. aeruginosa* 9110 on day 1, but the cell density of *M. aeruginosa* 9110 clearly decreased thereafter compared with that of the control (without Lzh-5). At the end of day 6, almost all *M. aeruginosa* 9110 cells (>91 %) had died. Thus, the algicidal activity of strain Lzh-5 ($t=6$ days) against *M. aeruginosa* 9110 was about 91.2 ± 6.3 %.

Algicidal mode of strain Lzh-5

As shown in SI Fig. S3, the cell-free supernatant of *Bacillus* sp. Lzh-5 had an algicidal effect on *M. aeruginosa* 9110 and a clearly visible zone of inhibition formed on the cyanobacterial lawn around the paper disc treated with the supernatant, whereas the suspension of washed bacterial cells and beef extract peptone medium (control) showed no algicidal effects. These results suggest that algicidal strain Lzh-5 attacked the *M. aeruginosa* 9110 cells indirectly by releasing algicidal compounds.

Extraction and purification of algicidal compounds

One fraction from a silica gel column exerted an algicidal effect and was subjected to semi-preparative high-performance liquid chromatography (HPLC; SI Fig. S4). As shown in SI Fig. S5, two fractions (fraction A, retention time=5.2–6.5 min; and fraction B, retention time=11.5–12.5 min) from the semi-preparative HPLC had algicidal effects. Fraction A was further purified with HPLC (SI Fig. S6), and S-5A (retention time=15–17 min) was confirmed to have algicidal effect on a cyanobacterial lawn test (SI Fig. S7). Fraction B was further purified by HPLC (SI Fig. S8), and fraction S-5B (retention time=25–27 min) was shown to have algicidal effect with the cyanobacterial lawn test (SI Fig. S9). The purified algicidal compounds S-5A and S-5B were collected for identification.

Identification of algicidal compounds S-5A and S-5B

The electrospray ionization (ESI) mass spectrum of S-5A was recorded in positive mode and yielded a deprotonated molecule at 155.0818 m/z ($M+H$)⁺, and its molecular formula was determined to be $C_7H_{10}N_2O_2$ from the ultra-high-performance liquid chromatography (UPLC)-time of flight (TOF) mass spectrometry (MS) data (SI Fig. S10). The electron ionization (EI) mass spectrum of S-5A, determined with gas chromatography (GC)-MS, indicated that ions at 154, 111, 83, and 70 corresponded to molecule M, $M-C_3H_7$, $M-C_4NH_9$, and $M-C_3NO_2H_2$ ions, respectively (SI Fig. S11a), which is similar to the mass spectrum of hexahydropyrrolo[1,2-a]pyrazine-1,4-dione (molecular weight [MW], 154; molecular formula, $C_7H_{10}N_2O_2$) in a GC-MS library (SI Fig. S11b), with a similarity index >850. The ¹H-NMR spectrum of S-5A is shown in SI Fig. S12, and the ¹H-NMR data were as follows: ¹H NMR (400 MHz, D₂O) δ 4.32 (s, 1H), 4.17 (dd, $J=17.3$, 2.6 Hz, 1H), 3.88 (d, $J=17.3$ Hz, 1H), 3.55 (dd, $J=8.7$, 4.8 Hz, 2H), 2.33 (dd, $J=8.5$, 3.2 Hz, 1H), 2.07 (dd, $J=7.3$, 3.2 Hz, 1H), 1.95 (m, 2H) (SI Fig. S12). The results of ESI, EI, and NMR indicated that S-5A is hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or “cyclo(Gly-Pro)” for short (Fig. 1).

