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The algicidal activity of *Aeromonas* sp. strain GLY-2107 against bloom-forming *Microcystis aeruginosa* is regulated by *N*-acyl homoserine lactone-mediated quorum sensing

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Summary

Cyanobacterial blooms have disrupted the efficient utilization of freshwater worldwide. A new freshwater bacterial strain with strong algicidal activity, GLY-2107, was isolated from Lake Taihu and identified as *Aeromonas* sp. It produced two algicidal compounds: 2107-A (3-benzyl-piperazine-2,5-dione) and 2107-B (3-methylindole). Both compounds exhibited potent algicidal activities against *Microcystis aeruginosa*, the dominant bloom-forming cyanobacterium in Lake Taihu. The EC_{50} values (concentration for 50% maximal effect) of 3-benzyl-piperazine-2,5-dione and 3-methylindole were 4.72 and 1.10 μg ml^{-1} respectively. Based on a thin-layer chromatography biosensor assay and ultra-performance liquid chromatography-coupled high resolution-tandem mass spectrometry (UPLC-HRMS/MS), the *N*-acyl homoserine lactone (AHL) profile of strain GLY-2107 was identified as two short side-chain AHLs: *N*-butyryl-homoserine lactone (C4-HSL) and *N*-hexanoyl-homoserine lactone (C6-HSL). The production of the two algicidal compounds was controlled by AHL-mediated quorum sensing (QS), and C4-HSL was the key QS signal for the algicidal activity of the strain GLY-2107. Moreover, 3-methylindole was found to be positively regulated by C4-HSL-mediated QS, whereas 3-benzyl-piperazine-2,5-dione might be negatively controlled by C4-HSL-mediated QS. This study suggests that a QS-regulated algicidal system may have potential use for the development of a novel control strategy for harmful cyanobacterial blooms.

Introduction

Eutrophication of freshwater bodies causes frequent outbreak of cyanobacterial blooms worldwide (Wu *et al.*, 2011; Lewitus *et al.*, 2012). Lake Taihu, the third largest freshwater lake (2338 km^2) in China, is a typical example; *Microcystis aeruginosa* is the dominant bloom-forming cyanobacterium in this lake (Ye *et al.*, 2011). *Microcystis* spp. blooms have provoked global concern due to their harmful impacts on the environment and human health (Otten *et al.*, 2012; Xiao *et al.*, 2014). Therefore, it is urgent to control harmful *Microcystis* blooms. To date, the high costs and secondary pollution of the available physical and chemical methods have prevented their widespread applications (Li *et al.*, 2014). Biocontrol technologies have drawn increasing attention and have been deemed an effective strategy for controlling harmful algal blooms due to their potential efficacy and eco-friendliness (Anderson, 1997; Zheng *et al.*, 2013). Algicidal bacteria have been found to play an important role in the regulation and termination of blooms in some lakes (Rashidan and Bird, 2001; Zhang *et al.*, 2012). Furthermore, algicidal bacteria and the corresponding algicidal substances have been shown to be effective for the control of harmful cyanobacteria (Manage *et al.*, 2000; Kim *et al.*, 2008; Li *et al.*, 2015; Lin *et al.*, 2016).

Through in-depth research of algae-bacteria interactions, it has been found that some algicidal bacteria must reach a threshold cell density before their algicidal activity can be observed. The strains J18/M01 (Mitsutani *et al.*, 1992) and A5Y (Imai *et al.*, 1993) of genus *Cytophaga*, *Flavobacterium* sp. strain 5N-3 (Fukami *et al.*, 1992), and *Flavobacteriaceae* strain S03 (Roth *et al.*, 2007) must reach a cell density of at least 10^6 cells ml^{-1} before they show any algicidal activity. Skerratt *et al.* (2002) found that *Bacillus* sp. strain ACEM32 was only algicidal in the stationary phase. Li *et al.* (2014) found that *Shewanella* sp.

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Lzh-2 must reach a threshold bacterial cell density of at least 10^8 cells ml^{-1} before any algicidal compound was detectable. These previous studies suggest that cell concentration-dependent quorum sensing may play an important role for algicidal bacteria in the algicidal process.

Quorum sensing (QS) is a cell-to-cell communication mechanism that relies on the production, release, and community-wide detection of the extracellular signal molecules in a cell-density-dependent manner and a subsequent response (Camilli and Bassler, 2006; Deng *et al*., 2011). At low bacterial cell density, each cell synthesizes a basal level of QS signals that diffuse or are transported into extracellular environments (Deng *et al*., 2011); after the QS signals reach a critical threshold concentration at high cell density, the accumulated signals bind to their cognate receptors and regulate a series of biological activities and cellular functions (Camilli and Bassler, 2006). To date, the best characterized QS signals are from the family of *N*-acylhomoserine lactones (AHLs), which are most common among Gram-negative bacteria (Berger *et al*., 2011). AHLs are synthesized by a LuxI type AHL synthase. The accumulated AHLs attain a threshold concentration and interact with their cognate LuxR type receptor, thus modulating a set of QS-controlled biological activities (Fuqua *et al*., 1996; Swift *et al*., 2001). Nakashima *et al*. (2006) found that the inhibited production of the algicidal pigment in erythromycin-treated γ-*proteobacterium* strain MS-02-063 can be recovered by exogenous homoserine lactone and that algicidal pigment production can be inhibited by β-cyclodextrin, which inhibits AHL activity. However, it remains unclear whether the algicidal strain produces AHL(s) and which types of AHLs are responsible for the regulation of the algicidal pigment production. Further in-depth research on the molecular mechanism of quorum sensing in the algicidal process of algicidal bacteria is necessary, and it may provide a means for the design of a novel effective control strategy for harmful cyanobacterial blooms.

In the present study, a potent algicidal bacterium, *Aeromonas* sp. strain GLY-2107, that produces two algicidal compounds was isolated from Lake Taihu. The role of AHL-mediated QS in the production of algicidal compounds by strain GLY-2107 was studied.

Results

Isolation and identification of algicidal bacterium producing AHL(s)

Strain GLY-2107 (Supporting Information Fig. S1a) with the strongest algicidal activity (*A*) ($A = 96.5\%$, $t = 6$ days) among the isolated twelve algicidal strains producing AHL(s), which were detected by the AHL biosensor *Chromobacterium violaceum* CV026, was selected for further study. The strain GLY-2107 was a Gram-negative, rod-shaped (0.6 to 1.2- by 2.0 to 2.5-μm) bacterium with a polar flagellum (Supporting Information Fig. S1b). Comparison of the 16S rRNA gene sequence with the sequence available in the GenBank database indicated that strain GLY-2107 most closely resembled the type strain *Aeromonas caviae* ATCC 15468^T (99.6% identity, GenBank accession number X74674). The phylogenetic tree based on the 16S rRNA gene sequences of strain GLY-2107 and type strains of other *Aeromonas* species supported a close relatedness to *Aeromonas caviae* ATCC 15468^T (Supporting Information Fig. S2). Meanwhile, the strain GLY-2107 was found to show positive catalase and oxidase activities, which are the common characteristics of the genus *Aeromonas* (Alperi *et al*., 2010; Aravena-Roman *et al*., 2013). The morphological, physiological and phylogenetic characteristics supported the classification of strain GLY-2107 as an *Aeromonas* strain. Therefore, the newly isolated strain was designated as *Aeromonas* sp. strain GLY-2107.

The algicidal effect of Aeromonas sp. strain GLY-2107on target species

As shown in Table 1, strain GLY-2107 showed strong algicidal effects on most of the tested cyanobacterial species, especially those isolated from Meiliang Bay of Lake Taihu. Strain GLY-2107 exhibited the strongest algicidal effect ($A = 96.5\%$) on *M. aeruginosa* 9110 among all the tested cyanobacterial species after six days of co-cultivation. Furthermore, strain GLY-2107 also showed high algicidal activity ($A \geq 80\%$, $t = 6$ days) against one eukaryotic algal strain from Lake Taihu, *Chlamydomonas* sp. BS3 ($A = 90.3\%$, $t = 6$ days).

Algicidal mode of Aeromonas sp. strain GLY-2107

As shown in Fig. 1, the culture of strain GLY-2107 showed a slightly higher algicidal activity ($A = 96.5\%$, $t = 6$ days) compared with its cell-free filtrate ($A = 86.2\%$, $t = 6$ days), while the algicidal activity of culture from strain GLY-2107 was far higher ($P < 0.01$) than that of washed cells from strain GLY-2107 ($A = 32.3\%$, $t = 6$ days). There was no significant ($P > 0.05$) difference between the algicidal activities of the cell-free filtrate and heat-treated cell-free filtrate ($A = 83.7\%$, $t = 6$ days). These results suggested that strain GLY-2107 exhibited algicidal activity mainly by excretion of heat-stable extracellular algicidal compounds.

Extraction and purification of the algicidal compounds

After the separation of the ethyl acetate extract of the cell-free culture filtrate of strain GLY-2107 using silica gel chromatography, one fraction in the effluent of the chromatographic column was found to be algicidal. The fraction was collected and then fractionated by semi-

Table 1. Algicidal effect of strain GLY-2107 against various algal and cyanobacterial strains.

Strain	Cell density or chl *a* concentration		Algicidal activity (*A*, %)
	Control	Treatment	
Microcystis aeruginosa 9110*	2.61 ± 0.28	0.09 ± 0.03	96.5 ± 1.1
Synechococcus sp.BN60*	8.20 ± 0.41	0.97 ± 0.22	82.2 ± 2.6
Chlorophyta sp. B1*	6.10 ± 0.20	0.38 ± 0.05	93.7 ± 0.8
Oscillatoria sp.BN35*	6.81 ± 0.16	0.60 ± 0.24	91.2 ± 3.5
Chlamydomonas sp. BS3*	5.73 ± 0.29	0.56 ± 0.07	90.3 ± 1.2
Chroococcus sp. FACHB-191	7.79 ± 0.32	4.98 ± 0.26	36.1 ± 3.3
Microcystis viridis FACHB-979	7.33 ± 0.36	0.64 ± 0.15	91.3 ± 2.1
Microcystis aeruginosa PCC7806	2.80 ± 0.13	0.31 ± 0.05	88.9 ± 1.9

*Isolated from Meiliang Bay of Lake Taihu. The cell densities of *Microcystis aeruginosa*, chl *a* concentrations of other cyanobacteria or alga, and algicidal activities of strain GLY-2107 were obtained after six days of co-cultivation. The data are the mean ± SD from triplicate biological replicates. Unit of cell density (for *Microcystis aeruginosa*): $\times 10^7$ cells ml^{-1}; unit of chlorophyll *a* (chl *a*) concentration (for other cyanobacteria or alga, except for *Microcystis aeruginosa*): mg l^{-1}. The calculation of the algicidal activity (*A*, %) was based on the following equation: $A = (1 - D_{t\text{-treatment}}/D_{t\text{-control}}) \times 100$, where $D_{t\text{-treatment}}$ and $D_{t\text{-control}}$ represent the cyanobacterial (or algal) cell density in the cultures with (treatment) or without (control) inoculation of strain GLY-2107, respectively, and *t* (day) is the inoculation time (here, $t = 6$ days). Except for *Microcystis aeruginosa*, the chl *a* concentrations were used as the equivalent cell densities for other cyanobacterial (or algal) species.

preparative high-performance liquid chromatography (HPLC), and two primary fractions, i.e. fractions A [retention time (RT): 18.5–20.5 min] and B (RT: 43.7–46.0 min) (Supporting Information Fig. S3), showed algicidal activities on cyanobacterial lawns. Subsequently, fine separation of fractions A and B was performed using further-purification HPLC. Finally, two purified substances with algicidal activity, i.e. 2107-A (RT: 93.0-105.0 min, Supporting Information Fig. S4a) and 2107-B (RT: 37.5-40.0 min, Supporting Information Fig. S4b), were obtained after further-purification HPLC.

