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Bacilysin from *Bacillus amyloliquefaciens* FZB42 Has Specific Bactericidal Activity against Harmful Algal Bloom Species

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Harmful algal blooms, caused by massive and exceptional overgrowth of microalgae and cyanobacteria, are a serious environmental problem worldwide. In the present study, we looked for *Bacillus* strains with sufficiently strong anticyanobacterial activity to be used as biocontrol agents. Among 24 strains, *Bacillus amyloliquefaciens* FZB42 showed the strongest bactericidal activity against *Microcystis aeruginosa*, with a kill rate of 98.78%. The synthesis of the anticyanobacterial substance did not depend on Sfp, an enzyme that catalyzes a necessary processing step in the nonribosomal synthesis of lipopeptides and polyketides, but was associated with the *aro* gene cluster that is involved in the synthesis of the *sfp*-independent antibiotic bacilysin. Disruption of *bacB*, the gene in the cluster responsible for synthesizing bacilysin, or supplementation with the antagonist *N*-acetylglucosamine abolished the inhibitory effect, but this was restored when bacilysin synthesis was complemented. Bacilysin caused apparent changes in the algal cell wall and cell organelle membranes, and this resulted in cell lysis. Meanwhile, there was downregulated expression of *glmS*, *psbA1*, *mcyB*, and *ftsZ*—genes involved in peptidoglycan synthesis, photosynthesis, microcystin synthesis, and cell division, respectively. In addition, bacilysin suppressed the growth of other harmful algal species. In summary, bacilysin produced by *B. amyloliquefaciens* FZB42 has anticyanobacterial activity and thus could be developed as a biocontrol agent to mitigate the effects of harmful algal blooms.

Eutrophication of surface waters has many undesirable effects and can lead to major water quality issues in freshwater and coastal systems (1). This phenomenon results in blooms of harmful algal species in freshwater lakes and brackish waters throughout the world. Moreover, the excessive growth of harmful algae, such as microalgae and cyanobacteria, often increases the production of inherent toxins such as microcystins and nodularins that cause acute poisonings of fish, birds, and mammals, including humans (2). For example, dogs died after they were exposed to a cyanobacterial bloom of *Microcystis aeruginosa* in Lake Amstelmeer (The Netherlands), and the concentration of microcystin in this lake was up to 5.27×10^3 μg g^{-1} of dry weight (3). In recent years, harmful algal blooms of eutrophic water in China have occurred frequently, including in Lake Taihu and Lake Chaohu, and *Microcystis* is thought to be the dominant bloom genera (4).

Many control techniques have been used to prevent and mitigate bloom problems, including yellow loess (5), clay (6), and chemical agents such as copper sulfate and hydrogen peroxide (7). However, each of the physical and chemical methods available to remediate eutrophic water is associated with certain disadvantages (8). Therefore, there is still a pressing need for environmentally friendly, cost-effective, and convenient bactericidal agents directed against cyanobacterial blooms in eutrophic lakes. Biological control agents such as bacteria, viruses, and protozoa are of particular interest (9).

A growing body of evidence suggests that some bacteria can inhibit the growth of red-tide algae effectively through direct or indirect attack. *Myxobacter* spp. (10), *Cytophaga* spp. (11, 12), and *Saprospira* spp. (13) can invade through cell walls into the interior of algal cells. Bacteria that act indirectly exert killing activity through the production of extracellular algicidal substances, such as the phenazine pigments and 1-methyl-β-carboline secreted by certain *Pseudomonas* spp. (14, 15), β-cyano-L-alanine from *Vibrio* spp. (16), and lactones produced by *Ruegeria pomeroyi* (17). Recent studies have demonstrated that *Bacillus* spp. can suppress the growth of harmful algal bloom species (9, 18, 19). In a previous study, Ahn et al. (18) revealed that the culture broth of *Bacillus subtilis* C1 containing 10 mg $liter^{-1}$ surfactin completely inhibited the growth of *M. aeruginosa*, although these researchers did not further isolate the surfactin from the culture broth or construct a mutant to verify its inhibitory activity. As such, the active compound(s) and mechanisms of action remain to be identified.

Some species from the genus *Bacillus*, such as *B. subtilis* and *B. amyloliquefaciens*, are plant-growth promoting bacteria (PGPR), and these species have been developed as biocontrol agents due to their ability to form heat- and desiccation-resistant spores. Several *Bacillus*-based commercial products are available, such as Quantum-400 (*B. subtilis* GB03), Serenade (*B. subtilis* QST713), and Rhizovital (*B. amyloliquefaciens* FZB42) (20, 21). *Bacillus* spp. produce a variety of bioactive metabolites that exert antagonistic actions against pathogens. Prominent classes of such compounds are the *sfp*-dependent lipopeptides and polyketides which need Sfp, a 4′-phosphopantetheinyl transferase to transfer 4′-phosphopantetheine from coenzyme A onto peptidyl carrier proteins in the nonribosomal peptide synthesis pathway (22). Lipopeptides consist of a lipid connected to a peptide, and these are largely

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amphiphilic membrane-active biosurfactants and also peptide antibiotics with mainly antifungal activity (23). Polyketides are biosynthesized through decarboxylative condensation, and these form a large family of secondary metabolites with antibacterial, immunosuppressive, antitumor, and other physiologically relevant bioactivities (24).