The ESI mass spectrum of S-5B was recorded in positive mode and yielded a deprotonated molecule at 197.1276 m/z ($M+H$)⁺ (SI Fig. S13), and the molecular formula was determined to be $C_{10}H_{16}N_2O_2$ with a UPLC-TOF MS analysis. The EI mass spectrum of S-5B (SI Fig. S14a), determined with GC-MS, indicated that ions at 196, 154, 125, 70, and 41

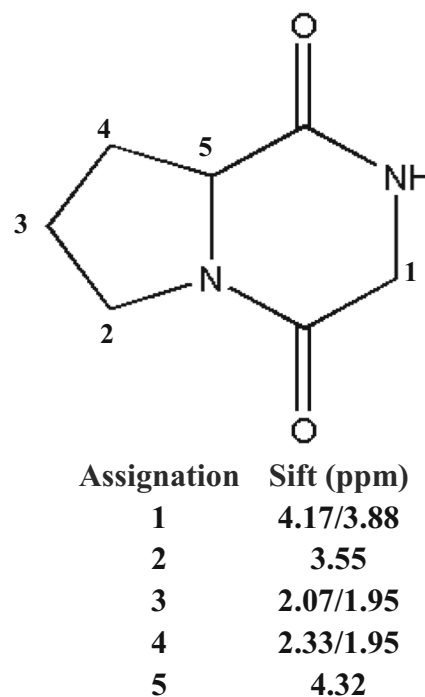


Fig. 1 Molecular structure of S-5A (Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione) and chemical shifts of ¹H signals

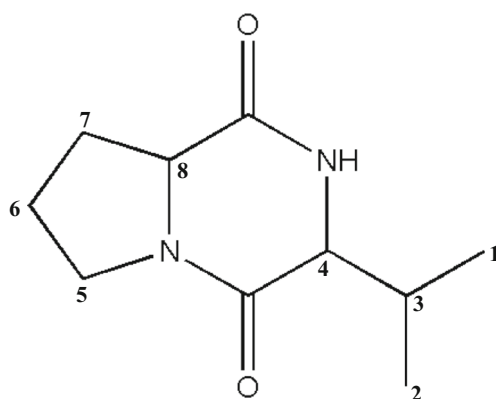
correspond to molecule M, M-C₃H₅, M-C₄NH₈, M-C₆NO₂H₇, and M-C₇N₂O₂H₁₀, respectively, which is similar to the mass spectrum of 3-isopropyl-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione (MW, 196; molecular formula, C₁₀H₁₆N₂O₂) in a previous report (Grant and G.. Sudhakar 1988) (SI Fig. S14b). The ¹H-NMR data for S-5B were as follows: ¹H NMR (400 MHz, D₂O) δ4.20 (m, 1H), 4.07 (s, 1H), 3.47 (m, 2H), 2.38 (dt, *J*=6.9, 4.6 Hz, 1H), 2.26 (m, 1H), 1.97 (d, *J*=7.5 Hz, 1H), 1.83 (t, *J*=4.8 Hz, 2H), 0.98 (d, *J*=7.2 Hz, 3H), 0.76 (d, *J*=6.9 Hz, 3H) (SI Fig. S15). According to these results, S-5B is 3-isopropyl-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, or “cyclo(Pro-Val)” for short (Fig. 2).

LD₅₀ of S-5A and S-5B against *M. aeruginosa* 9110

The growth of *M. aeruginosa* 9110 was clearly inhibited by a low concentration of S-5A (or S-5B), as shown in Fig. 3. The LD₅₀ values of S-5A and S-5B against *M. aeruginosa* 9110 were calculated from dose–response curves to be approximately 5.7 and 19.4 μg/ml, respectively.

Morphological changes in *M. aeruginosa* 9110 exposed to S-5A (or S-5B)

Compared with normal *M. aeruginosa* 9110 cells (Fig. 4a), clear morphological changes were observed in *M. aeruginosa* 9110 after exposure to S-5A (or S-5B) for 24 h. The cells were disrupted and some of the cellular contents were released after



Assignment	Sift (ppm)
1	0.76
2	0.98
3	2.38
4	4.07
5	3.47
6	1.83, 1.97
7	1.83, 2.26
8	4.20

Fig. 2 Molecular structure of S-5B (3-Isopropyl-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione) and chemical shifts of ¹H signals