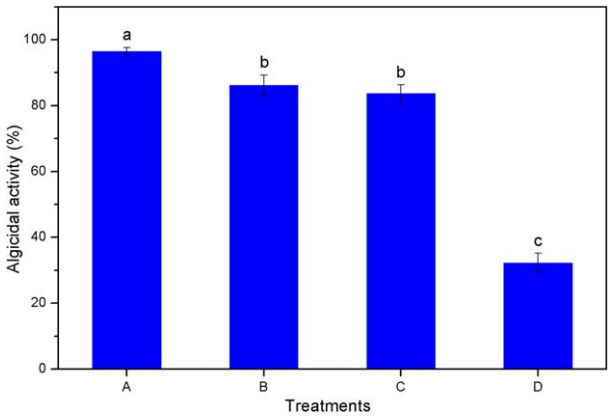

Fig. 1. Algicidal effects of differently treated *Aeromonas* sp. strain GLY-2107 cultures on *M. aeruginosa* 9110.
A. *M. aeruginosa* cultures with addition of *Aeromonas* sp. strain GLY-2107 cultures.
B. *M. aeruginosa* cultures with addition of *Aeromonas* sp. strain GLY-2107 cell-free filtrates.
C. *M. aeruginosa* cultures with addition of *Aeromonas* sp. strain GLY-2107 heat-treated cell-free filtrates.
D. *M. aeruginosa* cultures with addition of *Aeromonas* sp. strain GLY-2107 cells re-suspended in BG11medium. Data are the mean ± SD from three independent replicates. Mean values denoted by the same letter were not significantly different at $P < 0.05$.

Identification of the algicidal compounds

The structures of purified 2107-A and 2107-B were elucidated by high-resolution electrospray ionization mass spectrometry (ESI-MS), electron impact ionization mass spectrometry (EI-MS) and nuclear magnetic resonance (NMR).

The positive-mode high-resolution ESI-MS spectrum showed the mass-to-charge ratio (*m/z*) of 2107-A $(M+H)^+$ to be 205.0981 (Supporting Information Fig. S5a) and 2107-B $(M+H)^+$ to be 132.0807 (Supporting Information Fig. S5b), suggesting that the molecular formulas of 2107-A and 2107-B were $C_{11}H_{12}N_2O_2$ and C_9H_9N respectively. A comparison of the EI-MS spectra of 2107-A (Supporting Information Fig. S6a) and 2107-B (Supporting Information Fig. S6d) with data in the NIST Mass Spectral Library suggested that 2107-A and 2107-B were 3-benzyl-piperazine-2,5-dione and 3-methylindole, respectively, and that the similarity index (SI) between the EI-MS spectrum of 2107-A (or 2107-B) and the corresponding typical spectrum (Supporting Information Fig. S6c or S6f) was above 960. The EI-MS spectrum of 2107-A (or 2107-B) was good agreement with that of standard 3-benzyl-piperazine-2,5-dione (Supporting Information Fig. S6b) (or 3-methylindole, Supporting Information Fig. S6e). Meanwhile, the structures of 2107-A and 2107-B were further confirmed by ^1H NMR and ^{13}C NMR spectra analysis, and the NMR data of 2107-A (Supporting Information Fig. S7) and 2107-B (Supporting Information Fig. S8) were in agreement with those previously reported for 3-benzyl-

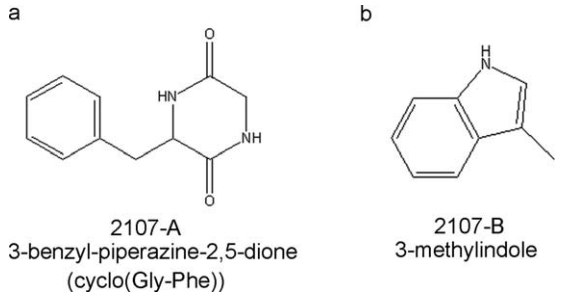

Fig. 2. Molecular structures of the algicidal compounds produced by *Aeromonas* sp. strain GLY-2107.

piperazine-2,5-dione (Coursindel *et al.*, 2010) and 3-methylindole(Morales-Ríos *et al.*, 1988) respectively.

Taken together, 2107-A and 2107-B were identified as 3-benzyl-piperazine-2,5-dione ["cyclo(Gly-Phe)" for short] (Fig. 2A) and 3-methylindole (Fig. 2B) respectively.

EC_{50} of cyclo(Gly-Phe) and 3-methylindole

In general, standard 3-methylindole exhibited a stronger algicidal activity against *M. aeruginosa* 9110 compared with that of standard cyclo(Gly-Phe), as shown in Fig. 3. The EC_{50} values (concentrations for 50% of maximal effect) of cyclo(Gly-Phe) and 3-methylindole, which were estimated from the dose-response curve, were 4.72 and 1.10 $\mu g\ ml^{-1}$ respectively.

Identification of AHL(s) produced by Aeromonas sp. strain GLY-2107

A thin-layer chromatography (TLC) overlay assay with biosensor *Chromobacterium violaceum* CV026 (responding to short-chain C4–C8 AHLs) (Fig. 4A) or *Chromobacterium violaceum* VIR24 (mainly responding to C6–C14 AHLs, although weakly responding to C4-AHL) (Fig. 4B) suggested that strain GLY-2107 produces two AHLs with a short *N*-acyl side-chain of four or six carbons. As shown in Fig. 4A and B, the mobility of one AHL of strain GLY-2107 corresponded to that of synthetic *N*-butyryl-homoserine lactone (C4-HSL), and the mobility of the other AHL of strain GLY-2107 corresponded to that of synthetic *N*-hexanoyl-homoserine lactone (C6-HSL). To further identify exactly which AHLs strain GLY-2107 produced, the cell free supernatant of strain GLY-2107 was analysed by ultra-performance liquid chromatography-coupled high-resolution tandem mass spectrometry (UPLC-HRMS/MS). The extracted ion chromatogram of either *m/z* at 172.09 [corresponding to the *m/z* of molecular ion $(M+H)^+$ of C4-HSL] or *m/z* at 200.12 [corresponding to the *m/z* of molecular ion $(M+H)^+$ of C6-HSL] from the total ion chromatography was a single peak and shared the same retention time with the standard C4-HSL or C6-HSL respectively (Supporting Information Fig. S9). Furthermore, the UPLC-HRMS/MS spectra of both *m/z* at 172.09 and *m/z* at 200.12 showed the typical fragment of AHLs at 102, which indicates the presence of a lactone ring (Lim *et al.*, 2014). As shown in Fig. 4C–F, they were in good agreement with those of standard C4-HSL or C6-HSL. No signals could be extracted from total ion chromatogram using the *m/z* corresponding to other AHLs. From the HRMS/MS spectra, it was confirmed that strain GLY-2107 produces two AHLs, i.e. *N*-butyryl-homoserine lactone and *N*-hexanoyl-homoserine lactone.

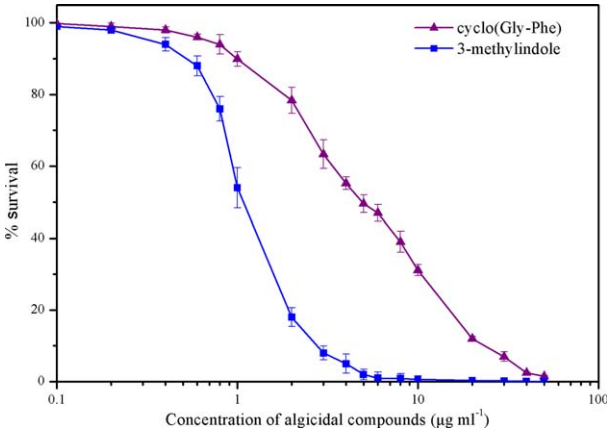

Fig. 3. Algicidal effects of identified algicidal compounds on *M. aeruginosa* 9110 after 24 h of exposure.

Identification and disruption of agyI and agyR genes in Aeromonas sp. strain GLY-2107

The sequence of the *agyI* gene showed 99% or 88% identity with that of the AHL synthase gene from *A. caviae* strain YL12 (GenBank accession number JOVP01000025.1: 783881.784510) or *A. hydrophila* strain A1 (GenBank accession number X89469.1: 24.647) respectively. The deduced protein sequence of AgyI showed 99% or 90% identity with that of AHL synthase from *A. caviae* strain YL12 (GenBank accession number KEP90884) or *A. hydrophila* strain A1 (GenBank accession number CAA61653) respectively. The sequence of the *agyR* gene showed 99% or 86% identity with that of the LuxR-type transcriptional regulator gene from *A. caviae* strain YL12 (GenBank accession number JOVP01000025.1: 784575.785357) or *A. hydrophila* strain A1 (GenBank accession number X89469: 710.1492) respectively. The deduced protein sequence of AgyR showed 99% or 92% identity with that of the LuxR-type transcriptional regulator from *A. caviae* strain YL12 (GenBank accession number KEP90885) or *A. hydrophila* strain A1 (GenBank accession number CAA61654) respectively.