The rhizosphere-colonizing *B. amyloliquefaciens* FZB42 is an environmental strain that has the impressive ability to stimulate plant growth while suppressing the growth of plant-pathogenic organisms. Genome analysis reveals that *B. amyloliquefaciens* FZB42 harbors an array of giant gene clusters involved in ribosome-dependent and nonribosomal peptide synthesis (25). The non-ribosomally synthesized lipopeptides (e.g., surfactin, fengycin, and bacillomycin D) and polyketides (e.g., bacillaene, difficidin, and macrolactin) exhibit potent antifungal, hemolytic, and antibacterial activities (24–26). Meanwhile, the ribosomally synthesized peptide antibiotics plantazolicin A and B show moderate nematicidal activity (27, 28). In the present study, we report that *B. amyloliquefaciens* FZB42 displays high inhibitory activity against *M. aeruginosa*. We managed to identify the anticyanobacterial substance present in the culture filtrates and clarify the underlying mechanisms responsible for the specific bactericidal activity against harmful algal bloom species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Twenty-four *Bacillus* strains of different origins were used in a first round of screening. *B. amyloliquefaciens* FZB42^T was kindly supplied by R. Borriss (ABiTEP GmbH, Berlin, Germany). Other strains were isolated from soil samples in Tibet, China. *Escherichia coli* DH5α (TaKaRa Bio, Inc., Dalian, China) was used as the host for all plasmids. *S. aureus* ATCC 9144 (29) was used as the assay organism in bacilysin determinations. Luria broth (30) was used for growing *S. aureus* and *E. coli*. Landy medium (31) was used to ferment all *Bacillus* isolates and mutants. For general bacilysin production, *B. amyloliquefaciens* FZB42 and associated mutants were grown in Perry and Abraham (PA) medium (32). When required, antibiotics were added to the following final concentrations: ampicillin (Amp) at 100 μg ml^{-1}, chloramphenicol (Cm) at 5 μg ml^{-1}, erythromycin (Erm) at 10 μg ml^{-1}, and kanamycin (Km) at 5 μg ml^{-1}.

Cyanobacterial culture. *M. aeruginosa* NIES-843, *A. flos-aquae* FACHB-1040, *Nostoc* sp. strain FACHB-1135, and *Anabaena* sp. strain FACHB-1383 were purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (China). All cultures were grown at 25 ± 1°C under white fluorescent lamps (60 μM m^{-2} s^{-1}, 16:8 h light-dark cycle). The cyanobacteria were cultured in sterilized BG11 medium (33) and transferred once a week to ensure that the experiments were always conducted with cultures during exponential growth phase.

Transformation, DNA manipulation, and transposon mutagenesis of *B. amyloliquefaciens* FZB42. The isolation and manipulation of recombinant DNA were performed using standard techniques. *E. coli* and *B. amyloliquefaciens* were transformed as described by Sambrook and Russell (34) and Spizizen (35), respectively. The transposon mutagenesis library was constructed using pMarA and Southern blotting was used to analyze the insertion copies of the pMarA plasmid into the selected transposon mutants of *B. amyloliquefaciens* FZB42, as described previously (36). All enzymes used in the present study were purchased from TaKaRa Bio.

Generation of *B. amyloliquefaciens* FZB42 mutant and complementation strains. The mutant *B. amyloliquefaciens* strain FZB42Δ*bacB* (devoid of bacilysin production) was obtained as follows. About 700-bp genomic regions upstream and downstream of the *bacB* gene were amplified from *B. amyloliquefaciens* FZB42 chromosomal DNA, respectively. The two gel-purified double-stranded DNA fragments were linked by a Cm^r resistance cassette and then ligated into pMD-18. The linearized plasmid was integrated into the genome of *B. amyloliquefaciens* FZB42 by double-crossover recombination, yielding the knockout mutant *B. amyloliquefaciens* FZB42Δ*bacB*.

For complementation, the entire *bacB* gene and two homologous recombination arms obtained from the *amyE* gene were amplified from the chromosomal DNA of *B. amyloliquefaciens* FZB42. Fragments were linked with a Km^r resistance cassette by overlap PCR, and then the sequence was ligated into pMD-18. Finally, the vector was transformed into the *B. amyloliquefaciens* FZB42Δ*bacB* mutant and selected on solid LB agar medium supplemented with 5 μg ml^{-1} Cm and 50 μg ml^{-1} Kan. The specific primers used above are listed in Table 1.

Preparation and assay of bacilysin activity. To obtain the pure bacilysin, culture filtrates of *B. amyloliquefaciens* FZB42 in PA medium were extracted twice in ice-cold ethanol. These extracts were subjected to Dowex 50WX8-200 separation (Sigma, USA) on a column equilibrated with 50/50 ethanol-water and eluted (after washing with water) by the application of 4% aqueous ammonium hydroxide (32). The eluate was immediately lyophilized to dryness and resuspended in 1 ml of water for loading onto the high-performance liquid chromatography-mass spectrometry (HPLC-MS). The sample was injected onto a ZoRBX Eclipse XDB-C18 column at a flow rate of 1 ml min^{-1}. A gradient of solvent A (0.1% [vol/vol] HCOOH) and solvent B (CH_3CN) was prepared; 100% solvent B was reached after 10 min, and this was held for 2 min (26). The retention time of bacilysin was 4.087 min, as detected by the absorbance at 230 nm and the expected molecular mass of 271 Da (26, 32). The eluate at the corresponding retention time was collected and rerun three times as described above. After lyophilization of the eluates, we obtained 1.81 g liter^{-1} of pure bacilysin (Fig. 1). Bacilysin in culture broths was determined by the paper-disc agar diffusion assay, and the antibiotic activity was estimated as previously described (37). *N*-Acetylglucosamine (Sigma), a known specific antagonist of bacilysin/anticapsin activity (38), was used to verify bacilysin activity on bioassay plates.