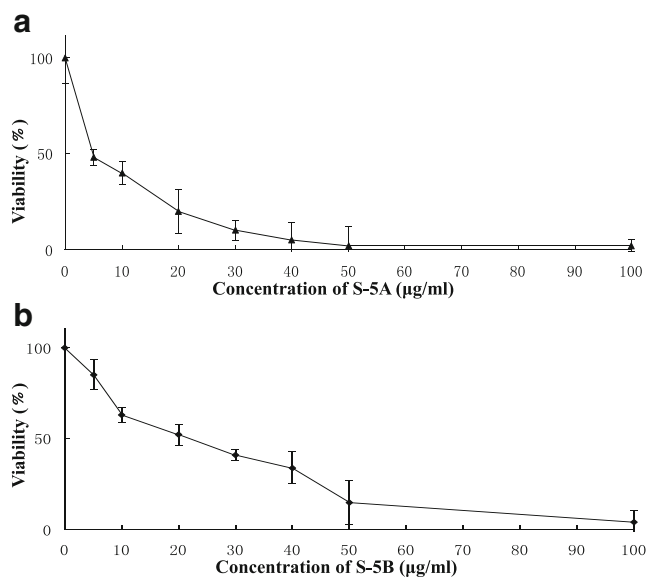


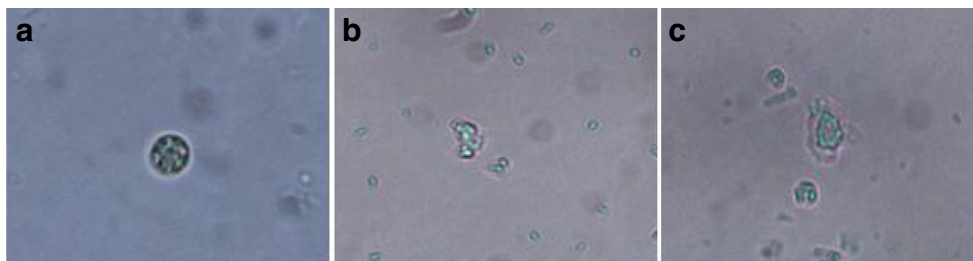
Fig. 3 The algicidal effects of algicidal compounds S-5A (filled triangles) and S-5B (filled diamonds) against *Microcystis aeruginosa* 9110. Different amounts of S-5A (or S-5B) were added to 10 ml exponential-phase cultures of *M. aeruginosa* 9110, respectively. The serial concentrations of S-5A or S-5B in the cultures ranged from 5 to 100 μg ml⁻¹. After incubation for 24 h, the viability of cyanobacteria was calculated by the following equation: $V(\%) = D_{t-treatment} / D_{t-control} \times 100$, where *V* indicates viability, *D*_{*t*-treatment} and *D*_{*t*-control} are viable cells of *M. aeruginosa* 9110 in cultures with and without S-5A (or S-5B) inoculation, respectively. The LD₅₀ value of S-5A and S-5B was calculated from the dose–response curve. Experiments were performed in triplicate. Error bars represent the SD

exposure to S-5A (10 μg ml⁻¹) for 24 h, as shown in Fig. 4b. Similar morphological changes were observed after treatment with S-5B (10 μg ml⁻¹) for 24 h, when the cells lost their integrity and the side walls of the cells damaged (Fig. 4c).

Dynamics of S-5A and S-5B concentrations, strain Lzh-5 cell densities, and the *M. aeruginosa* 9110 biomass in cocultures

The densities of *M. aeruginosa* 9110 cells, the densities of strain Lzh-5 cells, and the concentrations of S-5A and S-5B in cocultures of *M. aeruginosa* 9110 and *Bacillus* sp. Lzh-5 were determined, as shown in Fig. 5a, b. The cell density of *M. aeruginosa* 9110 in the cocultures was almost the same as the control (without Lzh-5 inoculation) at the end of day 1, and the mean cell density of strain Lzh-5 was 7.1×10^8 cells/ml; the concentration of S-5A was low (0.15 μg/ml) and that of S-5B was not detectable. At the end of day 2, the density of strain Lzh-5 increased to 1.8×10^9 cells/ml, S-5B was detected (0.26 μg/ml), and the concentration of S-5A increased to 0.47 μg/ml, with a corresponding increase in the algicidal activity of strain Lzh-5. From the end of day 2 to the end of day 6, the cell density of strain Lzh-5 increased and the concentrations of S-5A and S-5B increased, with an obvious reduction in the density of *M. aeruginosa* 9110 cells compared

