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The Selective Inhibitory Activity of a Fusaricidin Derivative on a Bloom-Forming Cyanobacterium, *Microcystis* sp.

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Fusaricidin analogs, produced by *Paenibacillus polymyxa*, were tested for selective control of a major bloom-forming cyanobacterium, *Microcystis* sp. Fusaricidin (A and B mixtures) and four analogs were isolated from *P. polymyxa* E681 and investigated for their inhibition of cyanobacterial cell growth. Among the four fusaricidin analogs, fraction 915 Da (designated as Fus901) showed growth inhibition activity for *Microcystis aeruginosa* but not for *Anabaena variabilis* and *Scenedesmus acutus*. Microcystin concentration decreased up to 70% and its content per cell also decreased over 50% after 3 days. Fusaricidin exhibited growth inhibition against Gram-positive bacteria but Fus901 did not. Molecular weights of fusaricidin A and B were 883 Da and 897 Da, whereas that of Fus901 was 915 Da. Structure analysis by a ring-opening method revealed a linear form for Fus901. Expression of the *pod* gene related to oxidative stress was increased 2.1-fold by Fus901 and that of *mcyD* decreased up to 40%. These results indicate that Fus901 exerts oxidative stress against *M. aeruginosa*. Thus, Fus901 can be used as a selective cyanobactericide without disturbing the ecological system and could help in decreasing the microcystin concentration.

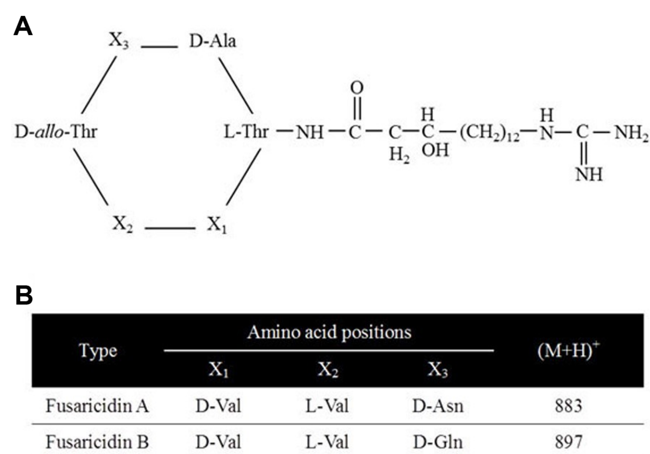
Keywords: Bloom control, cyanobacteria, fusaricidin, *Microcystis*, *Paenibacillus polymyxa*

Introduction

Freshwater blooms are generally formed by the uncontrolled growth of cyanobacteria, which can produce toxins such as microcystin (MC), nodularin, and anatoxin. MC is encoded by the *mcy* gene cluster [1]. The *mcyABCDEFGHIJ* genes are transcribed bidirectionally from a central promoter between *mcyA* and *mcyD*. Three peptide synthetases are encoded by *mcyABC*, *mcyD* encodes a modular polyketide synthase, *mcyE* and *mcyG* encode peptide synthetase and polyketide synthase, respectively, and *mcyJ*, *F* and *I* are putatively involved in tailoring, while *mcyH* is involved in toxin transport [2,3]. Polymerase chain reaction (PCR) amplification of the *mcyA*, *-B* and *-C* genes has been applied for the detection of potentially toxic *Microcystis* [4]. Two polyketide synthase modules of *mcyD* with another two polyketide synthases (*mcyE* and *mcyG*) are responsible for the synthesis of the unique Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid) that is used to detect MCs by the protein phosphatase inhibition (PPI) assay [3].

The toxicity of MCs, along with their environmental and economic impact, have triggered the development of methods for the treatment of these toxins [5]. Many chemical and physical methods, such as adsorption, chlorination, oxidation, and ozonation, have been applied to degrade stable MCs. However, until now, only a few biological methods have been reported.

