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Postharvest Biological Control of *Colletotrichum acutatum* on Apple by *Bacillus subtilis* HM1 and the Structural Identification of Antagonists

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Bacillus subtilis HM1 was isolated from the rhizosphere region of halophytes for its antifungal activity against *Colletotrichum acutatum*, the causative agent of anthracnose. Treatment of postharvest apples with the cell culture or with a cell-free culture supernatant reduced disease severity 80.7% and 69.4%, respectively. Both treatments also exhibited antifungal activity against various phytopathogenic fungi *in vitro*. The antifungal substances were purified and analyzed by acid precipitation, gel filtration, high-performance liquid chromatography, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Three compounds were identified as fengycin, iturin, and surfactin. The MALDI-TOF/TOF mass spectrum revealed the presence of cyclized fengycin homologs A and B, which were distinguishable on the basis of the presence of either alanine or valine, respectively, at position 6 of the peptide sequence. In addition, the cyclized structure of fengycin was shown to play a critical role in antifungal activity.

Keywords: Antifungal lipopeptide, *Bacillus subtilis*, cyclized fengycin

Introduction

Widespread use of synthetic chemicals to inhibit phytopathogens has decreased the incidence of plant disease. However, these chemicals have also caused serious biological problems, including pathogen resistance and the accumulation of chemical residues within the food chain [11, 14]. Recently, biocontrol has emerged as an alternative to chemical use, and naturally antagonistic microorganisms have been effective as agents against various pathogens [13]. Beneficial rhizobacteria are capable of antagonizing pathogens by competing for both niche and nutrients by direct mechanisms, such as the production of antifungal compounds or extracellular lytic enzymes, or through indirect mechanisms that stimulate the defense systems of the host plant [10, 17].

A number of *Bacillus* species are able to produce a wide spectrum of secondary metabolites that have diversity in both structure and function. Lipopeptides, one group of secondary metabolites synthesized *via* a nonribosomal process by large multienzyme complexes, have low toxicity and high biodegradability [7, 14–16]. They are also known to be powerful biosurfactants with potent antimicrobial, antiviral, and antitumor activities [12]. The cyclic lipopeptides, a kind of biosurfactants, which includes fengycin, iturin, and surfactin, have cyclized peptides or peptide macrolactones of amino acids with variable hydrophobic alkyl chains. The hydrophilic heads and lipophilic tails are responsible for their surface activity and membrane-binding capabilities [5]. Whereas iturin and fengycin have strong antifungal properties, surfactins are not toxic to pathogens by themselves but instead exhibit synergetic effects with the

antifungal activities of other lipopeptides such as iturin [5–7].

In this paper, *Bacillus subtilis* strain HM1 was isolated as a biocontrol agent for the anthracnose of apple caused by *Colletotrichum acutatum*. It exhibited a broad antagonistic spectrum on various fungal phytopathogens. Antagonistic substances extracted from the culture supernatant were purified and identified as lipopeptides, using chromatography, mass spectrometry, and chemical analysis. In addition, the structural homologs of the lipopeptides were distinguished by matrix-assisted laser desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS).

Materials and Methods

Microorganisms and Antagonistic Activity

The *B. subtilis* HM1 strain was isolated for its antifungal activity from the rhizosphere region of halophytes in Sunchon Bay, Korea. The strain was cultivated on Luria-Bertani (LB) medium (Difco Laboratories, USA) at 30°C. The antagonistic activity was evaluated against 10 species of fungal phytopathogens (*Alternate alternate, Aspergillus niger, Botrytis cinerea, C. acutatum, Colletotrichum coccodes, Diaporthe citri, Fusarium oxysporum, Penicillium digitatum, Penicillium expansum,* and *Penicillium italicum)*, which were all maintained on potato dextrose agar (PDA) at 27°C. *C. acutatum* was used as a target pathogen for the identification of the antifungal agent produced by strain HM1.