Fig. 4 Morphological changes of *M. aeruginosa* 9110 treated with S-5A (or S-5B) ($10 \mu\text{g ml}^{-1}$) for 24 h, respectively. **a** Normal *M. aeruginosa* 9110 cell. **b** *M. aeruginosa* 9110 cells treated with S-5A. **c** *M. aeruginosa* 9110 cells treated with S-5B



with the control. At the end of day 6, when the cell density of strain Lzh-5 was 9.4×10^9 cells/ml and the concentrations of S-5A and S-5B had reached 3.25 and 2.89 $\mu\text{g/ml}$, respectively, most *M. aeruginosa* 9110 (>92 %) had died.

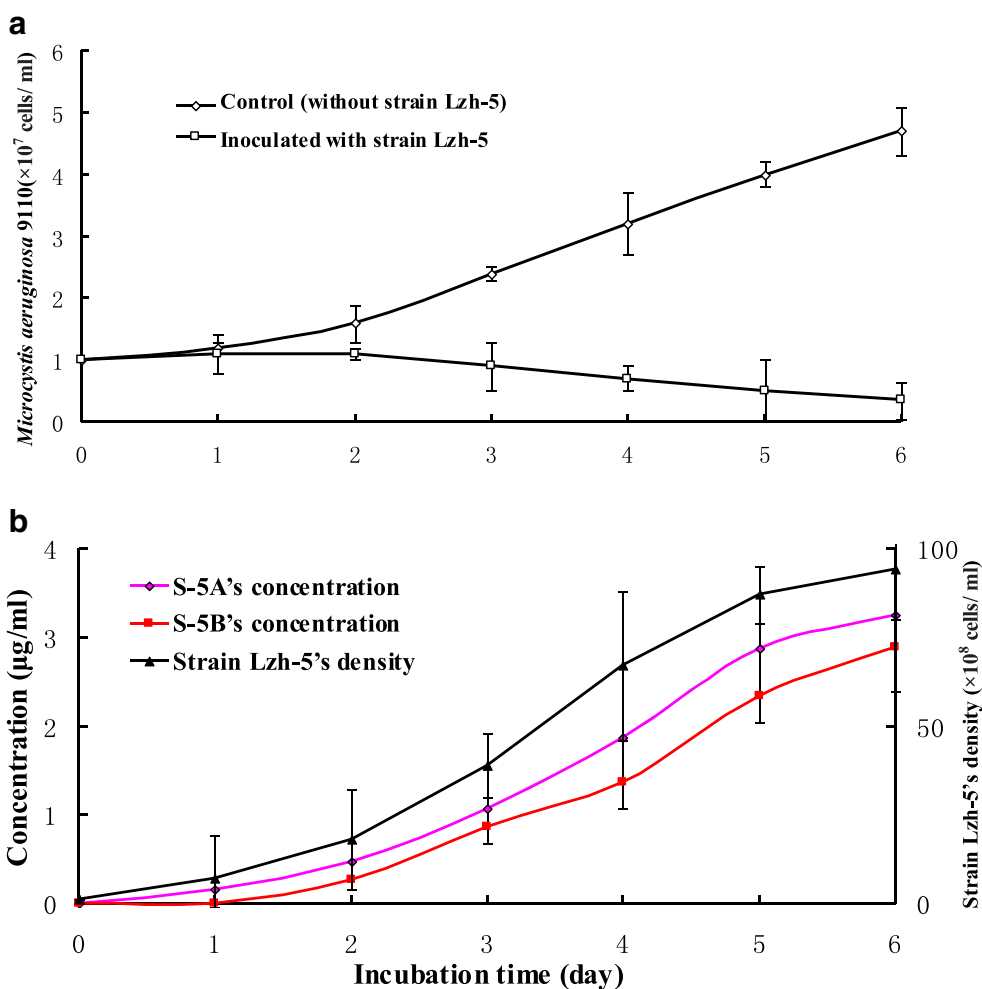
The cell densities of strain Lzh-5 and the concentrations of S-5A and S-5B in the bacterial suspensions of strain Lzh-5 were determined. As shown in SI Fig. S16, cell density of strain Lzh-5 was low, and S-5A and S-5B were not detected at the first 2 h. When cell density of strain Lzh-5 attained to 9.4×10^8 cells/ml at the end of 4 h, S-5A was detected (0.13 $\mu\text{g/ml}$). S-5B was not secreted (0.17 $\mu\text{g/ml}$) until cell density of strain Lzh-5 reached to 3.2×10^9 cells/ml at the end of 6 h. From the

