Isolation and NMR Analysis of Antifungal Fengycin A and B from *Bacillus amyloliquefaciens* subsp. *plantarum* BC32-1

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Natural antibiotics produced by microorganisms have generated considerable interest in various industries. Here we report the potential of antifungal lipopeptides produced by *Bacillus amyloliquefaciens* subsp. *plantarum* BC32-1 isolated from ultisols. The BC32-1 strain strongly inhibited mycelial growth of plant pathogenic fungi. Several lipopeptides were purified by organic solvent extraction and reversed-phase high-performance liquid chromatography (RP-HPLC). The purified lipopeptides contained iturin, fengycin, and surfactin-type lipopeptides, as confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses. Since fengycin-type lipopeptides showed strong antifungal activity, we characterized the chemical structures of fengycin A and B using LC-MS/MS and 2D NMR spectroscopy. This study will provide the structural basis for the development of new antifungal agents for the control of pathogenic fungal diseases.

Keywords: Antifungal lipopeptide, *Bacillus amyloliquefaciens*, Biocontrol, Biosurfactant, NMR

Introduction

Cyclic lipopeptides from microorganisms are the key components of antifungal reagents.[1] The amino acids in cyclic lipopeptides frequently possess the D-form and unnatural amino acids, probably to resist the action of proteases.[2] These lipopeptides consist of a hydrophobic, long alkyl chain linked to a cyclic polypeptide.[3] Because of these structural characteristics, these lipopeptides target the lipid membrane of pathogens and increase their permeability, thereby killing the pathogens. *Bacillus* strains produce a variety of antifungal cyclic lipopeptides, including iturin-, fengycin-, and surfactin-type lipopeptides.[1] These lipopeptides vary in their alkyl chain lengths and in the composition of their amino acid residues, thereby generating isoforms and consequently leading to a remarkable structural heterogeneity. Iturin-type lipopeptides, including iturin A, mycosubtilin, and bacillomycin, consist of a cyclic heptapeptide linked to a β-amino fatty acid from C_{14} to C_{17} and typically display a broad antifungal spectrum.[1,4] Lipopeptides belonging to the surfactin family consist of a β-hydroxyl fatty acid (C_{12}–C_{16}) linked to the *N*-terminal amino acid of a heptapeptide to form a cyclic lactone ring structure, and exhibit hemolytic, antiviral, antimycoplasma, and antibacterial activities.[1,5] Fengycin-type lipopeptides consist of a decapeptide with an internal lactone ring in the peptidic moiety and a β-hydroxyl fatty acid chain from C_{14} to C_{18} as linear, iso, or anteiso forms, which may be saturated or unsaturated.[6] These cyclic lipopeptides have attracted considerable attention as alternative biocontrol agents against plant fungal pathogens due to their strong and broad-spectrum antimicrobial activity, low toxicity, and biodegradability compared to those observed with chemical pesticides.[7] Ultisols, commonly known as red clay soils, present normally quite barren condition for agriculture and microbial growth since they are typically quite acidic having a pH of less than 5 and are typically deficient in major nutrients such as calcium and potassium.

In this study, we present the isolation of a bacterial strain *Bacillus amyloliquefaciens* subsp. *plantarum* BC32-1 from ultisols in the Jeonnam province of South Korea. The strain BC32-1 exhibited a strong antifungal activity and produced several cyclic lipopeptides identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Among them, fengycin A and B showed a strong antifungal activity against *Fusarium oxysporum* f. sp. *radicis-lycopersici*, a well-known soilborne fungus, some strains of which are pathogenic to plants and are difficult to control. The chemical structures of fengycin A and B were characterized by LC-MS/MS and 2D NMR spectroscopy.

Experimental

Microbial Strains and Culture Conditions. *B. amyloliquefaciens* subsp. *plantarum* BC32-1 was isolated from ultisols found in Haenam-gun, Jeonnam province, South Korea. Soil samples (1 g) were suspended in 9 mL of sterilized distilled water. The soil suspensions were serially diluted 10-fold and spread onto tryptic soy agar (TSA) plates. The plates were incubated at 37 °C for 1 day and maintained in TS broth at −70 °C as 20% glycerol stocks. The plant fungal pathogen *F. oxysporum* f. sp. *radicis-lycopersici* KACC 40031 was provided by the Korean Agricultural Culture Collection (KACC), Suwon, South Korea, and was incubated at 25–28 °C on potato dextrose agar (PDA).