Bactericidal activity. Bactericidal activity of *Bacillus* spp. on the cyanobacteria was investigated as previously reported (39). To obtain culture filtrates, the fermentation broth and PA culture medium of each strain was centrifuged at 12,000 × *g* for 20 min at 4°C and then filtered through 0.22-μm-pore-size Millipore membranes. Filtrates or bacilysin were inoculated into 30-ml algae cultures at the desired concentrations.

The chlorophyll *a* content was determined as previously described (40). The cyanobacterial cells incubated in the absence or presence of filtrates or bacilysin were harvested at the indicated times. Cell pellets were resuspended and extracted in 90% acetone for 24 h at 4°C. The samples were centrifuged at 10,000 × *g* for 10 min to remove cell debris, and then the chlorophyll *a* concentrations were determined by using the following equation: chlorophyll *a* concentration (μg liter^{-1}) = (11.47 × OD_{664}) − (0.40 × OD_{630}) (40), where OD_{664} is the optical density at 664 nm. The bactericidal activity was calculated by using the following equation: bactericidal activity (%) = (1 − *T*/*C*) ×100, where *T* (treatment) and *C* (control) are the chlorophyll *a* contents of *M. aeruginosa* with and without treatment, respectively (41). Small amounts of bacilysin were added into cyanobacterium cultures to obtain five concentration groups (1 to 10 mg liter^{-1}). The median effective concentration (EC_{50}) was calculated by the probit unit method using SPSS 16.0 software (39).

SEM and TEM studies. To investigate the changes in cell shape and ultrastructure of *M. aeruginosa*, cells treated with 15 mg liter^{-1} bacilysin for 2 h were centrifuged at 10,000 × *g* for 10 min before being washed twice with sodium phosphate buffer (50 mM, pH 7.2). The samples were then prefixed with 2.5% glutaraldehyde. For scanning electron microscopy (SEM) observation, samples were mounted on copper grids, sputter-coated with gold–palladium and examined with a Hitachi S-3000N scanning electron microscope (Hitachi, Tokyo, Japan). For transmission electron microscopy (TEM) observations, prefixed samples were washed three times with phosphate buffer, postfixed with 1% osmium tetroxide for 1 h, dehydrated in a graded series of ethanol solution, embedded in

TABLE 1 Oligonucleotide DNA primers used in this study

Function and primer	Sequence (5′-3′)	Purpose
Construction of mutants		
bacB1-F	CCTTGTTCCAATCGCTCAG	Construction of the site-directed mutant FZB42Δ*bacB*
bacB1-R	GTCGGAGATGTCACAAGAAA	Construction of the site-directed mutant FZB42Δ*bacB*
bacB2-F	AGAAAGCAGAACTTCCGTAT	Construction of the site-directed mutant FZB42Δ*bacB*
bacB2-R	CCTGAAGGGACAAGTAGTGAG	Construction of the site-directed mutant FZB42Δ*bacB*
amyE1-F	CCTCTTTACTGCCGTTATT	Complementation of the mutant FZB42Δ*bacB*
amyE1-R	ATGCCCGTAGTTAGAAGC	Complementation of the mutant FZB42Δ*bacB*
amyE2-F	ACAAGTTAGTCACATGGGTG	Complementation of the mutant FZB42Δ*bacB*
amyE2-R	TGCGGAAGATAACCATTCAAAC	Complementation of the mutant FZB42Δ*bacB*
bacB-F	GTCGGGAATGTCAATGCT	Complementation of the mutant FZB42Δ*bacB*
bacB-R	GTGACGACGTTGGAAGAT	Complementation of the mutant FZB42Δ*bacB*
Real-time PCR analysis		
16S-F	GGACGGGTGAGTAACGCGTA	Internal reference
16S-R	CCCATTGCGGAAAATTCCCC	Internal reference
glmS-F	TGTGCCTCCGATGTCAGT	Detection of the expression of *glmS*
glmS-R	ATGAAGTGACGATAACCCT	Detection of the expression of *glmS*
psbA1-F	GGTCAAGARGAAGAAACCTACAAT	Detection of the expression of *psbA*1
psbA1-R	GTTGAAACCGTTGAGGTTGAA	Detection of the expression of *psbA*1
mcyB-F	CCTACCGAGCGCTTGGG	Detection of the expression of *mcyB*
mcyB-R	GAAAATCCCCTAAAGATTCCTGAGT	Detection of the expression of *mcyB*
ftsZ-F	TCGCTGCTATTTCCTCGC	Detection of the expression of *ftsZ*
ftsZ-R	TGACTTCTCCCTGCATTTTCT	Detection of the expression of *ftsZ*

Epon 812, sectioned with an ultramicrotome (LKB-V, Sweden), and observed by using a Hitachi H-600 transmission electron microscope.

Real-time PCR analysis. For the determination of gene expression, *M. aeruginosa* was exposed to 4 mg liter^{-1} bacilysin for 1, 2, and 3 days. After incubation, 10 ml of algal culture was centrifuged at 10,000 × *g* for 10 min at 4°C to collect the algal cells. Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA was obtained by using reverse transcriptase (TaKaRa Bio) with oligo(T) primer. Real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa Bio) using a 7500 Fast Real-Time PCR detection system. The 16S rRNA gene was used as the internal reference for normalization. Primers for these genes are listed in Table 1.