To investigate the role of the *agyI-agyR* QS system in the algicidal activity of *Aeromonas* sp. strain GLY-2107, the

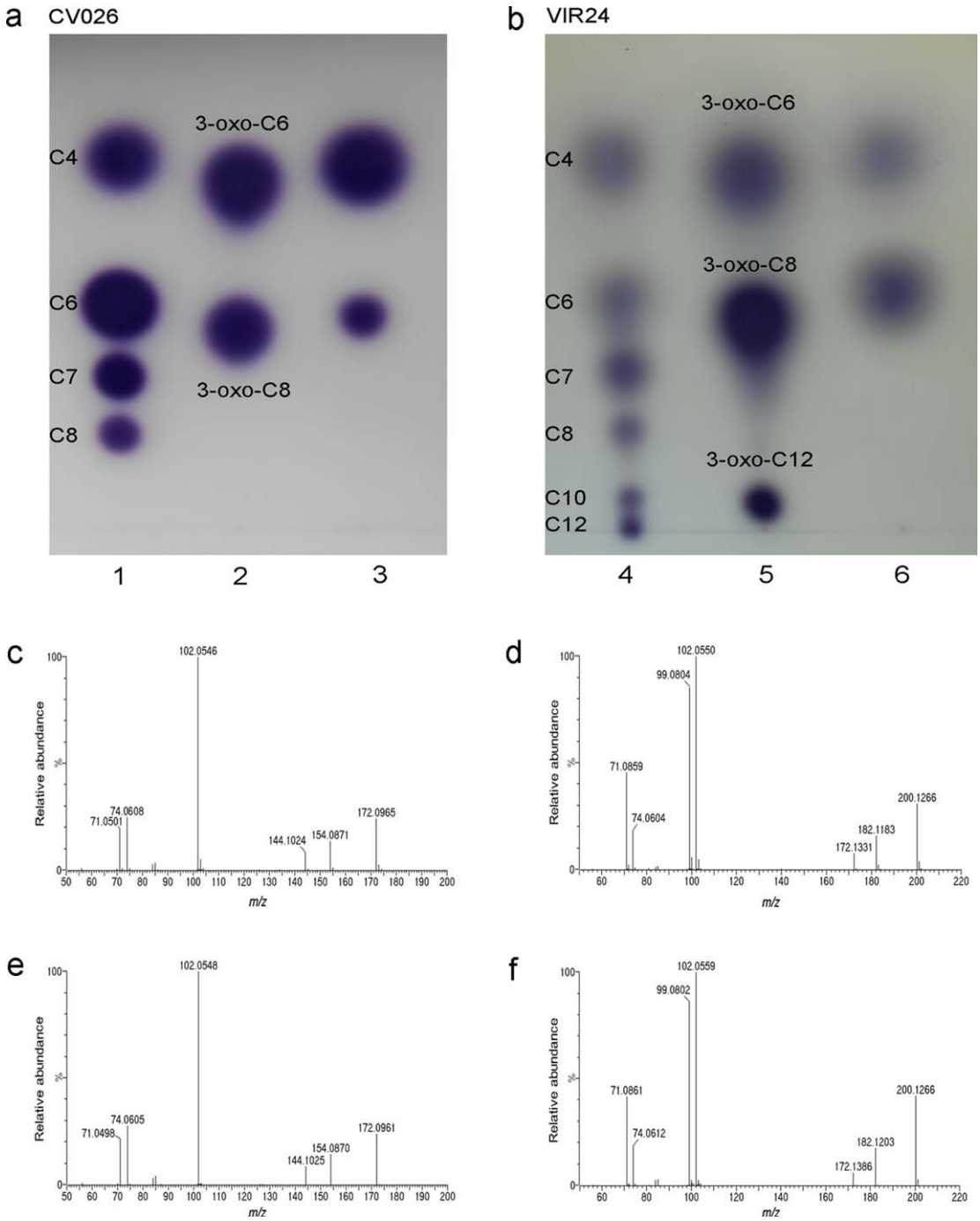

Fig. 4. Analysis of AHLs by pigment production in *C. violaceum* CV026 and VIR24 and MS/MS spectra of C4-HSL and C6-HSL standards/extracts. (A and B): analysis of AHLs produced by *Aeromonas* sp. strain GLY-2107 by TLC. AHLs were visualized as the induction of pigment production by *C. violaceum* CV026 (A) and VIR24 (B) after 24 h of incubation. Lane 1: Mixture of synthetic and fully reduced AHLs standards: C4 (C4-HSL), 2.9 nmol; C6 (C6-HSL) 0.25 nmol; C7 (C7-HSL) 0.23 nmol; C8 (C8-HSL) 2.20 nmol. Lane 2: Mixture of synthetic 3-oxo-HSL standards: 3-oxo-C6 (3-oxo-C6-HSL) 0.47 nmol; 3-oxo-C8 (3-oxo-C8-HSL) 4.15 nmol. Lane 3: AHL extracted from 5 ml of spent supernatant from *Aeromonas* sp. strain GLY-2107. Lane 4: Mixture of synthetic and fully reduced AHLs standards: C4 (C4-HSL), 146.12 nmol; C6 (C6-HSL) 0.50 nmol; C7 (C7-HSL) 0.17 nmol; C8 (C8-HSL) 0.11 nmol; C10 (C10-HSL) 0.39 nmol; C12 (C12-HSL) 0.17 nmol. Lane 5: Mixture of synthetic 3-oxo-HSLs standards: 3-oxo-C6 (3-oxo-C6-HSL) 0.23 nmol; 3-oxo-C8 (3-oxo-C8-HSL) 0.41 nmol; 3-oxo-C12 (3-oxo-C12-HSL) 0.25 nmol. Lane 6: AHL extracted from 100 ml of spent supernatant from *Aeromonas* sp. strain GLY-2107. (C–F): MS/MS spectra of authentic C4-HSL (C) and C6-HSL (d) standards as well as the extracted C4-HSL (e; *m/z* of molecular ion $(M+H)^+$: 172.09; retention time: 1.47 min) and C6-HSL (f; *m/z* of molecular ion $(M+H)^+$ 200.12; retention time: 2.84 min) from the spent supernatant of *Aeromonas* sp. strain GLY-2107 harvested after 24 h of incubation under 220 rpm at 28°C.

agyI and *agyR* genes were disrupted via homologous recombination as described in the Methods section. After a second crossover event, the mutants with disruption events in gene *agyI* (named *Aeromonas* sp. strain GLY-MI) or *agyR* (named *Aeromonas* sp. strain GLY-MR) were selected and verified by polymerase chain reaction (PCR) using the primer pairs *agyI*-For plus *agyI*-Rev or *agyR*-For plus *agyR*-Rev, respectively, and then sequenced. As shown in Supporting Information Fig. S10a and c, the use of genomic DNA from the mutants (strain GLY-MI or strain GLY-MR) as templates with the above-mentioned primer pairs resulted in the expected size PCR products (disrupted *agyI* gene (Δ*agyI*): 523bp; disrupted *agyR* gene (Δ*agyR*): 619bp); the PCR products from the mutants were obviously truncated compared with those from the wild-type strain (*agyI* gene: 630bp; *agyR* gene: 783bp). Furthermore, sequencing of the abovementioned PCR products confirmed that the *agyI* and *agyR* genes were disrupted successfully in the mutant strains GLY-MI and GLY-MR, respectively, as shown in Supporting Information Fig. S10b and d.

The agyI gene is in charge of the synthesis of AHLs in Aeromonas sp. strain GLY-2107

Through the AHL-bioassay analysis, it was found that the wild type (strain GLY-2107) and the *agyR* mutant (strain GLY-MR) can produce the AHLs. The sterile-filtered supernatants of these strains (Supporting Information Fig. S11b and c) exhibited a positive signal on the agar plates containing *C. violaceum* CV026. However, the *agyI* mutant (strain GLY-MI) lost the capacity for the production of AHLs, and the filtered supernatant of the *agyI* mutant (Supporting Information Fig. S11d) showed a negative signal on the agar plates containing *C. violaceum* CV026. Therefore, by functioning similarly to other LuxI-type AHL synthases genes, the *agyI* gene is in charge of the synthesis of C4- and C6-HSL in strain GLY-2107.

The C4-HSL-mediated agyI-agyR quorum sensing system modulates the production of algicidal compounds in Aeromonas sp. strain GLY-2107

To investigate the role of the *agyI-agyR* QS system in the algicidal function of *Aeromonas* sp. strain GLY-2107, the dynamics of the concentration of algicidal compounds (Fig. 5A and B) and dissolved organic carbon (DOC) (Fig. 6C), and the cell density of *M. aeruginosa* 9110 (Fig. 6A) and algicidal bacterial strains (Fig. 6B), were determined during the algicidal process against *M. aeruginosa* 9110 of the wild-type strain GLY-2107; the *agyI* mutant in the presence or absence of 10 μM C4-HSL or C6-HSL; and the *agyR* mutant with or without the pBBR-sup-*agyR* plasmid. As shown in Figs. 5 and 6, according to the fact that the

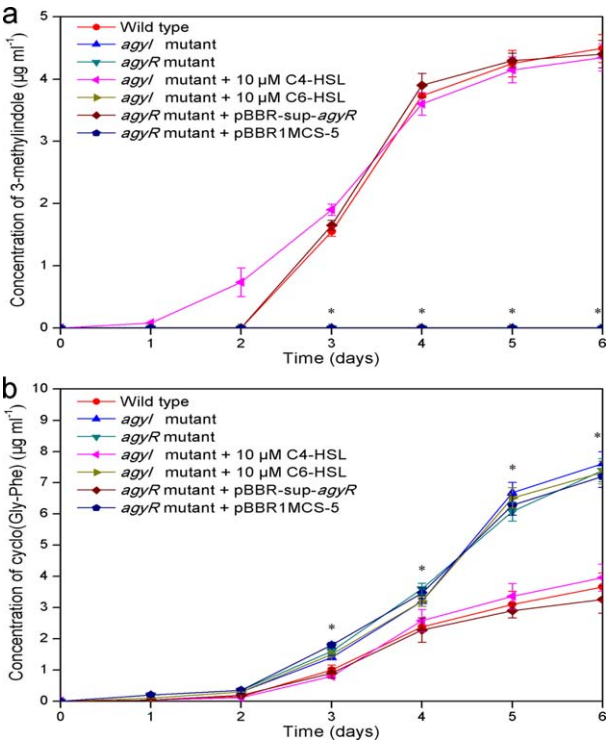

Fig. 5. Dynamics of the concentrations of the algicidal compounds [3-methylindole (A) and cyclo(Gly-Phe) (B)] during the algicidal process against *M. aeruginosa* 9110 of *Aeromonas* sp. strain GLY-2107; *agyI* mutant in the presence or absence of 10 μM C4-HSL or C6-HSL; and *agyR* mutant with or without pBBR-sup-*agyR* plasmid. The vertical bar represents the standard deviation of triplicate samples. The values of group II denoted by * were significantly ($P < 0.05$) different from those of group I at the same time point. Group I: wild type, *agyI* mutant + 10 μM C4-HSL, and *agyR* mutant + pBBR-sup-*agyR*; group II: *agyI* mutant, *agyI* mutant + 10 μM C6-HSL, *agyR* mutant, and *agyR* mutant + pBBR1MCS-5.

dynamics of the abovementioned parameters tended to vary during the algicidal process, the co-cultures of *M. aeruginosa* 9110 with algicidal wild type strain GLY-2107 or the derived mutants can be generally classified into two groups (group I: co-cultures in the presence of wild type strain GLY-2107, *agyI* mutant strain GLY-MI supplemented with 10 μM C4-HSL, and *agyR* mutant strain GLY-MR carrying pBBR-sup-*agyR*; group II: co-cultures in the presence of *agyI* mutant, *agyI* mutant supplemented with 10 μM C6-HSL, *agyR* mutant, and *agyR* mutant carrying pBBR1MCS-5). The dynamics of each parameter of the co-cultures in every group showed similar tendency to vary.