Screening and Identification of Antifungal Bacteria. The potential growth inhibition of bacterial isolates against *F. oxysporum* f. sp. *radices-lycopersici* KACC 40031 was tested on PDA medium. The bacterial culture broths were loaded on the edges of the plates, and mycelial plugs (5 mm) of the fungal pathogen were deposited at the center. Suppression of mycelial growth was estimated after further incubation for >3 days. Among the bacterial isolates, the BC32-1 strain exhibiting the strongest antifungal activity was selected. Generally, the gyrA gene sequence has been used for identification of *Bacillus subtilis* and related taxa. In this study, the gyrA nucelotide sequences of 24 strains retrieved from GenBank were analyzed. Genomic DNA was extracted using HiGene™ Genomic DNA Prep Kit for bacteria (Biofact Co., Daejeon, South Korea). The polymerase chain reactions (PCRs) were performed in 20 μL reaction mixtures containing 2 μL genomic DNA, 1.5 μL of each primer (5 pmol), 14 μL demineralized sterile water, and 1 μL PCR premix (Bioneer Co., Daejeon, South Korea). Primers gyr AF (5′-CAGTCAGGAAATGCGTACGTCCTT-3′) and gyr AR (5′-CAAGGTAATGCTCCAGGCATTGCT-3′) were used to amplify the *gyrase A* gene.[8] The PCR cycling conditions were as follows: an initial denaturation step at 95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, 53 °C for 40 s or 1 min, and 72 °C for 1 min. A final elongation step at 72 °C was performed for 10 min. The purified PCR products were sequenced using an ABI 3700 (Applied Biosystems, Foster City, CA, USA) automated DNA sequencer. The sequences retrieved from GenBank were aligned using Clustal X windows interface v. 2.1[9] and edited by BioEdit software v. 7.2.5 (Ibis Biosciences, Carlsbad, CA, USA). The alignment was manually refined using MEGA v. 6.0.[10] A neighbor-joining tree was constructed with Kimura's two-parameter distance model by using MEGA v. 6.0. One thousand bootstrap replicates were performed to assess the relative stability of the branches.

Extraction and Purification of Lipopeptides. The BC32-1 were cultured in LB broth for 16 h at 37 °C, and then the culture broth was centrifuged for 10 min at 8000*g*. The lipopeptides were extracted from the supernatant three times by using ethyl acetate and evaporated under vacuum. The crude extract was dissolved in methanol and filtered through a 0.2-μm non-pyrogenic hydrophobic membrane (Sartorius AG, Goettingen, Germany). The filtrates were subjected to reversed-phase high-performance liquid chromatography (RP-HPLC) equipped with a C_{18} column (Waters μBondapak® $C_{18;}$ 300 × 3.9 mm). The mobile phase components were 0.05% trifluoroacetic acid (TFA) in water and 0.05% TFA in acetonitrile. The products were eluted using a linear gradient of solvent B at a flow rate of 1 mL/min, developed from 5% to 95% for 50 min, and monitored by determining absorbance at 230 nm. The fractions were collected and lyophilized to assess their antifungal activity against *F. oxysporum* f. sp. *radicis-lycopersici*.

Determination of Minimal Inhibitory Concentrations (MIC). The antifungal activities of fengycin A and B against the *F. oxysporum* were tested in sterile 96-well 200-μL plates as follows. Aliquots (100 μL) of the *F. oxysporum* suspension at 1×10^4 cells/mL in molten PDA counted by a hemocytometer were added to 100 μL of the sample solutions. After incubation for 3 days at 27 °C, the MIC was determined by visual examinations based on the lowest concentration of sample solution in cells with no fungal growth. All independent experiments were repeated three times.

LC-MS Spectrometry. The lipopeptides were investigated by LC-MS analysis (API2000™, AB Sciex, Redwood City, CA, USA) by elution with a water/acetonitrile mobile phase containing 0.05% TFA using the same gradient as that used in RP-HPLC analysis. MS was performed using a triple-quadrupole spectrometer equipped with an electrospray ionization (ESI) source.