Statistical analysis. Each experiment was conducted in at least three independent replications. The data were statistically evaluated by using analysis of variance, followed by a Fisher least significant difference test ($P \leq 0.05$) using the SPSS v16.0 software (SPSS, Chicago, IL).

RESULTS

Screening of *Bacillus* strains for anti-*M. aeruginosa* activity. Twenty-four strains of *Bacillus* spp. were tested in competitive mixed-culture experiments for the ability to suppress the growth of *M. aeruginosa*. The anticyanobacterial ability was evaluated by measuring the content of chlorophyll *a* at 7 days. As shown in Fig. 2, all *Bacillus* strains inhibited the growth of *M. aeruginosa* to various extents. *B. amyloliquefaciens* FZB42, *Bacillus* sp. strain YBWC43 and *B. amyloliquefaciens* DJFZ40 showed most potent activities, but it was *B. amyloliquefaciens* FZB42 that displayed the greatest anti-*M. aeruginosa* activity (killing rate of 98.78%). After treatment with *B. amyloliquefaciens* FZB42, the water quality was restored (Fig. 2). Thus, these data suggest that metabolites may exist in the culture filtrates that exert anticyanobacterial activities.

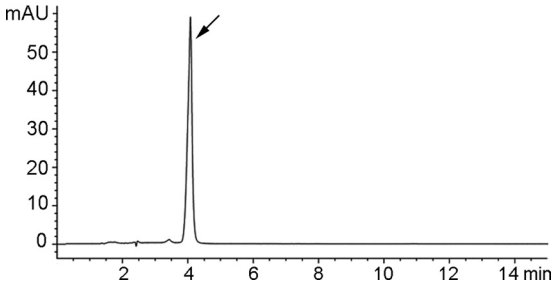

FIG 1 HPLC analysis of bacilysin from *B. amyloliquefaciens* FZB42. The retention time of bacilysin is 4.087 min.

The bactericidal substances against *M. aeruginosa* are Sfp independent. *B. amyloliquefaciens* FZB42 and other plant-associated strains of this species produce a broad spectrum of non-ribosomally synthesized antimicrobial lipopeptides and polyketides (25, 30). Sfp, a 4′-phosphopantetheinyl transferase, acts as the peptidyl carrier protein, and it is essential for the production of non-ribosomally synthesized lipopeptides and polyketides (22). Therefore, in order to confirm whether non-ribosomally synthesized lipopeptides and polyketides from *B. amyloliquefaciens* FZB42 were involved in suppressing the growth *M. aeruginosa*, we used a mutant deficient in the synthesis of Sfp (*B. amyloliquefaciens* CH03 [30]) to investigate whether this strain would suppress the growth of *M. aeruginosa*. At 7 days after the *M. aeruginosa* culture had been treated with *B. amyloliquefaciens* CH03 culture filtrate, the concentration of chlorophyll *a* was 245.8 μg liter^{-1}, which corresponded to an inhibitory effect of 94.39%. Thus, there was no difference in growth inhibition caused by *B. amyloliquefaciens* FZB42 and the CH03 strain. This indicates that nonribosomal lipopeptides and polyketides synthesized through the *sfp*-dependent pathway are not involved in the suppression of *M. aeruginosa* growth that and the anticyanobacterial effect of *B. amyloliquefaciens* FZB42 culture filtrate must be due to other metabolites (Fig. 3A and B).

Screening of mutant libraries and identification of anticyanobacterial related genes. To identify the anticyanobacterial agent,

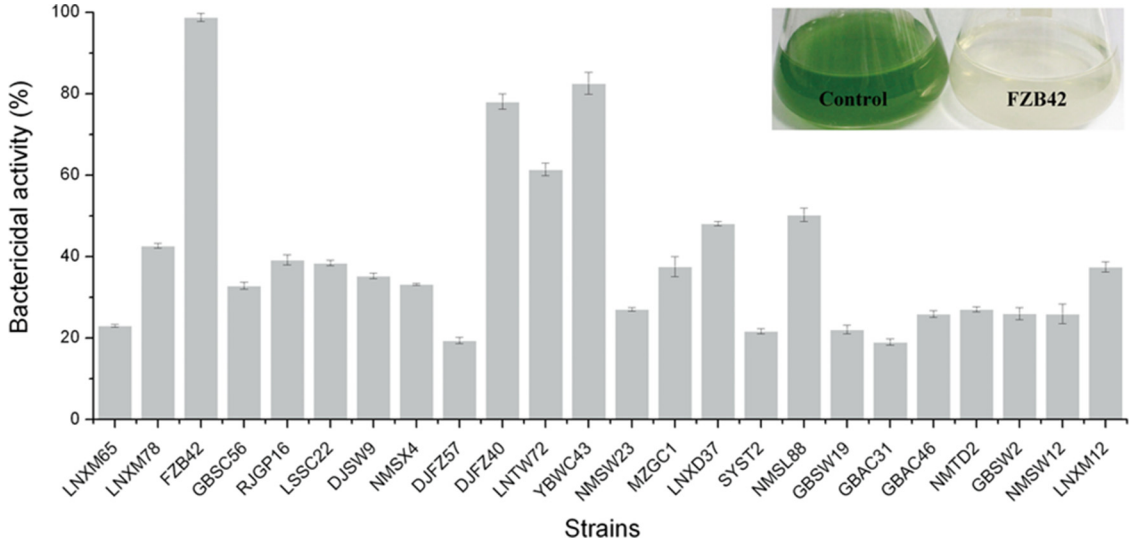

FIG 2 Suppressive activity of *Bacillus* strains against *M. aeruginosa*. The inset shows the effect of *B. amyloliquefaciens* FZB42 against an *M. aeruginosa* culture after 7 days (right). The control (left) is without *B. amyloliquefaciens* FZB42. The bactericidal activity was determined as described in Materials and Methods. All error bars represent the standard deviations.