Paenibacillus polymyxa is commonly found in many mineral deposits and the rhizosphere [6]. *P. polymyxa* strains produce two types of peptide antibiotics. One anti-bacterial group includes polymyxins and polypeptins. The other, which includes fusaricidin A, B, C, and D, is active against fungi and Gram-positive bacteria [7]. Fusaricidins have a ring structure composed of six amino acid residues in addition to 15-guanidino-3-hydroxypentadecanoic acid



Type	Amino acid positions			$(M+H)^+$
	X_1	X_2	X_3	
Fusaricidin A	D-Val	L-Val	D-Asn	883
Fusaricidin B	D-Val	L-Val	D-Gln	897

Fig. 1. (**A**) Primary structure of the fusaricidin-type lipopeptide antibiotics. X_1, X_2, and X_3 indicate three variable positions in fusaricidins. (**B**) Amino acid substitutions at three variable positions in previously reported fusaricidins and the molecular weights of the fusaricidins.

(GHPD). The general peptide sequence of the fusaricidins was determined to be L-Thr–X1–X2–D-*allo*-Thr–X3–D-Ala (Fig. 1A). A β-hydroxy fatty acid is attached to the N-terminal L-Thr via an amide linkage and the peptide is cyclized by an ester bond between the C-terminal D-Ala and the β-OH group of the N-terminal L-Thr. The antimicrobial activity of the fusaricidins varies depending on the amino acids at three variable positions (Fig. 1B) [8].

In this study, we report that one of the fusaricidin analogs has growth inhibition and can decrease intracellular MC content as well. The analog displayed no growth inhibition activity against Gram-positive bacteria. Simultaneously, structural analysis was also performed.

Materials and Methods

Strains and Culture Conditions

Anabaena variabilis NIES 23 and *Scenedesmus acutus* NIES 94 were obtained from the National Institute for Environmental Studies (NIES), Japan. *M. aeruginosa* KW was isolated from freshwater in Korea. The strains were grown in BG-11 liquid medium (pH 7.5) under 120 μmol Photons/m^2/s^1 provided by cool white fluorescent tubes at 27 ± 1°C. *Micrococcus luteus* (KCTC 1056), *Bacillus subtilis* (KCTC 1022), *Staphylococcus aureus* (KCTC 1621), *Staphylococcus aureus* (KCTC 1916), and *Escherichia coli* (KCTC 2443) were obtained from Korean Collection for Type Cultures (KCTC), and *Erwinia carotovora* was provided by Dr. Seung-Hwan Park (KRIBB, Korea). *P. polymyxa* E681, which was isolated from the roots of winter barley in the Republic of Korea, was cultured in Katznelson and Lochhead medium (KL medium) [9].

Purification of Fusaricidins and Its Analogs

P. polymyxa E681 was grown in 5 L of KL medium at 27°C until they reached the end of the stationary phase (24 h). The liquid culture medium was then centrifuged (12,000 ×*g*, 30 min), filtered through 0.2-μm filters and heated at 110°C for 10 min. The clarified culture medium was then applied to a CM-Trisacryl column (2 by 10 cm) equilibrated in 25 mM Tris-HCl (pH 8.5). A NaCl gradient of 100 ml (0 to 0.5 M) was used in the same buffer to elute the active components at a flow rate of 1 ml/min. A fraction (1 ml) was collected and tested for antagonistic activities. The active fractions were pooled, diluted three times with Milli-Q water, and applied to another CM-Trisacryl column (2 by 5 cm). Purified materials were eluted with 30 ml of 0.1 M NH_4OH at pH 11 and were concentrated and further purified with a C_{18} Sep-Pack cartridge (Waters, USA) by following the manufacturer's protocol. Active compounds were eluted from the cartridge in 100% methanol. The methanol was evaporated under reduced pressure and the antagonistic factor was obtained as a white powder that was soluble in methanol.

Mixtures of fusaricidin A and B (denoted as fusaricidin) and its four analogs were isolated from the methanol extract of *P. polymyxa* E681 by high-pressure liquid chromatography (HPLC). The methanol extract of the cell pellet was analyzed by LC/MS (Shimadzu, Japan) using a mixed solvent of water and acetonitrile containing 0.1% formic acid at a rate of 0.2 ml/min. To make the fusaricidin linearized, it was hydrolyzed by MeOH-H_2O-28% aqueous NH_3 (4:1:1, pH 9.0) or esterase for 24 h according to the method of Kuroda *et al.* [8].