Circular Dichroism (CD) Spectral Analysis. CD spectra were measured on a JASCO J-815 spectropolarimeter (JASCO, Tokyo, Japan) in methanol. Measurements were made at 20 °C using a quartz cell with 1 mm path length. The spectra were expressed as molecular ellipticity (θ) in deg/cm/dmol.

NMR Spectroscopy. NMR spectra were measured on a Bruker AVANCE600 (Bruker Biospin, Ettlingen, Germany) spectrometer equipped with an *xyz* gradient triple-resonance probe. The samples used for proton 2D NMR experiments were 5 mM fengycin dissolved in 99.9% DMSO-d_6. Two-dimensional DQF-COSY,[11] TOCSY,[12] and NOESY[13,14] spectra were acquired at 298 K. TOCSY spectra were recorded using a MLEV-17 pulse scheme with isotropic mixing time of 90 ms. NOESY spectra were recorded with mixing times of 200 and 300 ms. NMR data processing and analysis were performed using NMRPipe[15] and NMRView[16] software.

Results and Discussion

Screening and Identification of Antagonists. In this study, over 100 bacterial isolates were tested for their potential to inhibit the mycelial growth of *F. oxysporum* f. sp. *radicis-lycopersici*. Among them, the BC32-1 strain exhibited the strongest antifungal activity against the above-mentioned plant fungus (Figure 1). This indicated that the BC32-1 strain produces antifungal substances that inhibit the growth of plant pathogenic fungi. A comparative *gyrase A* gene sequence-based phylogenetic analysis placed strain BC32-1 in a clade

with *B. amyloliquefaciens* subsp. *plantarum*, with about 99.6% (885/889) pairwise similarities (Figure 2).

Identification and Purification of Lipopeptides. The lipopeptides generated in the culture supernatant were extracted using ethyl acetate and analyzed by RP-HPLC and LC-MS/MS. Nine fractions were obtained from RP-HPLC analysis (Figure 3), and each fraction was lyophilized for antifungal experiments. After fractionation, the active fractions were determined by monitoring their antifungal activity against *F. oxysporum* f. sp. *radicis-lycopersici*. An LC-MS analysis revealed a cluster containing several molecules at *m/z* 1032, 1046, and 1060 (iturin-type); 1464, 1478, 1492, and 1506 (fengycin-type); and 1009, 1023, and 1037 (surfactin-type) (Table 1). These masses revealed differences of 14 or

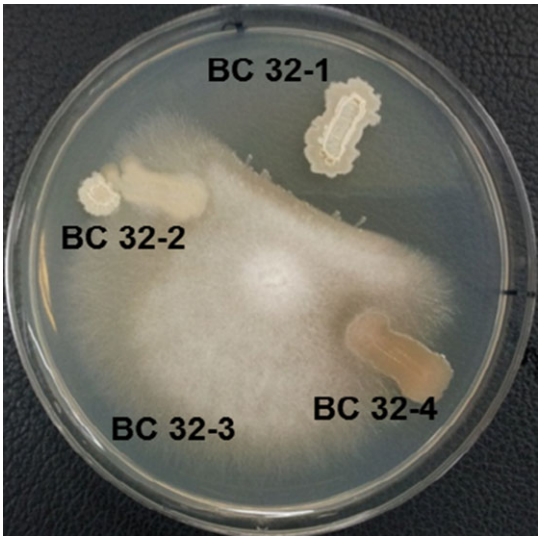

Figure 1. Growth-inhibitory activity of BC32-1 strain against *F. oxysporum* f. sp. *radices-lycopersici* KACC 40031 on PDA plate. Bacteria isolated from ultisols. Only the BC32-1 strain showed a strong antifungal activity.

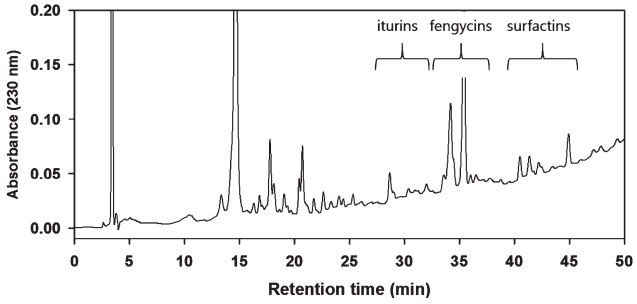

Figure 3. RP-HPLC chromatogram of compounds obtained by ethyl acetate extraction of *B. amyloliquefaciens* subsp. *plantarum* BC32-1 culture supernatant. Iturin, fengycin, and surfactin fractions are indicated.