2,000 TnYLB-1 transposon-inserted mutants were screened after *B. amyloliquefaciens* FZB42 was transformed with transposon-carrying pMarA. Two mutants, M436 (bactericidal activity 2.65%) and M1125 (bactericidal activity, 6.48%), were unable to inhibit the growth of *M. aeruginosa* (Fig. 3A and B). Insertion copy analysis by Southern blotting demonstrated that both mutants contained single insertions (data not shown). To identify the insertion sites, the inserted transposon and its flanking regions were cloned by inverse PCR and sequenced. Sequence analysis revealed that the *aroA* gene of M436 and *aroE* gene of M1125 had been disrupted by the TnYLB-1 transposon. The *aroA* gene encodes a bifunctional enzyme consisting of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase and chorismate mutase (42), while the *aroE* gene encodes 5-enolpyruvylshikimate-3-phosphate synthase (43, 44). Both genes are responsible for aromatic acid biosynthesis in many *Bacillus* spp. (42). All *aro* mutants are deficient in bacilysin biosynthesis, and it is known that the bacilysin pathway branches off the aromatic amino acid pathway at prephenate (29, 45, 46). Thus, we hypothesize that it is bacilysin produced by *B. amyloliquefaciens* FZB42 that has specific bactericidal activity against *M. aeruginosa*.

Construction of mutants and supplementation with an antagonist. Bacilysin, a dipeptide consisting of nonproteinogenic L-anticapsin and N-terminal L-alanine, is one of the simplest known peptide antibiotics, and it exhibits antifungal and antibacterial activities (38). In order to confirm the role of bacilysin in the anticyanobacterial activity, we constructed *B. amyloliquefaciens* FZB42Δ*bacB* that was deficient in bacilysin synthesis. *bacB*, the second gene in the bacilysin biosynthetic pathway, encodes an isomerase that catalyzes an allylic isomerization to generate a conjugated dienone (47–49). Furthermore, the *B. amyloliquefaciens* FZB42Δ*bacB* mutant was complemented with the entire *bacB* gene fused to the *amyE* gene. First, we investigated bacilysin production in the mutants and *Staphylococcus aureus* was used as an indicator strain because it is sensitive to this antibiotic (29). As shown in Fig. 3C, *B. amyloliquefaciens* FZB42Δ*bacB* did not cause an inhibition zone, and M436 and M1125 also showed no antagonistic action, thus indicating that all of these strains were devoid of bacilysin production. The level of bacilysin produced by the complemented transformant corresponded to that of the *B. amyloliquefaciens* FZB42 wild type. Second, we tested the anticyanobacterial activity of the mutants. Treatment of *M. aeruginosa* with the complemented transformant resulted in a bactericidal effect similar to that of the wild type, whereas *B. amyloliquefaciens* FZB42Δ*bacB* had no inhibitory effect since the bactericidal activity was just 1.45%, and the chlorophyll *a* content at 7 days was 4,756.5 μg liter^{-1}, which was not significantly different from the control (Fig. 3A and B). On the other hand, bacilysin obtained from *B. amyloliquefaciens* FZB42 wild-type culture filtrates showed inhibitory effects against *S. aureus* and *M. aeruginosa*, while supplementation with *N*-acetylglucosamine (10 mM), a known specific antagonist of bacilysin, abrogated the growth-inhibitory activities (Fig. 3). These data suggest that bacilysin produced by *B. amyloliquefaciens* FZB42 has specific bactericidal activity against *M. aeruginosa.*

Micro- and ultrastructural changes of *M. aeruginosa* caused by bacilysin. To determine the mechanism of inhibition of bacilysin, *M. aeruginosa* cells were examined before and after exposure by bacilysin by SEM. As shown in Fig. 4A and C, normal cells were intact, plump, and spherical with smooth exteriors; some cells showed segmentation. After treatment with bacilysin, the majority of cells were obviously depressed or distorted from their normal spherical shape and the integrity of cell wall was damaged (Fig. 4B and D). Moreover, TEM was used to observe changes at the ultrastructural level. In the absence of bacilysin, *M. aeruginosa* cells possessed an intact and very distinct cell wall and the space between the cell membrane and cell wall was uniform. The cytoplasm enveloped by the plasma membrane contained a large number of thylakoids with regularly scattered phycobilisomes, and there was a distinct nuclear area, vesicles, and other cell organelles (Fig. 4E). Cell damage caused by exposure to bacilysin is illustrated in Fig. 4F. In comparison to the untreated control, there was