Growth Inhibition Activity Assays

The growth inhibition activity of purified fusaricidin and its analogs was measured against Gram-positive and Gram-negative bacteria using the disk diffusion method. LB plates were spread with Gram-positive bacteria (*M. luteus* KCTC 1056, *B. subtilis* KCTC 1022, *S. aureus* KCTC 1621 and *S. aureus* KCTC 1916) and Gram-negative bacteria (*E. coli* KCTC 2443 and *E. carotovora*). Also, for polymyxin E activity, *P. polymyxa* E681 was cultured in KL medium at 30°C for 24 h. After centrifugation of the culture, the cell pellet was extracted with methanol. The growth inhibition of the mixture of cell extract and supernatant were measured using the disk diffusion method.

Chlorophyll-*a* and Cell Count

Isolated *M. aeruginosa* KW was cultured in BG-11 liquid medium (pH 7.5) under 120 μmol Photons/m^2/s^1 provided by cool white fluorescent tubes at 27 ± 1°C. The cell numbers of *M. aeruginosa* were counted using an optical microscope. Before counting, the cell suspensions of *M. aeruginosa* were briefly sonicated to disperse any aggregated cell clusters. To determine the chlorophyll-*a* fraction, samples were filtered using filter paper (Whatman GF/C), then extracted using chloroform-methanol (2:1 (v/v)) [10]. The concentration of chlorophyll-*a* was then measured by a Turner Quantech fluorometer (Barnstead/Thermolyne, USA) based on a

Table 1. Primers designed for real-time PCR.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
pod	GCCGTTTTCGATCAAGAGTT	GGATGGGATTGGACGTATTG
sodB	ACACACTTCCCCCTTTACCC	GCCGGTTTTGGTAACTTTGA
mcyD[a]	GGTTCGCCTGGTCAAAGTAA	CCTCGCTAAAGAAGGGTTGA
16S rRNA	GGACGGGTGAGTAACGCGTA[b]	CCCATTGCGGAAAATTCCCC[c]

[a]Kaebernick *et al*. (2000) [3]
[b]Urbach *et al*. (1992) [27]
[c]Nübel *et al*. (1997) [28]

predetermined standard curve programmed into the fluorometer. A blank sample of chloroform was measured and all the readings were readjusted with the blank sample reading.

MC Analysis

To determine the microcystin concentration by PPI assay, 5-ml sample aliquots were filtered through a Whatman GF/C filter. The microcystin in the filter papers was extracted with 5% acetic acid, purified with a Sep-Pak cartridge, and then diluted with 100% methanol. The PPI assay was performed as described by Ward *et al*. [11]. Briefly, the diluted protein phosphatase 1 (10 µl) was added to 25 µl of sample. After pre-incubation for 1 min, *p*-nitrophenol (100 µl) was added and measured after 22 min at 405 nm on a microplate reader (Sunrise, Tecan, USA). All enzyme assays were conducted in triplicate. Significant differences ($p < 0.05$) between the results were determined using a *t*-test.

RNA Extraction and Real-Time PCR

To study whether Fus901 could affect the expression of toxin-producing gene and antioxidant defense-related genes such as the peroxidase (*pod*) and superoxide dismutase (*sodB*) genes, qRT-PCR was carried out to determine the *mcyD*, *pod*, and *sodB* expression levels in Fus901-treated cells (BG11 media, 48 h incubation, final concentration 1 µg/ml) relative to the control cells and normalized with the expression of the reference gene, 16S rRNA, as described by Chini *et al*. [12].