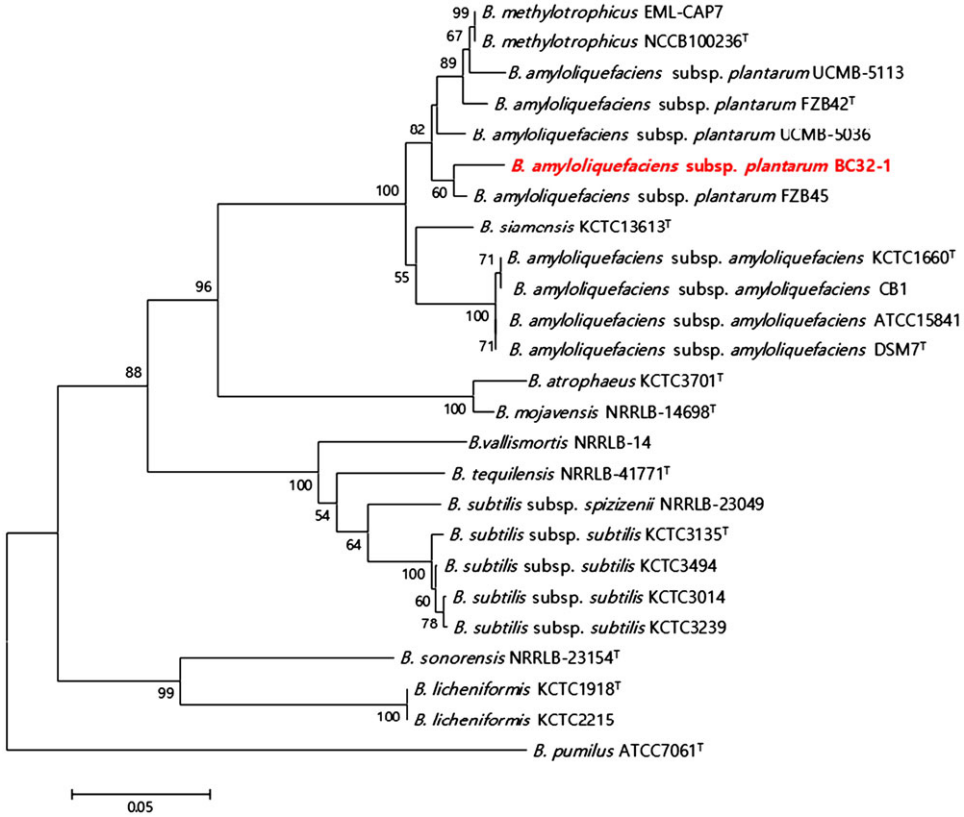

Figure 2. Neighbor-joining tree generated from alignments of *gyrase A* subunit gene sequences of 24 strains retrieved from GenBank used to infer the phylogenetic relationship between *B. amyloliquefaciens* and its subspecies *plantarum*. Bootstrap values >50%, based on 1000 replicates, are indicated at the branch points. The bar indicates substitution per nucleotide position. *B. pumilus* ATCC 7061^T was used as the outgroup.

28 Da, suggesting a series of homologous molecules having different lengths of fatty acid chains. Among them, the fengycin-type lipopeptides showed the antifungal activity against *F. oxysporum* f. sp. *radicis-lycopersici* (data not shown).

Identification of Fengycin A and B by LC-MS. The chemical structure of the fengycins of *m/z* 1478 and 1492 Da in fraction 5 was analyzed through a rapid and suitable LC-MS/MS, which allowed characterizing the composition and the structures of the lipopeptides.[17] Fengycin A can produce *m/z* 1080 and 966 fragment ions, and fengycin B produces *m/z* 1108 and 994 ions (Figure 4(a) and (b)). The fractions at *m/z* 1478 and 1492 were analyzed by the LC-ESI-MS system and showed the appearance of fragment ions at *m/z* 1080 and 966 from *m/z* 1478 and at *m/z* 1108 and 994 from the precursor at *m/z* 1492 (Figure 4(c)). The data indicate that fengycins at *m/z* 1478 and 1492 correspond to C_{17} fengycin A and C_{16} fengycin B, respectively.