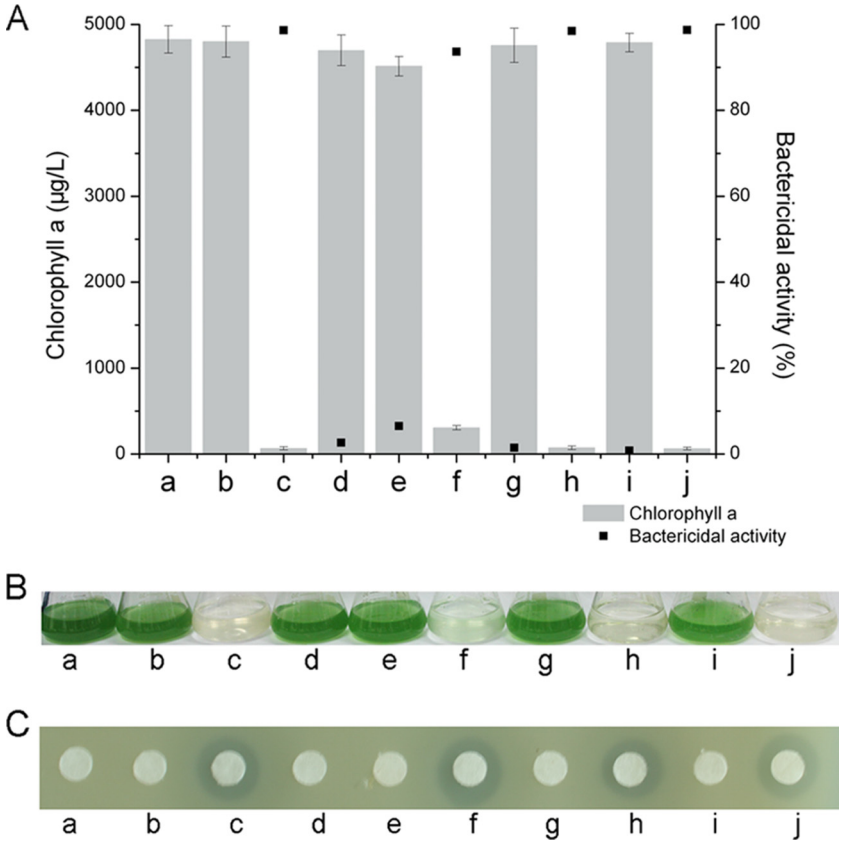

FIG 3 Detection of antagonistic action against *M. aeruginosa* (A and B) and *S. aureus* by paper disc agar diffusion assay (C). Columns: a, control; b, *N*-acetylglucosamine (10 mM); c, *B. amyloliquefaciens* FZB42; d, M436 (random mutant FZB42*aroA*::TnYLB-1); e, M1125 (random mutant FZB42*aroE*::TnYLB-1); f, CH03 (site-directed mutant FZB42Δ*sfp*); g, site-directed mutant FZB42Δ*bacB*; h, complemented FZB42Δ*bacB*Δ*amyE*::*bacB*; i, bacilysin supplemented with 10 mM *N*-acetylglucosamine; j, bacilysin. All error bars represent the standard deviations.

severe cell damage. The cell wall was partly ruptured, and the cytoplasm was condensed, resulting in slight plasmolysis. Furthermore, there were no thylakoids, and the cells had lost their basic structure.

Effect on *M. aeruginosa* gene expression after exposure to bacilysin. To explore the effects of bacilysin on cyanobacterial gene expression, we assessed the expression of *glmS*, *psbA1*, *mcyB*, and *ftsZ* in *M. aeruginosa* at 1, 2, and 3 days. *glmS* encodes L-glutamine: D-fructose-6-phosphate amidotransferase (known as glucosamine-6-phosphate synthase) that is important for the biosynthesis of peptidoglycan, a component of the bacterial cell wall (50). *psbA* encodes for the integral membrane protein D1 of photosystem II (51). *mcyB* encodes McyB, a protein involved in the synthesis of microcystins (52), while *ftsZ* encodes FtsZ, which is involved in cell division (53).

Real-time PCR analysis revealed that *glmS* showed a significant decrease in expression at all exposure times (35.3, 19.1, and 9.7% of the control values at day 1, 2, and 3, respectively). Meanwhile, transcript levels of *psbA1*, *mcyB*, and *ftsZ* were slightly downregulated after 1 day, while expression was reduced significantly after 2- and 3-day exposures (Fig. 5).

Bacilysin has potent bactericidal activity against other harmful algal species. To investigate whether bacilysin showed bactericidal activity against other harmful algal species, we examined effects against *Aphanizomenon flos-aquae*, *Nostoc* sp., and *Anabaena* sp. As shown in Table 2, bacilysin had potent growth inhibitory effects against each of these species. The chlorophyll *a* contents for treated cultures and controls at 7 days ranged from 103.5 to 591.3 μg liter^{-1} and 2.58 to 13.97%, respectively. The color of the cultures changed from blue-green to turbid white or pale green, and the bactericidal activity was >85%, with an EC_{50} of <5.0 mg liter^{-1}.

DISCUSSION

Harmful algal blooms have increased throughout the world, and these have caused serious problems in recent decades, such as the loss of aquaculture industries, environmental pollution, and damage to human health (54). Previous reports have demonstrated that many bacteria have a significant algicidal effect on several harmful algal bloom species (10, 11, 14, 17). Most algicidal bacteria isolated from the environment are characterized as belonging to the genera *Cytophaga*, *Saprospira*, *Pseudoalteromonas*, and *Alteromonas* (55). Recently, other studies have confirmed that some species of *Bacillus*, such as *B. subtilis* and *B. fusiformis*, can inhibit the growth of *M. aeruginosa* (18, 19). In the present study, we showed that 24 *Bacillus* strains exerted bactericidal activity against *M. aeruginosa*. Among these, *B. amyloliquefaciens* FZB42 exhibited the strongest suppressive effect, with a killing rate of 98.78%.