Antagonistic activity against pathogens was evaluated using the dual culture assay described by Yoshida *et al*. [19]. Briefly, a 5 day-old mycelia disk (1 cm diameter) of test pathogens was placed on the edge of PDA plates (90 mm). Bacterial suspensions or cell-free substances were equidistantly placed on the other side edge of the PDA plates after 48 h of pathogen inoculation. Plates were incubated at 27°C for 7 days, and the inhibitory effect on fungal growth was assessed by calculating the diameter of the inhibition zone (mm). The results were generated as the mean of three replicates.

Biological Control Activity on Fruit

Apples (*Malus pumila* var. *dulcissima* cv. Fuji) were purchased from a commercial garden. They were selected to be free of wounds and uniform with respect to physiological maturity stage and size. After washing with sterile water, the surface of selected fruit was disinfected by a consecutive immersion procedure using a solution of 70% ethanol and 1% sodium hypochlorite for 1 min. Excess solution on the surface was removed by drying, and uniform wounds ($3 \times 3 \times 3$ mm^3) were carefully made in 20 places on each apple fruit with the sterile tip of a needle. An aliquot (10 µl) of bacterial suspension (1×10^8 cells/ml) or cell-free culture supernatant was inoculated into each wound under aseptic conditions. One hour later, 10 µl of a spore suspension (1×10^5 spores/ml) of *C. acutatum* was inoculated in each wound and the apples were stored in plastic trays at 25 ± 2°C under relatively high humidity of 90 ± 5%. After 7 days of incubation, the infection rate and lesion diameters of the infected wounds were measured. The experiments were repeated three times and the results were recorded as the mean of the three replicates.

Extraction and Purification of Antifungal Substances

B. subtilis HM1 was cultivated on LB for 48 h at 30°C with shaking at 180 rpm. The cells were then removed by centrifugation at 12,000 ×*g* for 10 min. The obtained supernatant was treated with HCl to reach a pH of 2.0. This solution was stored at 4°C overnight to precipitate antifungal agents. The precipitated substances were collected by centrifugation at 12,000 ×*g* for 10 min and extracted using methanol. After the solvent was removed using a vacuum evaporator, the dried extracts were dissolved in methanol and filtered through a 0.2 µm cellulose acetate membrane filter (Sartorius Stedim Biotech, Germany).

Filtered materials were fractionated by size using gel filtration on a Sephadex LH-20 column (20 mm ID × 65.5 cm L; Sigma-Aldrich Co., USA). Absorbance was monitored at 210 nm during the process of collecting fractions at 1 ml/min with a fraction collector (BioFrac Fraction collector; Bio-Rad, USA). Fractions exhibiting antifungal activity were separated by reverse-phase high-performance liquid chromatography (HPLC) (JP/LC-20A; Shimadzu, Japan), as described by Chen *et al*. [3]. The fractionated extracts were further separated on a C_{18} column (5 µm particle diameter, 4.6 mm ID × 25 cm L; Advanced Chromatography Technologies, Scotland) with a mobile phase including water (solvent A) and acetonitrile (ACN, solvent B) both in 0.1% formic acid. The products were eluted with a linear gradient of solvent B at a flow rate of 1 ml/min [3]. Standard iturin A and surfactin were purchased from Sigma-Aldrich Co. (USA). The concentration of lipopeptides was determined by measuring protein concentrations with a Bio-Rad protein assay kit (Bio-Rad, USA).

Mass Spectrometric Analysis

MALDI-TOF mass spectra were recorded on a Voyage-DE STR BioSpectrometry Workstation (Applied Biosystem, USA). For mass spectrometric analysis, the isolated antagonists were mixed with equal volumes of matrix medium (10 g/l of α-cyano-4 hydroxycinnamic acid in 70% ACN containing 0.1% trifluoroacetic acid (TFA)). The sample was spotted onto a matrix plate and dried under air. Ions were generated using a 337 nm nitrogen laser and accelerated with a reflector voltage of 20 kV in pulsed ion extraction mode. The accuracy of measurements was improved by using a molecular mass gate of 350 Da to filter out matrix ions [15].