Antifungal Activity of Fengycin A and B. The antifungal activities of fengycins were investigated using the MIC of fengycin A and B against the *F. oxysporum*. The MIC value of fengycin A (450 μg/mL) is nine times larger than that of fengycin B (50 μg/mL), which indicates that the antifungal activity of fengycin B is much stronger than that of fengycin A.

Table 1. Summary of the types and molecular masses of lipopeptides produced by *B. amyloliquefaciens* subsp. *plantarum* BC32-1.

Fractions	Lipopeptide type	$[M + H]^+$
1	Iturin	1032
2		1046
3		1060
4	Fengycin	1464
5		1478
		1492
6		1506
7	Surfactin	1009
8		1023
9		1037

CD and NMR Analysis of Fengycin A and B. For CD experiments, fengycin A and B were dissolved in methanol instead of the NMR buffer DMSO, since DMSO has high absorbance in far UV and cannot be used for CD measurements. Figure 5 shows the CD spectra of fengycin A and B. In both cases, they show similar CD spectra displaying a negative minimum at around 200 nm, indicating a turn and extended backbone structure without regular secondary structures (not random coil). Complete proton resonance assignments for fengycin A and B in fraction 5 were determined using 2D NMR sequential assignment methods. Identification of the amino acid spin system was based on scalar coupling patterns observed in DQF-COSY and TOCSY experiments, complemented by the results of NOESY measurements. Sequence-specific assignments were obtained from NOESY spectra. Figure 6 shows the NH–$C^{\alpha}H$ fingerprint region of the NOESY spectrum, which contains sequential $d\alpha N(i, i+1)$ connectivity of fengycin A (black line) and fengycin B (red line). The structural difference between fengycin

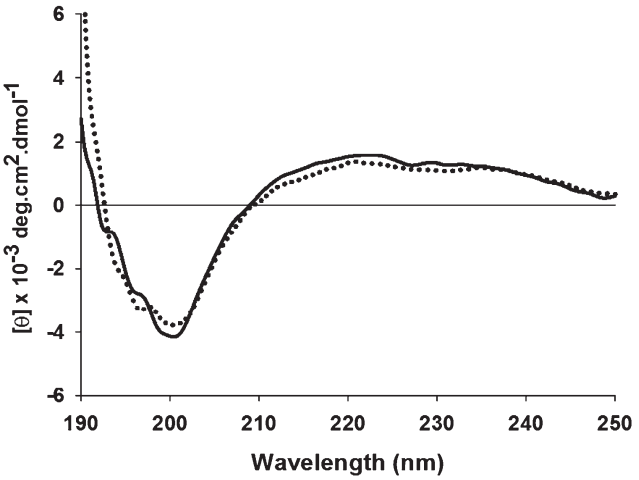

Figure 5. CD spectra of fengycin A (solid line) and fengycin B (dotted line) recorded in methanol at 20 °C.