As shown in Figs. 5B, 6A and B, during the first 2 days of the algicidal process, with the growth of the wild-type strain GLY-2107 and the derived mutants in the co-cultures, the concentration of cyclo(Gly-Phe) increased gradually. However, 3-methylindole could not be detected, except for in the co-culture in the presence of *agyI* mutant strain GLY-MI supplemented with C4-HSL (Fig. 5A). In this

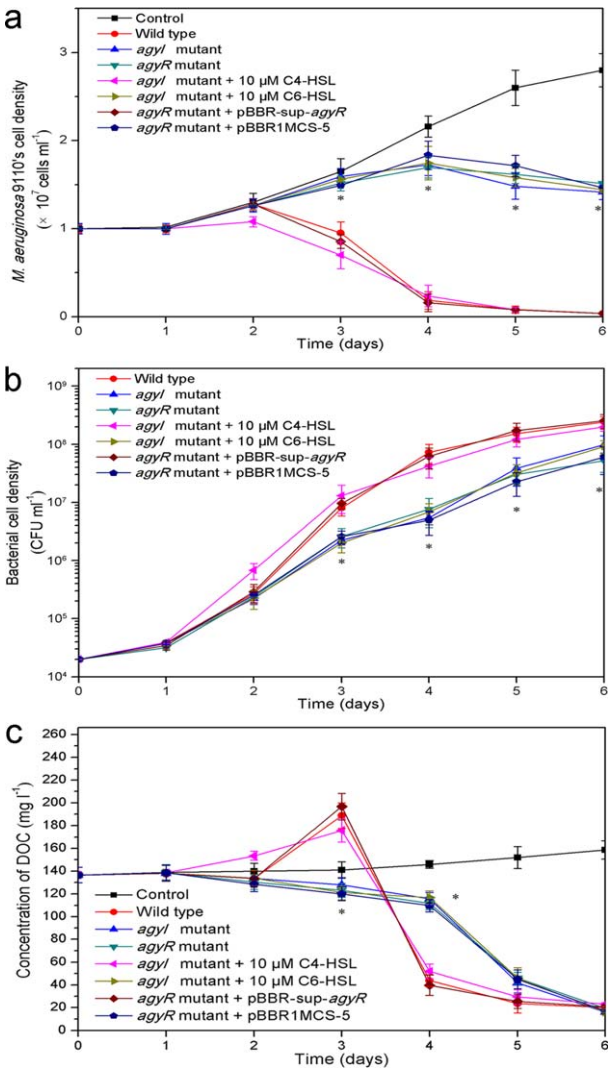

Fig. 6. Dynamics of the cell densities of *M. aeruginosa* 9110 (A) and the algicidal strains (B) and the concentration of dissolved organic carbon (DOC) (C) during the algicidal process against *M. aeruginosa* 9110 of *Aeromonas* sp. strain GLY-2107; *agyI* mutant in the presence or absence of 10 μM C4-HSL or C6-HSL; and *agyR* mutant with or without pBBR-sup-*agyR* plasmid. The vertical bar represents the standard deviation of triplicate samples. The values of group II denoted by * were significantly ($P < 0.05$) different from those of group I at the same time point. Group I: wild type, *agyI* mutant + 10 μM C4-HSL, and *agyR* mutant + pBBR-sup-*agyR*; group II: *agyI* mutant, *agyI* mutant + 10 μM C6-HSL, *agyR* mutant, and *agyR* mutant + pBBR1MCS-5.

culture, 3-methylindole could be detected on day 1; on day 2, its concentration increased to 0.73 μg ml^{-1}, leading to an obvious algicidal activity of 16.9% (Fig. 6A) and a significant ($P < 0.01$) increase in the concentration of DOC in the co-culture compared with that in the control (Fig. 6C). On day 2, as shown in Fig. 6A and C, no obvious algicidal activity could be observed in the other co-cultures, and the concentration of DOC decreased slightly compared with that in the control.

As shown in Fig. 6A, the algicidal capacities of mutants with an inactivated-QS system were significantly weakened relative to that of wild-type strain GLY-2107 ($P < 0.01$, day 3–6). In particular, after 3 days of co-cultivation, the algicidal activities of the strains in the co-cultures of group I were significantly ($P < 0.01$) higher than those of group II. After 3 days of co-cultivation, the tendencies of the dynamics to vary for all parameters in the co-cultures of group I were significantly ($P < 0.01$, except for DOC on day 5 and day 6) different from those in the co-cultures of group II, as shown in Figs. 5 and 6. In the co-cultures of group I, when the cell density of bacterial strains reached around 10^7 CFU ml^{-1} after three days of co-cultivation (Fig. 6B), 3-methylindole could be detected; its concentration continuously and rapidly increased over the following four days, ultimately increasing to 4.49, 4.35 and 4.42 μg ml^{-1} after 6 days of co-cultivation (Fig. 5A). The concentration of cyclo(Gly-Phe) in the three co-cultures of group I slowly increased from day 4 to day 6, and finally increased to 3.67, 3.96 and 3.22 μg ml^{-1}, respectively, after 6 days of co-cultivation (Fig. 5B). With the production of 3-methylindole and the increase of cyclo(Gly-Phe) in the three co-cultures of group I, on day 3 of the algicidal process, the cell density of *M. aeruginosa* 9110 decreased dramatically (Fig. 6A), while the concentration of DOC increased rapidly to peak values of 188.7, 175.9 and 196.8 mg l^{-1} respectively (Fig. 6C). From day 4 to day 6, with the rapid growth of the algicidal strains and the continuous increase in the concentration of two algicidal compounds in the co-cultures of group I, the cell density of *M. aeruginosa* 9110 decreased continuously, and the concentration of DOC fell dramatically. On day 6 of the algicidal process, the cell densities of the algicidal strains in the co-cultures of group I were on the order of 10^8 CFU ml^{-1} (Fig. 6B), while cells from *M. aeruginosa* 9110 were hardly detectable (Fig. 6A); the concentrations of DOC decreased to nearly 20 mg l^{-1} (Fig. 6C).

As for the co-cultures of group II, after 3 days of co-cultivation, with the growth of these algicidal mutants, the continuously increasing concentrations of cyclo(Gly-Phe) in these co-cultures were significantly ($P < 0.01$) higher than those in the co-cultures of group I at each time point and reached concentrations greater than 7.0 μg ml^{-1} after 6 days of co-cultivation, which was approximately 2.0 times higher than those in co-cultures of group I at the corresponding time point (Fig. 5B). However, 3-methylindole could not be detected, even when the cell densities of the algicidal mutants in the co-cultures of group II were on the order of 10^7 CFU ml^{-1} on day 6 of the algicidal process, as shown in Figs. 5A and 6B. With the increasing concentration of cyclo(Gly-Phe) in the co-cultures of group II, the cell densities of *M. aeruginosa* 9110 decreased slightly after four days of co-cultivation, but on day 6 of co-cultivation, the cell densities of *M. aeruginosa* 9110 were

still significantly ($P < 0.01$) higher compared with those in the co-cultures of group I and were approximately half of that in the control (Fig. 6A). Meanwhile, the concentrations of DOC in the co-cultures of group II continuously decreased from day 3 to day 6 with the growth of bacterial strains and reached values of approximately 20 mg l^{-1} on day 6 of the algicidal process (Fig. 6C).

Based on the results of the dynamics of algicidal activities, the concentrations of the algicidal compounds and DOC and the cell densities of the algicidal strain GLY-2107 and its derived mutants, it can be concluded that C4-HSL is the QS signal responsible for regulating the algicidal function of *Aeromonas* sp. strain GLY-2107 and that the C4-HSL-mediated *agyI-agyR* QS system positively regulates the synthesis of 3-methylindole but might negatively regulate the synthesis of cyclo(Gly-Phe).

Discussion

Aeromonas sp. strain GLY-2107 not only showed high algicidal activities against *Microcystis*, the dominant bloom-forming cyanobacteria in Lake Taihu (Ye *et al*., 2011), but also inhibited several other cyanobacterial species isolated from cyanobacterial blooms in Lake Taihu (Table 1), indicating that *Aeromonas* sp. strain GLY-2107 has potential for use in the control of outbreaks of cyanobacterial blooms in Lake Taihu. Bacterial algicidal activity functions in three main ways: penetration into the host cell, cell to cell contact, or production of extracellular compounds (Gerphagnon *et al*., 2015). *Aeromonas* sp. strain GLY-2107 exhibited algicidal activity mainly by the release of extracellular heat-stable compounds.

Some studies have demonstrated that quorum sensing may play an important role during the algicidal process of algicidal bacteria. Mayali and Doucette (2002) found that, during the algicidal process, *Cytophaga* strain 41-DBG-2 must attain a threshold cell density of at least 10^6 cells per milliliter before initiation of its algicidal activity. Paul and Pohnert (2011) found that the release of algicidal protein by the algicidal bacterium *Kordia algicida* was triggered in advance by cell-free bacterial filtrates. However, the key, definite signal molecule of the quorum sensing system that regulates the algicidal activities remains to be identified. Nakashima *et al*. (2006) found that exogenous addition of homoserine lactone can recover the production of the algicidal pigment in erythromycin-treated γ-*proteobacterium* strain MS-02-063 and that an AHL inhibitor can inhibit the production of algicidal pigments; this is just one report showing that the HSL-mediated QS system may control the algicidal activity of algicidal bacteria, but it remains unclear whether the algicidal strain produces AHL(s). The current study separated and identified the QS signal molecule (C4-HSL) responsible for the algicidal function of algicidal bacterium *Aeromonas* sp. strain GLY-2107 derived from Lake Taihu and verified that the algicidal process, efficacy and productions of algicidal compounds of this algicidal bacterium were controlled by the C4-HSL-mediated QS system.