The detailed structure was analyzed by MALDI-TOF/TOF MS (ABI 4700 Proteomic analyzer; Applied Biosystem, USA). The mass spectrometer was operated at a pulse voltage of 20 kV and 3,500 counts of intensity. Mass peak ionization was performed five times for each peak prior to acquiring data in the positive mode [15].

In order to determine the lactone linkage structure, the lipopeptide was treated with 1 N KOH and suspended overnight

at room temperature [18]. The excess KOH was removed by passing the solution through Pierce C_{18} Pipette Tips (100 µl bed; Thermo Scientific Inc., USA). The purified sample was then dissolved in 0.5% TFA-water. The bound sample was washed with 0.1% TFA in 5% ACN, and then eluted from the C_{18} cartridge using 0.1% TFA in 50% ACN. The mixture of eluted sample and matrix medium was dispensed directly onto a MALDI plate and MALDI-TOF/TOF MS was performed.

Results and Discussion

Several studies have reported the potential of *Bacillus* strains as biocontrol agents against plant fungal disease [5, 8, 14]. In this study, the antagonistic activity of strain HM1 was observed against phytopathogenic fungi, suggesting potent capability as a biological control agent for various plant fungal diseases (Table 1). In a dual culture assay, HM1 successfully inhibited the growth of *A. alternate*, *A. niger*, *B. cinerea*, *C. acutatum*, *C. coccodes*, *D. citri*, *F. oxysporum*, *P. digitatum*, and *P. expansum*, all of which cause losses in agricultural production.

Following the fungal inhibition test of HM1, an antagonistic assay was performed using a fungal phytopathogen, *C. acutatum*, which causes anthracnose disease. The suppression efficiency of the cells and the cell-free supernatant was estimated by counting the incidence of infection on apples relative to control. The cells and supernatant of *B. subtilis* HM1 prevented 80.7% and 69.4%, respectively, of anthracnose disease (Fig. 1).

In order to identify the substances responsible for antifungal activity, methanol extract from a cell-free supernatant of the culture was separated with HPLC with a reverse-phase C_{18} column (Fig. S1). Three main groups of peaks were observed. The retention times at 34 to 40 min and 72 to 78 min, respectively, were comparable to those for standard iturin A and surfactin, and one unknown group of peaks was observed broadly over 42 to 52 min. For further identification, the crude methanol extract was analyzed, based on molecular mass measured by MALDI-TOF MS. The mass spectral analysis revealed three clusters of different mass peaks (Table 2). Among these homologs, there was a 14 Da difference in molecular mass, indicating a difference in the carbon length of the fatty acids. The positive ions typically generated in MALDI-TOF MS consist of molecules with cation adducts, such as $[M+H]^+$, $[M+Na]^+$, or $[M+K]^+$ [15]. Subsequently, fengycin, iturin, and surfactin were assigned as additions of hydrogen, potassium, and sodium ions at typical molecular masses, as shown in Table 2 [15].

The relationship between physiological status of the HM1 strain and the production of antagonistic compounds was investigated by measuring cell growth, lipopeptides production, and antagonistic activity against *C. acutatum*. The antagonistic activity of a cell-free supernatant of the HM1 strain was detected in the transition state between the exponential and stationary phases of growth. Activity increased progressively during these phases, and reached the highest levels after 3 days of growth (Fig. 2). Considering the cell growth and the production of the antagonistic compound, cell cultures that were incubated for 2 days

Table 1. Antifungal activity of *B. subtilis* HM1 and its culture supernatant against various phytopathogens.