Total RNA was isolated with the RiboPure-Bacteria Kit (Ambion, USA) according to the manufacturer's instructions and treated with DNase I at 30°C for 30 min. First-strand cDNA synthesis, using 0.5 µg of RNA as a template, was carried out in a total volume of 20 µl using the iScript cDNA Synthesis Kit (Bio-Rad, USA) according to the manufacturer's instructions. Any residual DNA was eliminated by incubating RNA preparations for 5 min at 42°C with the gDNA Wipeout Buffer (Qiagen, Germany) and the cDNA synthesis reaction was carried out at 42°C for 15 min in duplicate with or without iScript reverse transcriptase, followed by 3 min incubation at 95°C to inactivate the enzyme. PCR primers for the three genes are listed in Table 1. Real-time PCR was performed in 20 µl of a reaction mixture containing 10 µl of iTaq SYBR Green Supermix with ROX (Bio-Rad, USA), 1 µl (10 pmol/µl) each of forward and reverse primers, 1 µl cDNA and 7 µl distilled water. The amplification reactions were performed by a Chromo4 Four-Color Real-Time PCR Detector (Bio-Rad, USA) under the following conditions: one cycle at 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 61°C for 1 min, and 72°C for 20 sec [13].

A threshold cycle (C_T) value was determined for each amplification plot. C_T values were standardized to 16S rRNA values. The quantitative PCR results were represented as the fold-change in target gene expression. The expression ratio was calculated based on the formula of $2^{-\Delta\Delta C_T}$ [14].

Results

Growth Inhibition of Fusaricidin Analogs

To investigate the growth inhibition, the four analogs and fusaricidin were added to *M. aeruginosa* KW culture (initial cell density $2.37 \pm 0.15 \times 10^7$ cells/ml, $n = 3$) at 1 µg/ml and incubated at 27°C for 3 days. Fraction 915 among the

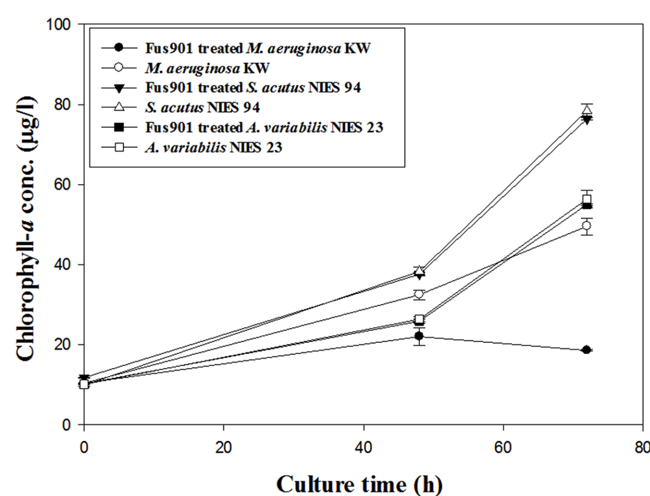


Fig. 2. The chlorophyll-*a* concentration of *A. variabilis* NIES 23, *S. acutus* NIES 94, and *M. aeruginosa* KW when Fus901 was added.

Controls are *A. variabilis* NIES 23, *S. acutus* NIES 94, and *M. aeruginosa* KW without Fus901 treatment.

four analogs showed potent growth inhibition against *M. aeruginosa* (data not shown). To investigate the specificity of fraction 915, another cyanobacterium, *A. variabilis* NIES 23 and a green alga, *S. acutus* NIES 94, were also treated. After 3 days, the growth of *A. variabilis* NIES 23 and *S. acutus* NIES 94 was not inhibited, but chlorophyll-*a* concentration of *M. aeruginosa* KW decreased from 49.6 μg/l to 18.6 μg/l (Fig. 2). Fraction 915 showing growth inhibition was designated as Fus901. Moreover, the Chlorophyll-*a* concentration of *M. aeruginosa* KW was also decreased when Fus901 was added in the middle of the growth phase (Fig. S3).

Decrease in MC Content by Fus901

After 3 days of cultivation, the intracellular MC concentration in Fus901-treated *M. aeruginosa* KW decreased up to 70%, while extracellular concentration showed no difference, when compared to control (Fig. 3A). These results indicated that Fus901 decreased MC content within *M. aeruginosa* cells. To confirm this, MC content per cell was determined after treatment with Fus901. The initial cell concentration of *M. aeruginosa* KW was 2.37 ± 0.15 $\times 10^7$ cells/ml and the cell concentrations of control and Fus901-treated samples after 72 h were 5.32 $\times 10^7$ and 2.77 $\times 10^7$ cells/ml, respectively. The MC content was 41.6 fg/cell in control, whereas that of Fus901-treated cells was 24.0 fg/cell (Fig. 3B).