Aeromonas sp. strain GLY-2107 can produce two algicidal substances, i.e. 3-methylindole and cyclo(Gly-Phe), and the EC_{50} value of 3-methylindole is far smaller than that of cyclo(Gly-Phe). Thus, the algicidal activity of the former is much stronger than that of the latter. 3-methylindole is an indole derivative produced by the bacterial metabolism of amino acids (Medzhitov *et al*., 2012); in this study, it is reported as an algicidal active substance for the first time. Before this study, Honeyfield and Carlson (1990) found that 3-methylindole is toxic to the growth of *Lactobacillus* sp. strain 11201. Li *et al*. (2014) found that another indole derivative, isatin, which is produced by *Shewanella* sp. Lzh-2, showed algicidal activity. Cyclo(Gly-Phe) belongs to the diketopiperazine family, which contains a large group of small molecules produced by a wide range of microbes that exhibit many valuable biological properties (Ortiz-Castro *et al*., 2011), including antibacterial (Fdhila *et al*., 2003), algicidal (Li *et al*., 2014; Guo *et al*., 2015; Li *et al*., 2015; Lin *et al*., 2016) and signal (sexual pheromone) (Gillard *et al*., 2013) activities. During the sexual reproduction of the diatom *Seminavis robusta*, diproline (proline-derived diketopiperazine) acting as the sexual pheromone of the attracting mating type (MT^- cells) can induce the migratory behavior of the migrating mating type (MT^+ cells) (Gillard *et al*., 2013). However, Zhu *et al*. (2013) found cyclo(Phe-Ala) and cyclo(Ala-Trp) isolated from marine bacterium *Pseudomonas putida* showed the anti-diatom activities. Although cyclo(Gly-Phe) was first identified to have algicidal activity against *M. aeruginosa*, some other diketopiperazine substances produced by other algicidal bacteria have been found to have algicidal activities against *M. aeruginosa*, such as: cyclo(Pro-Gly) (Li *et al*., 2014; Li *et al*., 2015; Lin *et al*., 2016), cyclo(Pro-Val) (Li *et al*., 2015), cyclo(Pro-Leu) (Guo *et al*., 2015) and cyclo(4-OH-Pro-Leu) (Guo *et al*., 2015). Cyclo(Pro-Gly) and cyclo(Pro-Val) can induce morphological changes and loss of cell integrity of *M. aeruginosa* (Li *et al*., 2015). Cyclo(4-OH-Pro-Leu) mainly interrupts the flux of electron transport in the photosynthetic system of *M. aeruginosa*, whereas cyclo(Pro-Leu) mainly inhibits the activity of intracellular antioxidases of *M. aeruginosa* (Guo *et al*., 2015). The EC_{50} values (concentration for 50% maximal effect) of cyclo(Pro-Gly), cyclo(Pro-Val), cyclo(Pro-Leu) and cyclo(4-OH-Pro-Leu) against *M. aeruginosa* (initial cell density: 1.0×10^7 cells ml^{-1}) were 5.7, 19.4, 2.7 and 1.3 μg ml^{-1} respectively. Compared with these known diketopiperazine algicidal substances, cyclo(Gly-Phe) showed stronger or similar algicidal activity, although algicidal mechanism of cyclo(Gly-Phe) against *M. aeruginosa* needs further in-depth investigation in the future.

On day 3 of algicidal process, the 3-methylindole was detectable in the co-culture of *M. aeruginosa* 9110 and wild-type algicidal strain GLY-2107, indicating that the concentration of the key algicidal QS signal molecule in this co-culture was above the threshold response concentration for inducing production of 3-methylindole. *AgyI* mutant did not produce 3-methylindole throughout the algicidal process. Although C4-HSL and C6-HSL could be degraded with time (half-lives of C4-HSL and C6-HSL were 1.06 and 2.57 days (Supporting Information Fig. S12), respectively, in the mixed medium), in the co-culture of *M. aeruginosa* 9110 and *agyI* mutant with exogenous C4-HSL or C6-HSL at an initial working concentration of 10 μM, the concentration of C4-HSL and C6-HSL on day 6 of algicidal process was 295.23 nM and 2.21 μM, respectively, which was 1.61 and 563.92 times higher than the corresponding concentration (C4-HSL: 182.81 nM; C6-HSL: 3.92 nM) in the co-culture of *M. aeruginosa* 9110 and wild-type algicidal strain GLY-2107 on day 3 of algicidal process. This indicated that during the algicidal process, the concentrations of the QS signal molecule in chemically complemented co-cultures of *agyI* mutant and *M. aeruginosa* 9110 were always higher than the threshold response concentration for inducing production of 3-methylindole. Thus, it was feasible for the chemical complementation of *agyI* mutant by the one-time application of exogenous C4-HSL or C6-HSL with a higher initial working concentration (10 μM) by reference to the previous complementary study on the AHL synthase gene mutants of *Aeromonas* spp. (Swift *et al.*, 1997; Lynch *et al.*, 2002).

Through knockout of AHL synthase and its cognate receptor in strain GLY-2107 and chemical or genetic complementation to the corresponding mutant, it was confirmed that in strain GLY-2107, the regulatory mode of the C4-HSL (algicidal signal molecule)-mediated QS system in the syntheses of algicidal compounds belongs to the classic AHL-based LuxIR-type QS system of Gram-negative bacteria. From previous reports, the C4-HSL-mediated AhyRI QS system positively modulates exoprotease production (Swift *et al.*, 1999), hemolysin (Bi *et al.*, 2007), and the type VI secretion system (Khajanchi *et al.*, 2009) in *A. hydrophila*. In *Pseudomonas aeruginosa*, the C4-HSL-mediated RhlRI QS system was found to positively control the production of toxic cyanide (Schuster *et al.*, 2003) and biofilm development (Favre-Bonte *et al.*, 2003) but to negatively control Type III secretion regulon expression (Bleves *et al.*, 2005). The current study confirmed that the synthetic process of 3-methylindole in *Aeromonas* sp. strain GLY-2107 was regulated by a C4-HSL-mediated QS system, and the regulatory mode might be completely opposite to that of cyclo(Gly-Phe). When the concentrations of these two algicidal compounds during the algicidal process were normalized to cell density of strain GLY-2107 and its QS mutants (Supporting Information Fig. S13), the results also supported this conclusion. During the first 2 days of the algicidal process, in the co-culture of *M. aeruginosa 9110* and *agyI* mutant strain GLY-MI with an initial C4-HSL concentration of 10 μM, the concentrations of 3-methylindole normalized to bacterial cell densities were above 1.1 pg CFU^{-1}, but the counterparts in other co-cultures were zero; however, during these 2 days, the concentrations of cyclo(Gly-Phe) normalized to bacterial cell densities in this co-culture were below 0.25 pg CFU^{-1}, and were significantly ($P < 0.01$) lower than the counterparts in other co-cultures at the same time point. The synthesis of 3-methylindole is positively regulated by the C4-HSL-mediated QS system. Moreover, 3-methylindole has higher algicidal activity, and thus it is the most important algicidal substance of *Aeromonas* sp. strain GLY-2107. Cyclo(Gly-Phe) might be negatively controlled by the C4-HSL-mediated QS system, and on the other hand, the possibility that 3-methylindole production consumed a considerable amount of energy thereby limiting cyclo(Gly-Phe) production might exist as well. In addition, cyclo(Gly-Phe) shows a lower algicidal activity, so cyclo(Gly-Phe) is the second most important algicidal substance of strain GLY-2107.

The DOC derived from the exudation or cell lysis of phytoplankton is a major energy source driving the growth of heterotrophic prokaryotes (Sarmento and Gasol, 2012). Mitsutani *et al.* (1992) found that *Cytophaga* sp. A5Y can utilize not only algal cells but also exudation from algae. In the current study, there was no obvious difference for the growth rates between the wild-type strain GLY-2107 and the derived mutants in the nutrient-rich full BEP medium; the cell densities of all strains reached the order of 10^9 CFU ml^{-1} after 24 h of cultivation (Supporting Information Fig. S14). However, during the algicidal process where the dilution of BEP medium were 1:100 in the bacterial-cyanobacterial co-cultures, the cell densities of strain GLY-2107 and the complementary (chemical or genetic) *agyI* and *agyR* mutants (group I) began to increase rapidly with the lysis of cyanobacterial cells from day 2 of co-cultivation and was significantly ($P < 0.01$) higher than those of the *agyI* and *agyR* mutants (group II) after 6 days of co-cultivation (Fig. 6B). This phenomenon could be attributed to the fact that after two days of co-cultivation, in the co-culture with the strain in group I, the lysis of cyanobacterial cells (Fig. 6A) caused by the production of 3-methylindole (Fig. 5A) and cyclo(Gly-Phe) (Fig. 5B) resulted in an increased concentration of DOC (Fig. 6C); this increase provided more energy to drive the rapid growth of the strains in group I. After 3 days of co-cultivation, the decrease in DOC concentration may be because the consumption rate of DOC by the rapidly proliferating algicidal bacteria was higher than the production rate of DOC from the exudation and lysis of cyanobacterial cells.

Table 2. All the bacterial strains used in this study.

Strains	Relevant characteristics[a]	Source or reference
Aeromonas sp. strain GLY-2107	Wild-type strain with algicidal activity isolated from Lake Taihu, China	This study
Aeromonas sp. strain GLY-MI	*Aeromonas* sp. strain GLY-2107 mutant obtained by deletion of the *agyI* gene	This study
Aeromonas sp. strain GLY-MR	*Aeromonas* sp. strain GLY-2107 mutant obtained by deletion of the *agyR* gene	This study
Aeromonas sp. strain GLY-MR (pBBR1MCS-5)	*Aeromonas* sp. strain GLY-MR harboring the plasmid pBBR1MCS-5	This study
Aeromonas sp. strain GLY-MR (pBBR-sup-*agyR*)	*Aeromonas* sp. strain GLY-MR harboring the plasmid pBBR-sup-*agyR*	This study
Escherichia coli DH5α	F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 hsdR17*(r_K^- m_K^+)λ^-	(Hanahan, 1983)
Escherichia coli S17-1	*thi-1 proA hsdR17* (r_K^- m_K^+) *recA1*, *tra* gene of plasmid RP4 integrated in chromosome	(Simon *et al*., 1983)
Chromobacterium violaceum CV026	ATCC 31532 derivative, *cviI*::Tn*5xylE*, Km^R; An AHL biosensor produces a purple pigment in response to short chain AHL (C4-C8).	(McClean *et al*., 1997)
Chromobacterium violaceum VIR24	An in-frame deletion mutant of the *cviI* gene encoding AHL synthase in ATCC 12472[T]; An AHL biosensor strongly responds to medium-chain-length AHLs (C6–C14, although weakly to C4), and then produces a purple pigment.	(Someya *et al*., 2009)

a. Km^R: kanamycin resistance.

Although the lysis of cyanobacterial cells could result in the release of harmful cyanotoxins along with dissolved organic matter (DOM) into the water column, the disadvantage is not unique to algicidal bacteria and the biologically derived algicidal compounds. Other approaches such as traditional, physical and chemical manipulations are also subject to this disadvantage (Shao *et al*., 2013). Control of cyanobacteria before they form blooms can reduce environmental health risks caused by the release of DOM and cyanotoxins (Shao *et al*., 2013), lessen the influence on the microbial communities and biogeochemical cycles, and avoid water column hypoxia resulting from the excessive prolification of bacteria with the excessive release of DOM. Thus, the most recommended approach may be to preemptively control cyanobacteria at the early stages of prolification. In this study, both cyclo(Gly-Phe) and 3-methylindole are biodegradable (Yin and Gu, 2006; Perzborn *et al*., 2013), which means that they may be largely environmentally friendly when used to control cyanobacterial blooms. The unravel of regulation mechanism of AHL-mediated QS on the synthesis of these two algicidal compounds by *Aeromonas* sp. strain GLY-2107 may provide help to the design and optimization of the strategy for the practical application of algicidal strain GLY-2107 (such as immobilization) in the future.