In *B. amyloliquefaciens* FZB42, numerous gene clusters are devoted to the nonribosomal synthesis of secondary metabolites that

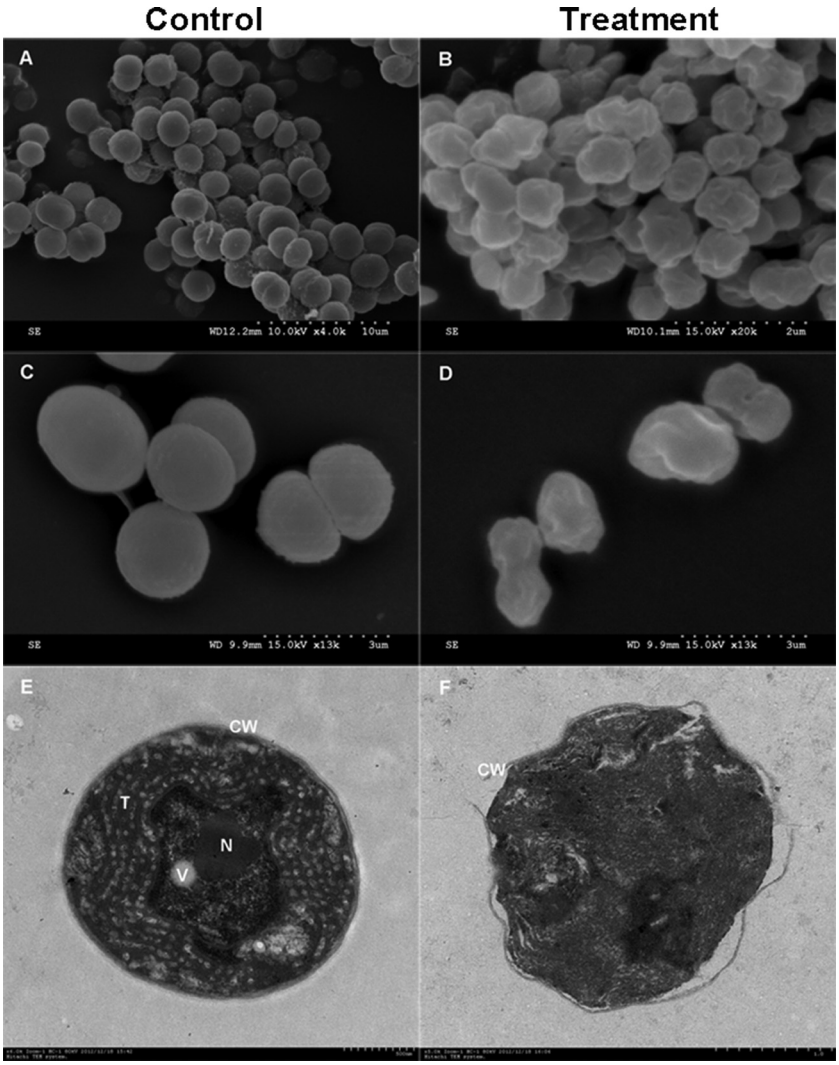

FIG 4 Micro- and ultrastructural changes of *M. aeruginosa* in the presence of 15 mg liter^{-1} bacilysin for 2 h. (A) Normal *M. aeruginosa* cells at 10 kV × 4.0 k; (B) damaged *M. aeruginosa* cells at 15 kV (magnification, 20,000×); (C) normal cells at 15 kV (magnification, 13,000×): (D) damaged cells at 15 kV (magnification, 13,000×); (E) a control *M. aeruginosa* cell at (magnification, 6,000×); (F) a damaged *M. aeruginosa* cell (magnification, 5,000×). CW, cell wall; N, nuclear area; T, thylakoid; V, gas vesicle.

are dependent on the Sfp phosphopantetheinyl transferase (25). Four types of these metabolites, surfactin and polyketides (difficidin, macrolactin, and bacillaene), are known for their antibacterial activity (24, 30). So, as a first step to determine whether these compounds were involved in the antagonistic effects on cyanobacteria, we used an Sfp mutant strain of *B. amyloliquefaciens* FZB42 (CH03), which is unable to synthesize antibacterial surfactin and polyketides. Surprisingly, the suppressive activity against *M. aeruginosa* was almost unaffected, suggesting that metabolites other than surfactin and polyketides were involved in the antagonistic activity. To further identify the substance, we prepared a mutant library and performed site-specific mutagenesis. The results demonstrated that bacilysin, produced independently of Sfp, was the bactericidal substance produced by *B. amyloliquefaciens* FZB42 that acted against harmful algal bloom species.