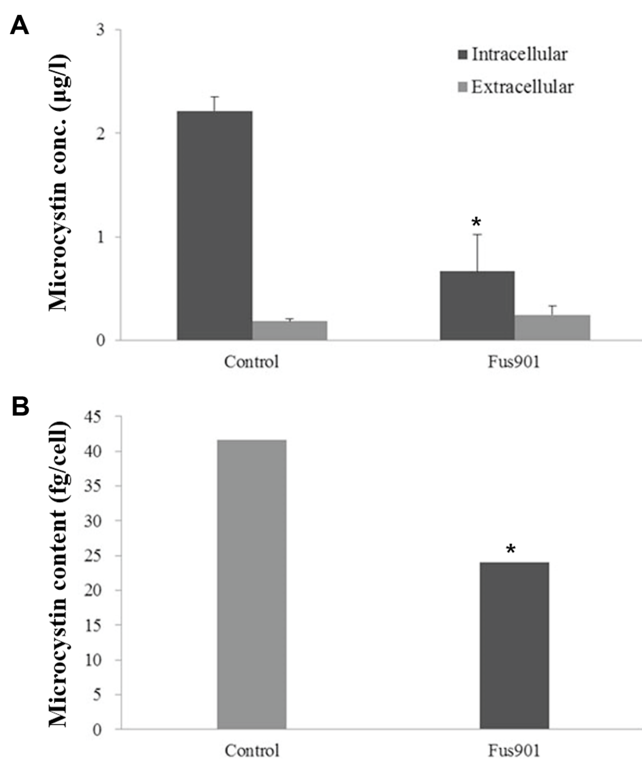


Fig. 3. Effect of Fus901 on MC production by *M. aeruginosa* KW.

(**A**) Comparison of total MC concentration between control and treatment. (**B**) Comparison of MC content per cell. Data are means ± SD ($n = 3$). *$p < 0.05$ indicate significant differences compared with the corresponding controls.

Growth Inhibition Activity of Fus901

Growth inhibition activity was tested against four Gram-positive bacteria (*M. luteus*, *B. subtilis*, and *Staphylococcus* sp. KCTC 1621 and 1916) and two Gram-negative bacteria (*E. coli* and *E. carotovora*). Various concentrations (0.625, 1.25, 2.5, 5.0, and 10 mg/ml) of Fus901 were tested to check its growth-inhibiting activity on the *M. luteus* (Fig. S4). Fusaricidin and Fus901 were tested at 1 mg/ml concentration and crude extract (20 mg/ml) of polymyxin E produced by *P. polymyxa* E681 was also used. Polymyxin E is known to have strong growth inhibition activity against Gram-negative bacteria [15]. Whereas fusaricidin showed strong growth inhibition activity on the tested Gram-positive bacteria, Fus901 completely lost its growth inhibition activity against the Gram-positive bacteria (Fig. 4A). Similar to fusaricidin, Fus901 showed no growth inhibition activity on *E. coli* or *E. carotovora* (Fig. 4B).

Structure Analysis of Fus901

Fus901 and fusaricidin linearized by a ring-opening method were analyzed by LC/MS/MS. The ring-opening

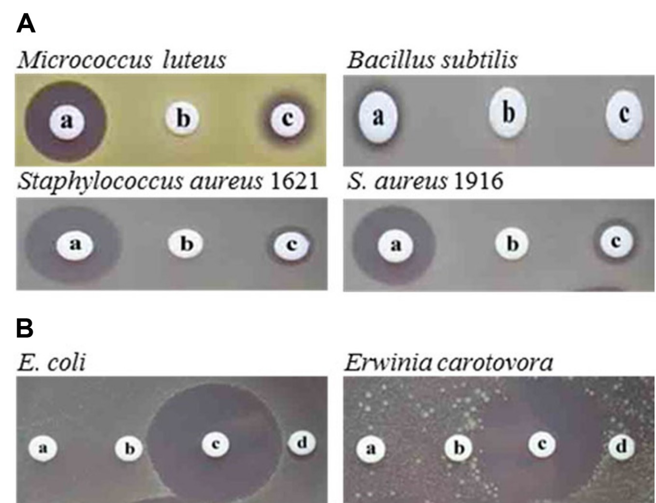


Fig. 4. Growth inhibition activity of Fus901 compared to the activity of intact fusaricidin.