Fungal pathogen	Antifungal activity[a]	
	Cell suspension	Cell-free supernatant
Alternate alternate	13.5 ± 1.5	5.50 ± 0.50
Aspergillus niger	11.0 ± 2.0	6.00 ± 1.00
Botrytis cinerea	10.0 ± 1.0	2.50 ± 0.50
Colletotrichum acutatum	13.5 ± 2.5	4.50 ± 1.50
Colletotrichum coccodes	11.0 ± 1.5	4.00 ± 2.00
Diaporthe citri	11.0 ± 1.5	5.00 ± 1.00
Fusarium oxysporum	10.0 ± 1.0	4.00 ± 1.00
Penicillium digitatum	5.0 ± 2.0	4.00 ± 1.00
Penicillium expansum	8.0 ± 3.0	3.00 ± 1.00
Penicillium italicum	1.7 ± 1.0	1.25 ± 0.25

[a]Semi diameter (cm), that of mycelium growth of test pathogen was subtracted from that of mycelium growth of negative control. Sterilized LB was treated as a negative control.

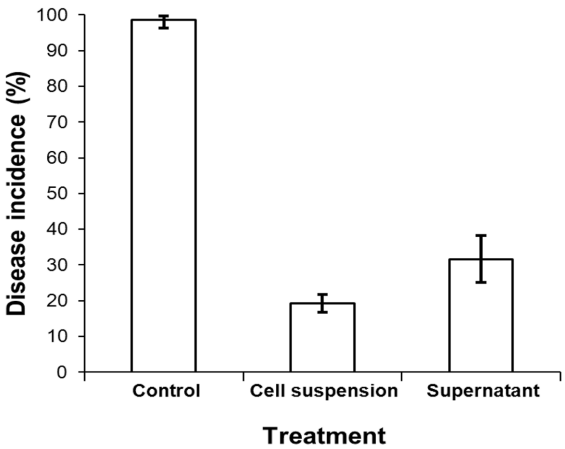

Fig. 1. Severity of anthracnose disease in apples after treatment with *Bacillus subtilis* strain HM1 or its culture supernatant.
Collectotrichum acutatum was used as the fungal pathogen.

Table 2. Assignment of main mass spectrum of the lipopeptides analyzed by MALDI-TOF mass spectrometry.

Metabolite	Mass (m/z)	Assignment
Crude lipopeptides	1044.6005	C_{14} surfactin, $[M+Na]^+$
	1058.6131	C_{15} surfactin, $[M+Na]^+$
	1060.5863	C_{14} surfactin, $[M+K]^+$
	1074.5780	C_{15} surfactin, $[M+K]^+$
	1061.5812	C_{14} iturin, $[M+H]^+$
	1075.5810	C_{15} iturin, $[M+H]^+$
	1449.5585	C_{15} fengycin, $[M+H]^+$
	1463.5685	C_{16} fengycin, $[M+H]^+$
	1477.5688	C_{17} fengycin, $[M+H]^+$
	1485.5338	C_{16} fengycin, $[M+Na]^+$
	1491.5678	C_{18} fengycin, $[M+H]^+$
	1501.5070	C_{16} fengycin, $[M+K]^+$
	1505.5329	C_{19} fengycin, $[M+H]^+$
	1515.5127	C_{17} fengycin $[M+K]^+$

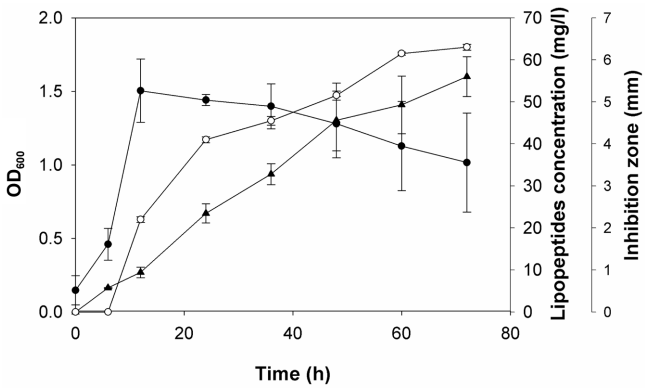

Fig. 2. Production of antagonistic compounds during the growth of strain HM1 in LB medium.
Closed circle, cell density measured as absorbance at a wavelength of 600 nm; open circle, antagonistic activity against *C. acutatum*; closed triangle, lipopeptide concentration.

were used in subsequent studies.