Experimental procedures

Cyanobacterial and algal strains, and cultivation conditions

The *Microcystis aeruginosa* 9110 (maintained as a unialgal axenic culture with actidione at a concentration of 50 μg ml^{-1}, and deposited in the China General Microbiological Culture Collection Center (CGMCC) under accession no. CGMCC 9118), *Synechococcus* sp. BN60 (CGMCC 9117), *Chlorophyta* sp. B1, *Chlamydomonas* sp. BS3 and *Oscillatoria* sp. BN35 were isolated from Meiliang Bay of lake Taihu (Tian *et al*., 2012; Li *et al*., 2014). *Chroococcus* sp. FACHB-191, *Microcystis viridis* FACHB-979 and *Microcystis aeruginosa* PCC7806 were purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB-collection), Chinese Academy of Science, China. *Chlorophyta* sp. B1 and *Chlamydomonas* sp. BS3 are algae, others are cyanobacterium. All of the above-mentioned cyanobacterial and algal strains used in this study were maintained in sterilized BG11 medium (Li *et al*., 2014) and incubated at 25°C under 40 μmol photons $(m^2 \cdot s)^{-1}$ and a 12 h:12 h (light:dark) cycle (Tian *et al*., 2012).

Bacterial culture conditions

All the bacterial strains used in study were listed in Table 2. All *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium, unless otherwise indicated. *Chromobacterium violaceum* CV026 and VIR24 were grown at 28°C in LB medium. *Aeromonas* sp. strain GLY-2107 and its mutants were grown at 28°C in beef extract peptone (BEP) medium (peptone 10 g l^{-1}, NaCl 5 g l^{-1} and beef extract 3 g l^{-1}), unless otherwise indicated. When necessary, the appropriate antibiotics were added into the medium at the following concentrations: ampicillin, 100 mg l^{-1}; chloramphenicol, 30 mg l^{-1}; gentamycin, 25 mg l^{-1}; and kanamycin, 50 mg l^{-1}.

Chemicals

Standard *N*-acyl homoserine lactones were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Taq DNA polymerase, all restriction endonucleases and T4-DNA DNA ligase were purchased from Takara (Dalian, China). The chemicals used for chemical analysis and all culture media preparations, as well as the standard 3-benzyl-piperazine-2,5-dione and 3-methylindole used for the dose response bioassays on *M. aeruginosa* 9110 were purchased from Sigma (St. Louis, MO, USA), if not specifically indicated.

Calculation of the algicidal activity and percent survival

The calculations of the algicidal activity (A, %) and percent survival were based on the following equations (Tian *et al*., 2012; Li *et al*., 2014): $A = (1 - D_{t\text{-treatment}}/D_{t\text{-control}}) \times 100$ and percent survival $= D_{t\text{-treatment}}/D_{t\text{-control}} \times 100$, where $D_{t\text{-treatment}}$ and $D_{t\text{-control}}$ represent the cyanobacterial cell density in the treatment and control, respectively, and t (day or hour) is the inoculation time. The determination of the cell density of *M. aeruginosa* (cells ml^{-1}) was performed using a hemocytometer under a light microscope (BH-2, Olympus, Japan; Magnification: ×400). For sample with a cell density ranging from 5×10^6 to 1×10^7 cells ml^{-1}, cells were counted in the four corner squares and the centre square in the central grid of a hemocytometer, and the number of the counted cells ranged from 100 to 200. When the cell density in the sample was above 1×10^7 cells ml^{-1}, the sample was diluted to a cell density ranging from 5×10^6 to 1×10^7 cells ml^{-1}. For samples containing $< 5 \times 10^6$ cells ml^{-1}, cells were counted in all 25 squares of the central grid. Three technical replicates were counted to ensure accurate representation of density. As for other cyanobacterial species, the concentrations (mg l^{-1}) of chlorophyll *a* (chl *a*) as the equivalent cell densities were measured spectrophotometrically. A 100-ml volume of cyanobacterial or algal culture was filtered through 0.2-μm-nominal-pore-size polycarbonate membrane filter (25-mm diameter; Millipore). The filter was ground in 6-ml chilled 90% acetone, and then extracted, at 4°C, for 24 h in the dark. The homogenate was centrifuged for 10 min at 12 000 × *g* and 4°C. The supernatant was brought to 10 ml in a volumetric flask with 90% acetone, generating the extraction for further spectrophotometric analysis. Chl *a* concentration ($C_{Chl\ a}$) in the tested culture was calculated following the equation (Lorenzen, 1967; Carrere *et al*., 2004): $C_{Chl\ a}$ (μg l^{-1}) $= [11.64\ (OD_{663} - OD_{750}) - 2.16(OD_{645} - OD_{750}) + 0.1\ (OD_{630} - OD_{750})]\ V_e/V_f\delta$, where OD_λ = optical density at wavelength λ(nm), V_e (ml) = volume of extraction, V_f (l) = volume of culture filtered, and δ (cm) = path length of cuvette (here, $\delta = 1$ cm).

Isolation, screening, and identification of algicidal bacteria producing AHL(s)

The water samples used for the isolation of algicidal bacteria were collected using a Ruttner Standard Water Sampler, at the Taihu Ecosystem Research Station (31°24′N, 120°13′E) in hypertrophic Meiliang Bay located in the northeast part of Lake Taihu in October 2012. The sampled water was immediately transferred into sterile bottles and subsequently transported to the laboratory in a mini-icebox. The isolation, screening and identification of algicidal bacteria from the water samples were performed according to the method provided in the Supporting Information Methods S1. After the algicidal strains against *M. aeruginosa* 9110 ($A \geq 90\%$, $t = 6$ days) were isolated, the algicidal strains producing AHL(s) were screened using the AHL biosensor *C. violaceum* CV026 from the obtained algicidal strains. The *C. violaceum* CV026 was firstly streaked into "L" shape on the LB agar (1.5%, wt/vol) plate; then, the tested algicidal strains were parallelly streaked along one side of the L-shape streak of *C. violaceum* CV026. After 24 h of cultivation at 28°C, it was observed whether the purple pigment was produced by the *C. violaceum* CV026. Strain GLY-2107 showed the strongest algicidal activity among the strains producing AHL(s). Thus, strain GLY-2107 was selected for the further research. The 16S rRNA gene sequence data of the strain GLY-2107 has been submitted to the GenBank database under accession number KP717443. Furthermore, the strain has been deposited in the China General Microbiological Collection Center (CGMCC) under accession number CGMCC-8979.

The algicidal effect of Aeromonas sp. strain GLY-2107on target species

The investigation of algicidal effect of strain GLY-2107 was performed according to the procedure described by Tian and colleagues (2012) with some modifications. The target species included *Microcystis aeruginosa* 9110, *Synechococcus* sp. BN60, *Chlorophyta* sp. B1, *Chlamydomonas* sp. BS3, *Oscillatoria* sp. BN35, *Chroococcus* sp. FACHB-191, *Microcystis viridis* FACHB-979 and *Microcystis aeruginosa* PCC7806. Strain GLY-2107 was grown in the sterile BEP medium for 24 h at 28°C and 220 rpm. Bacterial cells were then harvested by centrifugation at 3000 × *g* and 25°C for 10 min, and the pellets were washed twice with sterile BG11 medium and re-suspended in sterile fresh BEP medium to generate a 1000-fold dilution of bacterial suspension. Subsequently, 1-ml bacterial suspensions were added to 99-ml exponential-phase tested cyanobacterial or algal cultures (Table 1) and incubated at 25°C under cyanobacterial growth conditions. In the cocultures, initial cell concentration of strain GLY-2107 was approximately 2.0×10^4 CFU ml^{-1}. Meanwhile, 1-ml BEP medium was added to the 99-ml tested cyanobacterial or algal cultures, acting as the corresponding control. During the investigation of algicidal activities of strain GLY-2107 on different cyanobacterial or algal species, the same batch of bacterial cell suspension was used. The algicidal activities were measured, after 6 days of cocultivation.

Investigation of algicidal mode of strain GLY-2107 against Microcystis aeruginosa 9110

The investigation of algicidal mode against *M. aeruginosa* 9110 was performed according to the procedure described by Lin and colleagues (2014) with some modifications. The culture of strain GLY-2107 (grown at 28°C and 220 rpm, for 24 h, in BEP medium) was centrifuged for 20 min at 12 000 × *g* and 25°C, and the supernatant was subsequently filtered using a sterile 0.22 μm polycarbonate filter to yield the cell-free filtrate. The preparation of heat-treated cell-free filtrate was performed through autoclaving at 121°C for 20 min. The procedure for preparation of bacterial cells re-suspended in sterile BG11 medium were as follows: the bacterial cells were pelleted by centrifugation for 10 min at 3000 × *g* and 25°C, washed twice with sterile BG11 medium, and then re-suspended in an equal amount of sterile BG11 medium. An aliquot of 1 ml of bacterial cultures, washed bacterial cells, cell-free filtrates, and heat-treated cell-free filtrates was added to 99-ml exponential-phase *M. aeruginosa* 9110 cultures, respectively, and then incubated at 25°C under cyanobacterial growth conditions. As a control, 1-ml BEP medium was added to 99-ml *M. aeruginosa* 9110 culture. After 6 days exposure, the algicidal activities in various treatments were measured. The bacterial cultures, washed bacterial cells, cell-free filtrate and heat-

treated cell-free filtrate used for the investigation of algicidal mode of strain GLY-2107 against *M. aeruginosa* 9110 were from the same batch of bacterial culture.

Separation and purification of bacterial algicidal compounds

The separation and purification of algicidal compounds was completed using the method described in our previous work (Li *et al.*, 2014) with some modifications. During every chromatographic separation step, effluent fractions were collected according to the peak shape, and the effective peak was obtained by estimating the algicidal activity of each effluent fraction. When the peak shape was not good at an effluent time, the eluent was collected every half an hour for silica-gel column chromatography or every two minutes for high performance liquid chromatography (HPLC). In the whole isolation process of algicidal compounds, all effluent fractions of each step were collected, evaporated, and re-dissolved in either methyl alcohol (for further purification and identification) or 0.2% DMSO aqueous solution (for detection of algicidal activity). Subsequently, algicidal activities of the solutions of residues were monitored with the cyanobacterial-lawn method (Tian *et al.*, 2012; Li *et al.*, 2014). The cyanobacterial lawn was prepared as described by Li and colleagues (2014), and incubated under the cyanobacterial growth conditions.

In general, the process of separation and purification of algicidal compounds mainly included four steps as follows.

First, strain GLY-2107 was grown in BEP liquid medium for 24 hours at 28°C and 220 rpm, and the culture was then centrifuged at 12 000 × g for 20 min to gather the supernatant. After the supernatant was extracted three times with an equal volume of ethyl acetate, the organic phase was pooled, and evaporated to dryness under reduced pressure at 30°C. Subsequently, the residue was re-dissolved in the methyl alcohol and filtered with a 0.22 μm membrane filter.