(**A**) Growth inhibition activity against Gram-positive bacteria, (**B**) Gram-negative bacteria. a, fusaricidin 1 mg/ml; b, Fus901 1 mg/ml; (A)-c and (B)-d, methanol; (B)-c, crude extract (20 mg/ml) of polymyxin E produced by *P. polymyxa* E681.

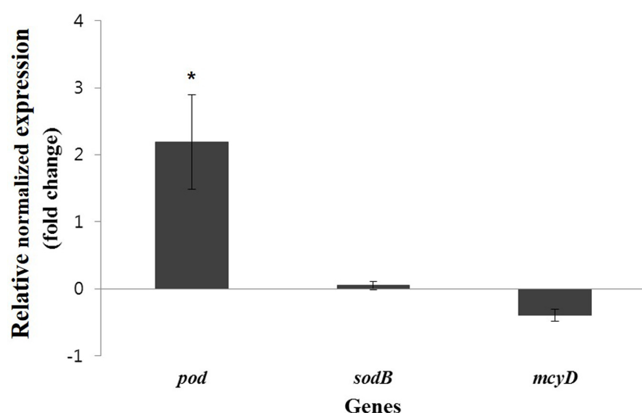


Fig. 5. Level of *mcyD*, *pod*, and *sodB* mRNA expression in *M. aeruginosa* KW as a response to Fus901 by real-time PCR.
The error bar is the mean ± SD (*$p < 0.05$).

method was carried out by addition of NaOH and esterase. The methanol extract of the Fus901 was analyzed by LC/MS/MS. The $(M+H)^+$ ion peak of Fus901 was 915 at a retention time of 8.23 min (Fig. S1A), while those of fusaricidin were 883 and 897 at retention times of 10.04 min and 10.42 min, respectively (Figs. S1B and S1C). Like Fus901, the $(M+H)^+$ ion peak of ring-opened fusaricidin by treatment of esterase and NaOH was 915 with retention times of 10.20 min and 10.70 min (Figs. S1B and S1C). From the LC/MS/MS results, Fus901 was identified as a linearized form with a broken ester bond (Fig. S2).

Effect of Fus901 on *mcyD* Transcription and Antioxidant Defenses

A modular polyketide synthase is encoded by *mcyD*, involved in the synthesis of the β-amino acid Adda that is responsible for the toxicity of the microcystins [2]. Moreover, McyD is essential in microcystin synthesis and the lack of this protein results in the absence of microcystin synthesis [3]. Thus, *mcyD* was considered for expression studies.

Relative transcriptional change by Fus901 based on the housekeeping gene, 16S rRNA, is shown in Fig. 5. The level of *pod* transcript increased 2.1-fold when *M. aeruginosa* KW was grown under Fus901. Meanwhile, *sodB* transcript level hardly changed. The level of *mcyD* transcript slightly decreased (up to 40%) and this result corresponded with the decrease of MC content.

Discussion

Several control techniques for algal bloom have been developed such as the use of yellow loess [16] and clay [17]. Even though effective, yellow loess and clay can cause secondary effects on other aquatic organisms. The application of chemical cyanobactericidal agents such as copper sulfate [18] and hydrogen peroxide [19] are also effective in controlling cyanobacterial blooms within a short time, but these chemicals can inhibit the entire phytoplankton community and cause water quality deterioration [20]. Recent studies have focused on the identification of bacteria capable of inhibiting or degrading algal blooms in marine and freshwater environments [21]. Application of biological agents, such as bacteria [22], viruses [23] and planktonic ciliates [24], in aquatic systems, faces difficulty posed by the demands of high bacterial cell density. Five strains (HYY0510-SK04, HYY0511-SK09, HYK0512-SK12, HYK0512-PK04 and HYY0512-PK05) isolated by Kang *et al*. [22] degraded *Stephanodiscus hantzschii* cells when those bacteria were inoculated at a concentration of $\geq 10^7$ cells/ml. Moreover, these bacteria showed growth inhibition activity against several algae and cyanobacteria. Mayali and Doucette [24] isolated *Cytophaga* strain 41-DBG2 from Gulf of Mexico waters which showed algicidal activity against *Karenia brevis* (Dinophyceae), when added at $\geq 10^6$ cells/ml.