The initial purification of crude lipopeptides using Sephadex LH-20 yielded 120 fractions that showed *in vitro* antagonistic activity. The lipopeptide fractions showing the activities were separated into two groups, consisting of either fengycins or iturins mixed with surfactin, based on MALDI-TOF MS determination. HPLC with a reverse-phase C_{18} column was used for the subsequent purification step. The lipopeptides were successfully separated into three groups consisting of fengycins, iturins, and surfactins (Fig. S2). The antifungal activities against *C. acutatum* were observed with eluted fengycin and iturin, but not with surfactin. Lipopeptides, specifically combinations of surfactins and iturin-like peptides [7] and surfactin and fengycin-like peptides [8], have been reported to act in a synergistic manner. Therefore, surfactin seems to play a supplementary role with respect to antagonistic activity in the HM1 strain.

Fengycins have been classified into A and B groups depending upon the amino acid sequence of the peptide moiety. The peptide sequences of fengycin A isoforms are composed of L-Glu, D-Orn, L-Tyr, D-Thr, L-Glu, D-Ala, L-Pro, L-Gln, D-Tyr, and L-Ile, whereas fengycin B isoforms substitute Val for Ala at position 6 [2, 4]. Both isoforms are cyclic lipopeptides composed of 10 amino acids and a β-hydroxy fatty acid [1, 9]. We were unable to compare the eluted fengycin with a standard substance as it was not commercially available. The structure determination was therefore performed using a MALDI-TOF/TOF mass spectrometer on protonated molecular ion peaks $[M+H]^+$ at m/z 1449.8, 1464.8, 1478.8, 1491.8, 1492.8, and 1505.8 generated from MALDI-TOF MS of purified fengycin (Table 3). The fragmentation profile of the ions at m/z 1449.8, 1464.8, 1478.8, and 1491.8 contained the fingerprint product ion peaks at m/z 1080 and 966, whereas the product ion peaks at m/z 1108 and 994 were identified in the MALDI-TOF/TOF mass spectrum of the precursor ions m/z 1492.8 and 1505.8. These two pairs of ion peaks at m/z 1080 and 966, and m/z 1108 and 994 originated from fengycin A and B, respectively.

Table 3. Identification of fengycin A and fengycin B homologs.

Mass peak (m/z)	Fengycin homologs	Fingerprint product ions	Amino acid at position 6
1449.7787	C_{15} FengycinA	m/z 1080 and m/z 966	Ala
1464.7866	C_{16} FengycinA	m/z 1080 and m/z 966	Ala
1478.7960	C_{17} FengycinA	m/z 1080 and m/z 966	Ala
1491.7923	C_{18} FengycinA	m/z 1080 and m/z 966	Ala
1492.7968	C_{16} FengycinB	m/z 1108 and m/z 994	Val
1505.7967	C_{17} FengycinB	m/z 1108 and m/z 994	Val