Second, the filtrate was separated using silica-gel column chromatography (commercial silica gel, Qingdao Haiyang Chemical Group Co., 200–300 mesh; 1 × 50 cm; UV detection at 254 nm). The column was eluted with methanol/chloroform (50:50, vol/vol) at a flow rate of 1 ml min^{-1}. Algicidal effect of each effluent fraction was determined using the cyanobacterial-lawn method.

Third, the algicidal effluent fraction from silica-gel column was applied to a semi-preparative C18 reverse-phase column (Supersil^{TM} C18-EP, 5 μm, 10.0 × 250 mm, Dikma, China) and chromatographed on a HPLC system (1260 Infinity, Agilent, USA) using UV-Vis detector (G1314F, Agilent, USA) at 210 nm. The column was eluted with a linear gradient of methanol/water (5%–100%, vol/vol) for 60 min at a flow rate of 4 ml min^{-1}. Algicidal effect of each effluent fraction was estimated with the cyanobacterial-lawn method.

Finally, the effective fractions with algicidal activities obtained from semi-preparative HPLC column were further separated and purified on a further-purification HPLC column (Supersil^{TM} C18-EP, 5 μm, 4.6 mm × 250 mm, Dikma, China; flow rate: 1 ml min^{-1}) using UV-Vis detector (G1314F, Agilent, USA) at 210 nm. After several rounds of further-purification HPLC, the purified algicidal substances were collected for the elucidation of molecular structure.

Identification of bacterial algicidal substances, and quantitative assays of the algicidal substances produced by strain GLY-2107 and its mutants using UPLC-MS during the algicidal process

Identification of the algicidal substances and the quantitative assays of the algicidal substances in the co-cultures were performed according to the experimental procedures described in our previous work (Li *et al.*, 2014) with some modifications as provided in the Supporting Information Methods S1.

Dose-response bioassays of cyclo(Gly-Phe) and 3-methylindole against M. aeruginosa 9110

To investigate the efficiency of cyclo(Gly-Phe) and 3-methylindole, the dose-response bioassays were conducted using an initial cyanobacterial density of 1×10^7 cells ml^{-1} (cells in logarithmic growth) according to the method described in our previous study (Li *et al.*, 2014), with slight modifications. Stock solutions of standard cyclo(Gly-Phe) and 3-methylindole were prepared by dissolving them in dimethyl sulfoxide (DMSO). An aliquot (20 μl) of each stock solution (0.05, 0.10, 0.20, 0.30, 0.40, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 2.5, 5.0, 10.0, 15.0, 20.0 or 25.0 mg ml^{-1}) of standard cyclo(Gly-Phe) or 3-methylindole was added into 9.98 ml of *M. aeruginosa* 9110 culture to provide a final concentration of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2, 2.5, 3, 4, 5, 10, 20, 30, 40 or 50 μg ml^{-1}. For the control, an equal amount of DMSO was added instead of the solution of the standard algicidal compound. The growth of *M. aeruginosa* was not influenced by DMSO below a concentration of 0.3% (vol/vol) (Yi *et al.*, 2012). The relevant percent survival was determined after 24 h of incubation under cyanobacterial growth conditions. The EC_{50} values were obtained from the corresponding dose-response curves by probit analysis (Zhang *et al.*, 2013).

AHL profiles analysis

The culture of *Aeromonas* sp. strain GLY-2107 or a mutant (grown in BEP medium at 28°C and 220 rpm for 24 h) was centrifuged at 25°C and 12 000 × *g* for 10 min, and the supernatant was subsequently filtered using a sterile 0.22 μm polycarbonate filter to yield a cell-free spent supernatant for the AHL profiles analysis. The spent supernatant was extracted three times with an equal volume of ethyl acetate. Then, the organic phase was separated, and dried under reduced pressure in a rotary evaporator at 30°C. After the solvent completely evaporated, the residue was dissolved in methanol for further analyses, including paper disc diffusion assay, thin layer chromatography (TLC) and tandem MS (MS/MS), and then, the extracts were concentrated 2000-fold. TLC analysis was performed according to the methods described by Shaw and colleagues (1997) with slight modifications. 2.5 μl (detection using *Chromobacterium violaceum* CV026) or 50.0 μl (detection using *Chromobacterium violaceum* VIR24) of extract was loaded onto a TLC plate (20 × 20 cm TLC aluminum sheets; RP-

Table 3. Plasmids used in this study.

Plasmids	Relevant characteristics[a]	Source or reference
pMD18-T	*bla* TA cloning vector, Amp^R	TaKaRa
pMD18-T-*agyI*	A PCR amplified fragment containing whole length of *agyI* gene from *Aeromonas* sp. strain GLY-2107 genome was cloned into pMD18-T, Amp^R	This study
pMD18-T-*agyR*	A PCR amplified fragment containing whole length of *agyR* gene from *Aeromonas* sp. strain GLY-2107 genome was cloned into pMD18-T, Amp^R	This study
pMD-T-m*agyI*	A in-frame deletion mutant allele of *agyI* gene was cloned into pMD18-T, Amp^R	This study
pMD- T-m*agyR*	A in-frame deletion mutant allele of *agyR* gene was cloned into pMD18-T, Amp^R	This study
pDM4	Suicide plasmid for gene knockout, Cm^R	(Milton *et al.*, 1996)
pDM-m*agyI*	A in-frame deletion mutant allele of *agyI* gene confirmed by sequencing from pMD-m*agyI* as SphI/XbaI fragment was subcloned into pDM4, Cm^R	This study
pDM-m*agyR*	A in-frame deletion mutant allele of *agyR* gene confirmed by sequencing from pMD-m*agyR* as SphI/XbaI fragment was subcloned into pDM4, Cm^R	This study
pBBR1MCS-5	Broad-host-range vector for gene complementation, Gm^R, $P_{(lac)}$	(Kovach *et al.*, 1995)
pBBR-sup-*agyR*	A PCR amplified fragment containing whole length of *agyR* from pMD18-T-*agyR* was cloned into pBBR1MCS-5, Gm^R	This study

a. Amp^R: ampicillin resistance; Cm^R: chloramphenicol resistance; Gm^R: gentamycin resistance.

18 F254 S) (Merck, Germany) which was then developed with a methanol/water (60:40 vol/vol) mobile phase. The TLC plates were subsequently air-dried, overlaid with a 100-ml LB agar seed with 10 ml of overnight culture of the biosensor *C. violaceum* CV026 or *C. violaceum* VIR24, and incubated at 28°C for 24 h. The AHL biosensors VIR24 (Someya *et al.*, 2009) and CV026 (McClean *et al.*, 1997) are *cviI* mutants of *Chromobacterium violaceum* ATCC 12472^T and ATCC 31532, respectively, and produce the purple pigment violacein mainly in response to long-chain (C6–C14 side-chains, although weakly to C4) and short-chain (C4–C8) AHLs respectively.

The AHLs were identified by the high-resolution mass spectrum analysis using a Waters ACQUITY ultra performance liquid chromatography (UPLC) system equipped with a binary solvent delivery manager and a sample manager, coupled with a Waters Micromass quadrupole time of flight (Q-TOF) Premier Mass Spectrometer equipped with an electrospray interface (Waters Corporation, Milford, MA, USA). The analytes were separated on a C18 RP-column (ACQUITY BEH-C18 1.7 mm, 2.1 × 100 mm, Waters Co.) and eluted by a gradient mobile phase prepared from water containing 0.1% (vol/vol) formic acid (solution A) and acetonitrile (0.1% (vol/vol) formic acid) (solution B) with a flow rate of 0.4 ml min^{-1}. The gradient program was as follows: 5.0%–15.0% solution B for 0.5 min, 15.0%–40.0% solution B for 3.0 min, 40.0%–60.0% solution B for 1.5 min, 60.0%–85.0% solution B for 1.0 min and 85.0%–100% solution B for 1.0 min. Total ion chromatogram (TIC) and the mass spectrum of the selected ions were obtained in positive electrospray ionization mode. The capillary and cone voltages were set to 3.0 kV and 15 V respectively. The source temperature was 115°C and the desolvation temperature was 350°C with the desolvation gas flow rate of 600.0 l h^{-1}. The cone gas flow was 50.0 l h^{-1}. The data were collected between 50 and 1,000 *m/z* with an alternating collision energy, at 4.0 eV for precursor ion information generation and a collision profile from 5.0 to 20.0 eV for fragment ion information. The ion chromatograms of the AHLs with unmodified or modified (either keto or hydroxyl modifications at position 3) acyl chains (4 to 14 carbons in length) were extracted from TIC. If one of the abovementioned target ions was present in the TIC and shared the same chromatographic retention time with the corresponding authentic AHL standard, the MS/MS spectrum of the target ion was acquired and further confirmed by comparison with that of the corresponding authentic AHL standard. The mass spectra data were acquired and processed using the MassLynx v4.0 software (Waters Corporation, Milford, MA, USA). The synthetic AHLs used as standards included *N*-butyryl-, hexanoyl-, heptanoyl-, octanoyl-, decanoyl-, dodecanoyl- and tetradecanoyl-homoserine lactone (C4-, C6-, C7-, C8-, C10-, C12- and C14-HSL) and *N*−3-oxo-hexanoyl-, octanoyl-, decanoyl-, dodecanoyl- and tetradecanoyl-homoserine lactone (3-oxo-C6, C8-, C10-, C12- and C14-HSL).

Cloning of the agyI and agyR genes in Aeromonas sp. strain GLY-2107, construction of the agyI and agyR mutants of Aeromonas sp. strain GLY-2107, and construction of the complementation strain of the agyR mutant

The cloning of *agyI* (*Aeromonas* sp. strain GLY−2107 LuxI-type autoinducer synthase gene) *and agyR* (*Aeromonas* sp. strain GLY−2107 LuxR-type transcriptional regulator gene) genes encoding a LuxI-LuxR type QS system with an AHL synthase AgyI and a LuxR-type transcriptional regulator AgyR from *Aeromonas* sp. strain GLY-2107, construction of the *agyI* and *agyR* mutants of *Aeromonas* sp. strain GLY-2107, and construction of the complementation strain of the agyR mutant were completed according to the experimental procedures as provided in the Supporting Information Methods S1. The sequence data of *agyI* and *agyR* genes has been submitted to the GenBank database under accession numbers KT372887 and KT372888 respectively. All the plasmids and primers used in this study were listed in Table 3 and Supporting Information Table S1 respectively.