Several aquatic plants produce metabolites inhibiting the growth of algae and cyanobacteria. These substances are known as allelochemicals. Many allelochemicals have been isolated and identified. Nakai *et al*. [25] reported that macrophyte *Myriophyllum spicatum* released allelopathic polyphenols, which inhibited the growth of *M. aeruginosa* at 1.26 mg/l. However, none of these methods can specifically control algal blooms without causing problems to other organisms in aquatic environments.

Tandem mass spectrophotometry (MS/MS) has been used for elucidating the primary structures of peptides or proteins. In this study, to investigate the structural differences in the four analogs derived from fusaricidin, we purified them by HPLC. The $(M+H)^+$ ion peaks of fusaricidin A, B, C, and D were 883, 897, 947, and 961, respectively. But, those of the four analogs were 915, 929, 943, and 957, respectively (data not shown). Because fusaricidin A and B comprise a large proportion of the fusaricidin produced by *P. polymyxa* E681, we made a ring-opened fusaricidin (A and B mixture) by NaOH treatment and compared those structures with Fus901 by MS/MS analysis. The $(M+H)^+$ ion peak of ring-opened fusaricidin was 915 and this molecular weight of m/z 915 corresponded with that of Fus901. Ring-opened fusaricidin was probably made by the ring-opening process between

the β-carbon atom and the oxygen atom at the β-position of threonine (Fig. S2A asterisk).

Fusaricidin B showed strong growth inhibition activity against a wide variety of fungi and also had low minimum inhibitory concentration values against Gram-positive bacteria (*S. aureus* and *M. luteus*) with 1.56 μg/ml. On the other hand, it showed relatively low sensitivity against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) [26]. However, Fus901 did not show growth inhibition activity against Gram-positive bacteria or Gram-negative bacteria at 1 μg/ml. This indicated that Fus901 is an eco-friendly cyanobactericide that does not disturb other aquatic microorganisms.

A few studies have focused specifically on the MC biosynthesis genes and have shown the effects of single or combined factors on MC synthesis [3]. The current study selected *mcyD* as representative of decreased MC production and used real-time PCR to analyze the transcript levels of this gene under Fus901 treatment. Expression of *mcyD* transcript level was slightly decreased (up to 40%) by Fus901. A decrease in *mcyD* transcript level resulted in the reduction of MC synthesis. In photosynthetic organisms, environmental stress can create oxidative stress through overproduction of reactive oxygen species, which induce the expression of the superoxide dismutase (SOD) and peroxidase (POD) genes. Under oxidative stress, plants and microalgae respond by increasing antioxidant defenses such as SOD and POD enzymes. Fus901 increased *pod* gene transcription in *M. aeruginosa*, whereas *sodB* transcript level did not change. These results indicated that Fus901 creates oxidative stress in *M. aeruginosa* and consequently inhibits the growth of *M. aeruginosa* while also reducing the MC production.

Four fusaricidin analogs were isolated and purified from *P. polymyxa* E681. One analog, designated Fus901, exhibited highly species-specific growth inhibition on *M. aeruginosa*. The growth of *M. aeruginosa* was inhibited and the MC concentration also decreased up to 70% when *M. aeruginosa* was grown in 1 μg/ml of Fus901. MC content also decreased from 41.6 fg/cell to 24.0 fg/cell. Real-time PCR analyses of the *mcyD* gene under Fus901 revealed that the level of *mcyD* transcript slightly decreased (0.4-fold) as compared to the control. The level of *pod* transcript increased in response to Fus901. The data indicated that Fus901 induced oxidative stress in *M. aeruginosa*. Fus901, unlike fusaricidin, showed no growth inhibition against Gram-positive bacteria. Therefore, Fus901 appears to be a promising cyanobactericide that does not disturb the aquatic ecosystem.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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