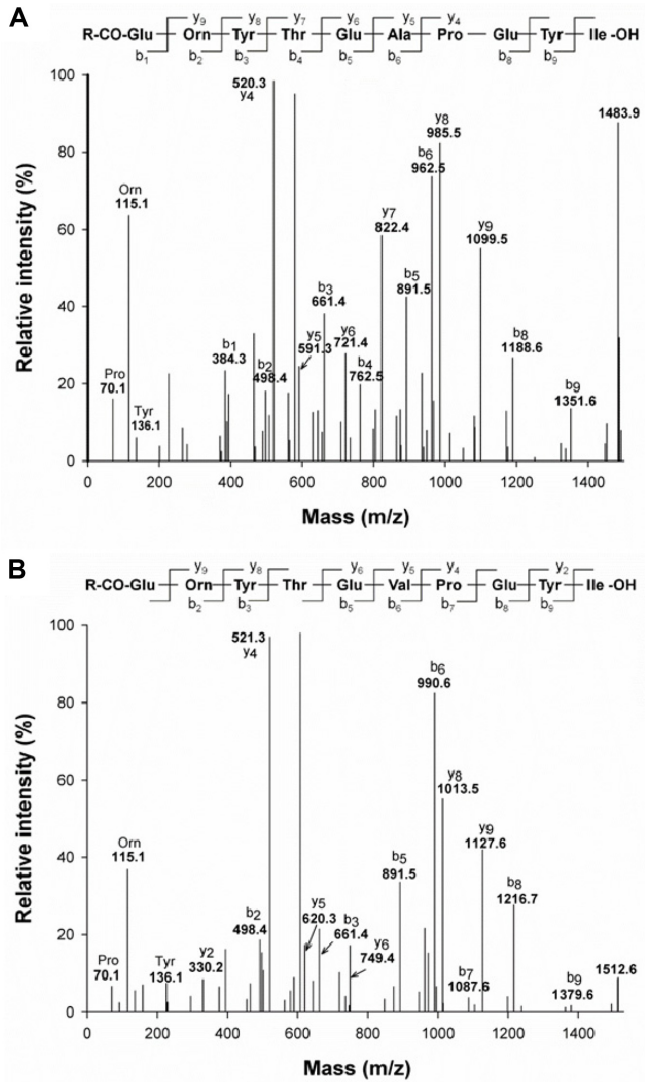

Fig. 3. MALDI-TOF/TOF mass spectrum of protonated ions of *m/z* 1483.9 (**A**) and *m/z* 1512.6 (**B**), showing the typical fragments of fengycins A and B.

In order to explore the amino acid sequences of the fengycin isoforms, purified fengycins were hydrolyzed with KOH and then analyzed *via* MALDI-TOF MS. The most abundant protonated molecular ion peaks were compared with those on the spectrum of intact fengycin. The product ion peaks at *m/z* 1468.7, 1483.9, 1496.8, 1509.8, 1512.6, and 1524.8 in the spectrum were observed, indicating that KOH-treated fengycins gained approximately 18 Da in molecular mass compared with those of intact fengycins (data not shown). This implies that the intact fengycin had a cyclic structure, and that the lactone linkage was hydrolyzed by KOH, thereby generating a linear structure.

In addition, the MALDI-TOF/TOF mass spectrum of *m/z* 1483.9 was consistent with the putative mass of the amino acid composition of fengycin A (Glu-Orn-Tyr-Thr-Glu-Ala-Pro-Glu-Tyr-Ile; Fig. 3A) [2]. The ionized mass peaks of *m/z* 1512.6 corresponded to the amino acid sequence Glu-Orn-Tyr-Thr-Glu-Val-Pro-Glu-Tyr-Ile (Fig. 3B) [2]. Based on these sequence determinations, the HM1 strain was proven to simultaneously produce fengycin isoforms A and B, which had a different amino acid, alanine or valine, at peptide position 6. In dual culture assay, the antagonistic activity of fengycin against *C. acutatum* was completely abolished along with linearization by hydrolysis. Consequently, the cyclic structure was considered to play a key role in antagonistic activity. These analyses demonstrated the existence of at least four homologs of fengycin A (C_{15} to C_{18}), and two homologs of fengycin B (C_{16} and C_{17}) (Table 3).

Consequently, the *B. subtilis* HM1 strain produced the lipopeptides iturin, surfactin, and fengycin, which corresponded with antifungal activity. Whereas iturin and fengycin displayed efficient antifungal properties against various fungal phytopathogens, surfactin did not show this activity. In particular, MALDI-TOF/TOF MS determined that fengycin in a cyclic form possessed activity and consisted of both A and B isoforms, which differed at amino acid composition 6. The results of this study suggest that the antagonistic lipopeptide producer, *B. subtilis* HM1, has great potential in the biocontrol of plant fungal diseases and for industrial uses.

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