end of 8 h to the end of 24 h, the concentrations of S-5A and S-5B increased. At the end of 24 h, the cell density of strain Lzh-5 was 2.15×10^{10} cells/ml and the concentrations of S-5A and S-5B had reached 7.68 and 6.41 $\mu\text{g/ml}$, respectively.

Algicidal range and antibacterial effect of S-5A and S-5B

The algicidal effect of S-5A and S-5B against the cyanobacterial species was shown in SI Table S3. S-5A and S-5B both had algicidal effect against *M. aeruginosa* 9110, *M. aeruginosa* PCC7806, *M. viridis* FACHB-979, *Chroococcus* sp. FACHB-193, and *Oscillatoria* sp. FACHB-

Fig. 5 Dynamics of cell density of *M. aeruginosa* 9110, cell density of strain Lzh-5, and concentration of S-5A and S-5B in the cocultures of *M. aeruginosa* 9110 and strain Lzh-5. **a** Cell density of *M. aeruginosa* 9110 inoculated with Lzh-5 (squares) (initial concentration of Lzh-5 in the cocultures, 1×10^8 cells/ml) and control (without strain Lzh-5) (diamonds). **b** Concentration of S-5A (filled diamonds) and S-5B (filled squares); cell density of strain Lzh-5 (filled triangles). Experiments were performed in triplicate. Error bars represent the SD



1083, but had no algicidal effect against *Synechococcus* sp. BN60 and *Anabaena* sp. FACHB-1140.

S-5A and S-5B had no antibacterial effect against the test strains of bacteria when their concentrations were 1, 10, 20, and 50 $\mu\text{g ml}^{-1}$, respectively. After S-5A and S-5B's concentration was up to 100 $\mu\text{g ml}^{-1}$, respectively, S-5A and S-5B exerted antibacterial effect against *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *V. cholerae*, but still had no antibacterial effect against *Shewanella* sp., *Sphingobacterium* sp., and *Stenotrophomonas* sp. (SI Table S4).

Discussion

The algicidal modes of algicidal bacteria can be summarized as either direct (bacterial and algal cell contact) or indirect (the release of algicidal compounds) (Mayali and Azam 2004). The algicidal mode of *Bacillus* sp. Lzh-5 is indirect, attacking the cyanobacterial cells with the release of two algicidal compounds, S-5A (cyclo[Gly-Pro]) and S-5B (cyclo[Pro-Val]). Both cyclo(Gly-Pro) and cyclo(Pro-Val) displayed strong algicidal activity against *M. aeruginosa* (Fig. 3), one of the dominant organisms in the water blooms of Lake Taihu (Ye et al. 2011). *Bacillus* sp. Lzh-5 showed similar or stronger algicidal activity against *M. aeruginosa* than other algicidal bacteria isolated from freshwater (Jung et al. 2008; Li et al. 2012; Ren et al. 2010; Shunyu et al. 2006; Yang et al. 2013) (data in SI Table S1). These results indicate that *Bacillus* sp. Lzh-5 has potential utility for controlling the outbreaks of cyanobacterial blooms in Lake Taihu.