Bacilysin (L-alanyl-[2,3-epoxycyclohexanone-4]-L-alanine) is a dipeptide antibiotic that contains an L-alanine residue and the nonproteinogenic amino acid L-anticapsin. This nonribosomal dipeptide is synthesized by the *bacABCDEFG* gene cluster and generated independently of the Sfp pathway (32, 47–49). Many studies have demonstrated that bacilysin is active against a wide range of bacteria (38). Bacilysin, together with difficidin produced by plant-associated *B. amyloliquefaciens*, is efficient for controlling fire blight disease caused by *Erwinia amylovora* (26). Bacilysin also exhibits a certain antifungal activity against the yeast (38). Although bacilysin is antimicrobial, its inhibitory activity against harmful algal bloom species has not been reported previously. In the present study, we demonstrated that bacilysin significantly inhibited the growth of *M. aeruginosa* with an EC_{50} of 4.13 mg liter^{-1}. Moreover, bacilysin was shown to exert specific bactericidal activity against *A. flos-aquae*, *Nostoc* sp., and *Anabaena* sp. Compared to other algicidal compounds, bacilysin exerts similar potency of anti-algal effects (Table 3). Thus, our results suggest that bacilysin not only acts as a bactericide but also possesses significant inhibitory effects against cyanobacteria and microalgae.

Numerous studies on the mode of action of bacilysin have

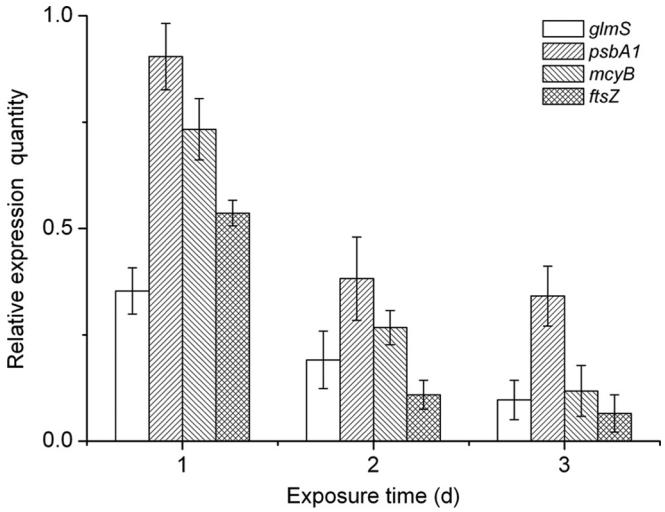

FIG 5 Real-time PCR analysis of expression of *glmS*, *psbA1*, *mcyB*, and *ftsZ* in *M. aeruginosa* cells in response to 4 mg liter^{-1} bacilysin for 1, 2 and 3 days. Values were normalized to the levels of the 16S rRNA gene, an internal reference gene. The *y* axis values represent the mean expression ± the standard deviations ($n = 3$) relative to the control.

demonstrated that its antibacterial activity depends on the anticapsin moiety, which is released by an intracellular peptidase (61) after bacilysin is taken up into susceptible cells by a distinct peptide permease system (62). Anticapsin behaves as a glutamine analogue, and reaction of its epoxide group with a thiol group of glucosamine synthase results in its covalent linkage to the enzyme, thus blocking its function and therefore bacterial peptidoglycan or fungal mannoprotein biosynthesis. This inhibition leads to cell protoplasting and lysis (49, 61, 63–65). In the present study, the mechanism of action of bacilysin against *M. aeruginosa* was clarified. We found that bacilysin produced by *B. amyloliquefaciens* FZB42 primarily affected the cell wall, as evidenced by microscopic and ultramicroscopic observations. The fast breakdown of the cell wall and plasma membrane led to increased cell permeability and the efflux of intracellular components (see Fig. S1 and S2 in the supplemental material). In addition, the transcript levels in *M. aeruginosa* of four target genes (*glmS*, *psbA1*, *mcyB*, and *ftsZ*) involved in the synthesis of peptidoglycan, the photosynthesis system, microcystins, and cell division were downregulated, which suggests that the metabolism of *M. aeruginosa* was significantly inhibited by bacilysin. A similar phenomenon was observed that ginkgolic acids extracted from *Ginkgo biloba* exocarp cause pleiotropic effects on *M. aeruginosa* such as destruction of the cellular structure, induction of oxidative damage, and reduced photosynthesis (58).

TABLE 2 Bactericidal activity of bacilysin against various harmful algalbloom species[a]

Algal species	Mean amt of chlorophyll *a* (μg liter^{-1}) ± SD at day 7		Bactericidal activity (%)	EC_{50} (mg liter^{-1})
	Control	Treatment		
Anabaena sp.	4,234.1 ± 154.8	591.3 ± 34.7	86.04	5.01
A. flos-aquae	4,012.4 ± 201.3	103.5 ± 15.3	97.43	4.29
Nostoc sp.	4,893.5 ± 161.7	229.4 ± 21.4	95.32	4.51

[a] Values are means from five independent experiments.

TABLE 3 Comparison of the activity of bacilysin to the activities of other algicidal compounds

Algicidal compound	Target species	Initial algae density	EC_{50} (mg liter^{-1})	Source or reference
Bacilysin	*M. aeruginosa*	10^6	4.13	This study
Artemisinin	*M. aeruginosa*	10^6	3.2	39
Sinapic acid	*M. aeruginosa*	10^4–10^5	4.9	56
Copper sulfate	*M. aeruginosa*	10^6	0.3	57
Ginkgolic acids	*M. aeruginosa*	10^7	2.03	58
Rhamnolipid	*P. dentatum*	10^4–10^5	5.00	59
HPA3	*P. minimum*	10^4	16 μM	60

In conclusion, the present study provides direct evidence for the remarkable anticyanobacterial effects of bacilysin produced by *B. amyloliquefaciens* FZB42 and the mechanism by which this compound acts against algal cell walls. These results might provide an alternative method for managing harmful algal blooms, although further research has to confirm whether bacilysin could be applied safely to eutrophic lakes and reservoirs.

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