Investigation of the role of the agyI-agyR QS system in the algicidal process of Aeromonas sp. strain GLY-2107

The bacterial suspensions of the wild-type strain GLY-2107, *agyI* mutant (strain GLY-MI), *agyR* mutant (strain GLY-MR), complemented *agyR* mutant strain GLY-MR (pBBR-sup-*agyR*) and the corresponding control strain GLY-MR(pBBR1MCS-5) used in this section were prepared as follows: first, each strain was grown separately in sterile BEP medium for 24 h at 2°C and 220 rpm; then, the cells of each strain were harvested by centrifugation at 3000 × *g* and 25°C for 10 min, and the pellets were washed twice with sterile BG11 medium and re-suspended in sterile fresh BEP medium to generate a 1000-fold dilution of the bacterial suspension. Subsequently, an aliquot (1.0 ml) of each bacterial suspension was added to 99.0 ml of *M. aeruginosa* 9110 culture to yield an initial cyanobacterial density of 1.0×10^7 cells ml^{-1} and an initial bacterial cell density of 2.0×10^4 CFU ml^{-1}.

A complementation study of the *agyI* mutant (strain GLY-MI) was performed by exogenous addition of synthetic C4-HSL or C6-HSL with a working concentration of 10 μM, as described in the previous complementary studies on the AHL synthase gene mutants of *Aeromonas* spp. (Swift *et al.*, 1997; Lynch *et al.*, 2002). Stock solutions of the standard C4-HSL and C6-HSL used for the complementation study were prepared by dissolving them in sterile distilled BG11 medium (pH = 7.1). A 20.0-μl fresh stock solution (50 mM) of C4-HSL or C6-HSL and a 1.0-ml bacterial suspension of strain GLY-MI were added to 98.98 ml of *M. aeruginosa* 9110 culture to yield a working AHL concentration of 10 μM, an initial cyanobacterial density of 1.0×10^7 cells ml^{-1} and an initial bacterial cell density of 2.0×10^4 CFU ml^{-1}. Meanwhile, as a control, 1.0 ml of BEP medium was added to 99.0 ml of *M. aeruginosa* 9110 culture instead of the bacterial suspension.

Both the co-cultures and control were incubated at 25°C under cyanobacterial growth conditions. During the algicidal process, aliquots from both co-cultures and the control were sampled daily to determine the cell densities of *M. aeruginosa* 9110, the algicidal bacterial strain GLY-2107 and the derived mutants as well as the concentrations of the algicidal compounds and DOC.

Determination of DOC

After the sampled culture was centrifuged at 4000 × *g* and 25°C for 10 min, the supernatant was filtered through a 0.22-μm cellulose acetate membrane filter. Next, the concentration of DOC in the filtrate was measured by a TOC-500 total organic carbon analyzer (Shimadzu, Japan).

Data analysis

In this study, the data were generated from three replicates and are presented as the means ± standard deviation. One-way analysis of variance (ANOVA) was conducted with SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA). The comparisons between the means were performed using Duncan's Multiple Range Test. The probit analysis and regression analysis were performed with SPSS v 20.0 (IBM Corp., Armonk, NY, USA).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Methods S1. Isolation, screening and identification of algicidal bacteria. Determination of bacterial cell density. Identification of bacterial algicidal compounds, and quantitative analysis of algicidal compounds secreted from GLY-2107 and its derived mutants by UPLC-MS during the algicidal process. Cloning *agyI* and *agyR* genes in *Aeromonas* sp. strain GLY-2107, construction of *agyI* and *agyR* mutants of *Aeromonas* sp. strain GLY-2107, and construction of the complementation strain of the *agyR* mutant. Quantitative analysis of AHLs using UPLC-MS. AHL half-life experiment.

Fig. S1. Detection of *N*-acyl-homoserine lactones (AHLs) production by strain GLY-2107 with biosensor *Chromobacterium violaceum* CV026 (a) and transmission electron microscopic observation of the strain GLY-2107 after 24 h of incubation at 28°C (b).

Fig. S2. The neighbor-joining phylogenetic tree derived from the 16S rRNA gene sequences showing relationship of strain GLY-2107 (shaded) with all other described species of genus *Aeromonas*. GenBank sequence accession numbers are given in parentheses after species name. Numbers at nodes indicate bootstrap values $>$ 50% (percentages of 1000 replicates). The scale bar indicates 0.002 substitution per nucleotide position.

Fig. S3. Semi-preparative high performance liquid chromatography (HPLC) of an algicidal fraction from the silica gel column using a Supersil^{TM} C18-EP column (5 μm, 10.0 $\times$ 250 mm) and with a gradually increasing ratio methanol/water (5%-100%, vol/vol) as the mobile phase (a). Algicidal effect of fraction A (retention time (RT) = 18.5-20.5 min), B (RT = 43.7-46.0 min), and control (all other fractions) from the semi-preparative HPLC on a cyanobacterial-lawn,

respectively (b). The cyanobacterial-lawns were incubated at 25°C for 2 days under 40 μmol photons $(m^2 \cdot sec)^{-1}$ and a 12h: 12 h (light: dark) cycle, and RT means retention time.

Fig. S4. Further-purification HPLC of the fraction A (a) and fraction B (b) using a Supersil™ C18-EP column (5 μm, 4.6 mm × 250 mm). The mobile phase of further-purification HPLC of the fraction A was 100% water. The mobile phase of further-purification HPLC of the fraction B was a gradually increasing ratio methanol/water (40%-80% methanol). Arrows indicate the effective peaks of secondary active fractions 2107-A (RT = 93.0-105.0 min (a)) and 2107-B (RT = 37.5-40.0 min (b)) on the cyanobacterial-lawn. The cyanobacterial-lawns were incubated at 25°C for 2 days under 40 μmol photons $(m^2 \cdot sec)^{-1}$ and a 12h: 12 h (light: dark) cycle, and RT means retention time.

Fig. S5. Positive-mode ESI mass spectrum of 2107-A (a) and 2107-B (b) recorded on LC/MS spectrometers with a ZORBAX Extend-C18 column (Agilent Technologies HPLC 1290-MS 6230, USA). "Frag = 105.0 V" means the voltage on the fragmentor was 105.0 V.

Fig. S6. EI mass spectra of 2107-A (a), standard 3-Benzyl-piperazine-2,5-dione (b), 2107-B (d) and standard 3-methylindole (e) by GC/MS analysis and typical spectra of 3-Benzyl-piperazine-2,5-dione (c) and 3-methylindole (f) in the GC/MS library. The structure of 3-Benzyl-piperazine-2,5-dione **or** 3-methylindole is inserted in the corresponding typical mass spectrum from GC/MS library.

Fig. S7. ^{1}H-NMR (a) and ^{13}C NMR (b) spectra of 2107-A in DMSO-d_6 determined using an NMR spectrometer (600 MHz, Avance III, Bruker, Switzerland) at 298K.

Fig. S8. ^{1}H NMR (a) and ^{13}C NMR (b) spectra of 2107-B in $CDCl_3$ determined using an NMR spectrometer (600 MHz, Avance III, Bruker, Switzerland) at 298K.

Fig. S9. UPLC-ES-QTOF analysis of authentic AHLs standards and extractive from *Aeromonas* sp. strain GLY-2107 culture. Total ion current chromatogram of authentic AHLs standards (a); Extracted ion chromatograms with *m/z* 172.09 (b) and *m/z* 200.12 (c) from total ion current chromatography of extractive from *Aeromonas* sp. strain GLY-2107 culture.

Fig. S10. Confirmation of *agyI* and *agyR* mutants by PCR analysis (a and c) and sequencing (b and d). (a) Primers *agyI*-For and *agyI*-Rev were utilized, and the PCR products were separated in a 1.5% agarose gel. Lane M, DNA molecular weight marker; lane 1, PCR product from chromosomal DNA of wild type strain (stain GLY-2107); Lane 2: PCR product from chromosomal DNA of *agyI* mutant strain (stain GLY-MI). (b) Sequencing results of the sequence near the deletion mutation site in the mutant *agyI* gene of strain GLY-MI (up), and the sequence of *agyI* gene (indicated in black box) in wild type strain GLY-2107 (down). The region shaded gray was replaced by an EcoR I site (indicated in red box) in the mutant *agyI* gene of strain GLY-MI. (c) Primers *agyR*-For and *agyR*-Rev were utilized, and the PCR products were separated in a 1.5% agarose gel. Lane M, DNA molecular weight marker; lane 3, PCR product from chromosomal DNA of wild type strain (stain GLY-2107); Lane 4: PCR product from chromosomal DNA of *agyR* mutant strain (stain GLY-MR). (d) Sequencing results of the sequence near the deletion mutation site in the mutant *agyR* gene of strain GLY-MR (up), and the sequence of *agyR* gene (indicated in black box) in wild type strain GLY-2107 (down). The red arrow indicated the deletion mutation site, where the region shaded gray was deleted in the mutant *agyR* gene of strain GLY-MR.

Fig. S11. *Chromobacterium violaceum* CV026 paper disc diffusion assay. (a) addition of 5 μl methanol solution of extractive from 10 ml BEP medium to paper disc on the LB agar plate containing *Chromobacterium violaceum* CV026 as control; (b) addition of 5 μl methanol solution of extractive from 10 ml *Aeromonas* sp. strain GLY-2107 culture (220 rpm, 28°C, 24 h) to paper disc on the LB agar plate containing *C. violaceum* CV026; (c) addition of 5 μl methanol solution of extractive from 10 ml *agyR* mutant strain GLY-MR culture (220 rpm, 28°C, 24 h) to paper disc on the LB agar plate containing *C. violaceum* CV026; (d) addition of 5 μl methanol solution of extractive from 10 ml *agyI* mutant strain GLY-MI culture (220 rpm, 28°C, 24 h) to paper disc on the LB agar plate containing *C. violaceum* CV026.

Fig. S12. Changes in concentrations of C4-HSL and C6-HSL in the mixed medium (BEP medium: BG11 medium (pH=7.1) = 99: 1, vol/vol), during the six days of incubation at 25°C under 40 μmol photons $(m^2 \cdot sec)^{-1}$ and a 12h: 12 h (light: dark) cycle.

Fig. S13. During the algicidal process against *M. aeruginosa* 9110, dynamics of the concentrations of the algicidal compounds (3-methylindole (a) and cyclo(Gly-Phe) (b)) normalized to bacterial cell densities of *Aeromonas* sp. strain GLY-2107; *agyI* mutant in the presence or absence of 10 μM C4-HSL or C6-HSL; and *agyR* mutant with or without pBBR-sup-agyR plasmid. The vertical bar represents the standard deviation of triplicate samples. The values from the co-culture in presence of *agyI* mutant and 10 μM C4-HSL, which were denoted by *, were significantly ($P < 0.01$) different from those from other co-cultures at the same time point.

Fig. S14. Growth curves of *Aeromonas* sp. strain GLY-2107, *agyR* mutant strain GLY-MR, strain GLY-MR carrying either pBBR-sup-*agyR* or pBBR1MCS-5, *agyI* mutant strain GLY-MI, and strain GLY-MI in the presence of either 10 μM C4-HSL or 10 μM C6-HSL. The cultures were grown at 28°C and 220 rpm in Beef extract peptone medium. The vertical bar represents the standard deviation of triplicate samples.

Table S1. Oligonucleotide primers used in this study.