This is the first study to show that algicidal compound cyclo(Pro-Val), which was found in extracts of the sponges *Tedania ignis* (Stierle et al. 1988a) and *Alternaria alternata* (Stierle et al. 1988b), has algicidal activity against *M. aeruginosa*. Algicidal compound cyclo(Gly-Pro) was also not only secreted by *Bacillus* sp. Lzh-5 in this study, but also produced by the algicidal bacterium *Shewanella* sp. Lzh-2 (Li et al. 2014), also isolated from Lake Taihu. Both cyclo(Gly-Pro) ($\text{LD}_{50}=5.7 \mu\text{g/ml}$) and cyclo(Pro-Val) ($\text{LD}_{50}=19.4 \mu\text{g/ml}$) had stronger or similar algicidal activity compared to other reported algicidal compounds, such as prodigiosin (which is a well-known anti-algal pigment secreted by marine bacteria, and had algicidal activity against *Chattonella marina* (Nakashima et al. 2006a)), isatin (which was secreted by *Shewanella* sp. Lzh-2 and had algicidal activity against *M. aeruginosa* (Li et al. 2014)), and argimicin A (Imamura et al. 2000). Algicidal compounds cyclo(Gly-Pro) and cyclo(Pro-Val) not only showed strong algicidal activity against *M. aeruginosa*, but also significantly inhibited the growth of several other cyanobacterial species (SI Table S3). In terms of their biological safety, both cyclo(Gly-Pro) and cyclo(Pro-Val) are biodegradable

(Perzborn et al. 2013), which means that they may be largely environmentally friendly when used to control cyanobacterial blooms.

Cyclo(Gly-Pro) and cyclo(Pro-Val) are both diketopiperazines (cyclic dipeptides), which are the simplest peptide derivatives, found in various natural sources, including plants, fungi, and bacteria (Fdhila et al. 2003; Prasad 1995; Stark and Hofmann 2005). Formation of the diketopiperazines was thought to be catalyzed by nonribosomal peptide synthetases (NRPSs) and cyclodipeptide synthetases (CDPSs) (Borthwick 2012). NRPS-dependent pathways are widespread in bacteria and fungi, but only albonoursin, pulcherriminic acid, and mycocyclosin were found to be catalyzed by CDPS (Belin et al. 2012). It is interesting to note that cyclo(Gly-Pro) probably derives from cyclo(Pro-Val) with the thermal loss of a propene molecule (Grant and G. Sudhakar 1988), which means that they may utilize the same biosynthetic route.

In previous report, prodigiosin and isatin could inhibit cell division of *C. marina* (Nakashima et al. 2006a) and *M. aeruginosa* 9110 (Li et al. 2014), respectively, and 2-hydroxy-12-oleanene-3, 28-O-D-glucopyranosyl were shown to disrupt the antioxidant systems of *M. aeruginosa* cells (Luo et al. 2013). In this study, both cyclo(Gly-Pro) and cyclo(Pro-Val) caused cell-wall damage and cell disruption, as shown in Fig. 4, suggesting that they also share the same algicidal mechanism.

In cocultures of *M. aeruginosa* 9110 and *Bacillus* sp. Lzh-5, cyclo(Gly-Pro) and cyclo(Pro-Val) were not detected until the density of strain Lzh-5 cells exceeded 7.1×10^8 cells/ml and 1.8×10^9 cells/ml, respectively (Fig. 5). The algicidal compounds (isatin and cyclo[Gly-Pro]) of another algicidal bacterium isolated from Lake Taihu, *Shewanella* sp. Lzh-2 (Li et al. 2014), were only detected when the bacterial cell density reached to 1.5×10^9 cells/ml and 5.3×10^8 cells/ml, respectively. This suggests that there is a threshold bacterial cell density that regulates the secretion of algicidal compounds. Compared with the results in Fig. 5 and SI Fig. S16, it suggested that the production of these two algicidal compounds was not upregulated with the presence of *M. aeruginosa*, and the concentrations of algicidal compounds were related to the cell density of algicidal bacteria after the algicidal bacterial cell density reached to the threshold. In the present study, we also showed that the algicidal bacterial density and the concentrations of algicidal compounds correlated positively with the algicidal activity after the algicidal bacterial cell density exceeded the threshold.

In conclusion, our results show that the algicidal bacterium *Bacillus* sp. Lzh-5 and its two algicidal compounds are potentially useful for controlling cyanobacterial blooms in Lake Taihu. Future work should focus on the algicidal mechanisms of these two algicidal compounds.

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