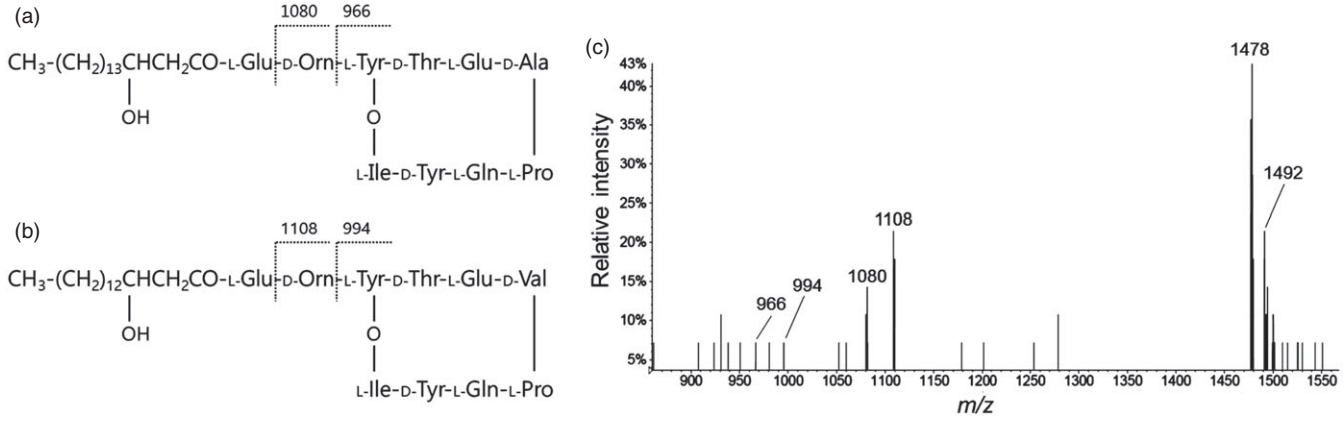

Figure 4. Structures and MS spectra of the fengycin A and B homologues. (a) and (b) Cyclic structures and proposed fragmentations of fengycin A (product ions *m/z* 1080 and 966) and B (product ions *m/z* 1108 and 994), respectively. (c) MS spectra of the protonated molecules $[M + H]^+$ of fengycin A at *m/z* 1478 and fengycin B at *m/z* 1492, respectively.

A and B is only the single amino acid at residue 6 (Ala in fengycin A and Val in fengycin B). In NOESY spectra, the Ala6 of fengycin A and Val6 of fengycin B were clearly identified, and almost all proton chemical shifts of fengycin A and B were assigned from proton 2D NMR spectra (Table 2). Since prolines do not have amide protons in their backbone, NMR peaks of Pro7 were missing in the finger print region but assigned based on the NOEs in the NOESY spectrum.

Significance of Bacterial Lipopeptides as Antifungal Agents. Bacterial antagonists have been considered ideal candidates because of their rapid growth, ease of handling, and aggressive colonizing characteristics. Bacilli are gram-positive, endospore-producing bacteria that are resistant to heat and desiccation, a trait that is required for their successful field application. The newly isolated *B. amyloliquefaciens* subsp. *plantarum* BC32-1 produces various types of lipopeptides that could inhibit the growth of foreign bacteria and fungi. In particular, the BC32-1 strain produces fengycin-type lipopeptides, which showed strong antifungal activity against the plant pathogenic fungus *F. oxysporum* f. sp. *radicis-lycopersici*, suggesting that the BC32-1 strain may be a potential biocontrol agent. The high antifungal activity exhibited by this strain is consistent with the known properties of fengycin-type lipopeptides. In addition to fengycins, the BC32-1 strain produced iturins and surfactins. Iturins typically exhibit broad-spectrum antifungal activity, while surfactins exhibit strong biosurfactant activity rather than antifungal activity. In spite of their weak antifungal activity, surfactins display a strong synergistic action in combination with iturins. In addition, surfactins seem to be the key inhibitors of biofilm formation by other bacteria, thereby contributing to the protective action.

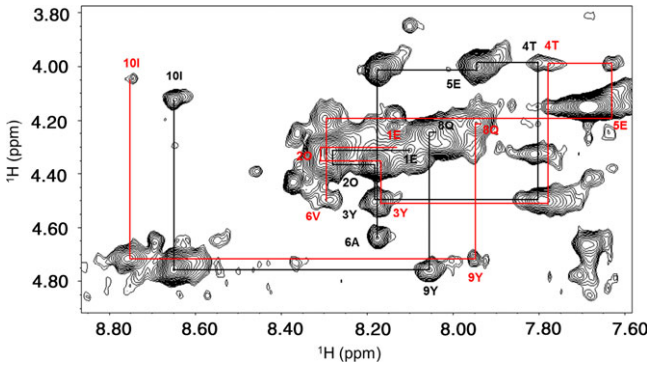

Figure 6. Sequential $d\alpha N(i, i+1)$ NOE connectivities for fengycin A and B in NOESY spectrum observed with a mixing time of 200 ms at 298 K. Intra-residue NH-C$^{\alpha}$H cross-peaks are labeled with the residue number of fengycin A in black and fengycin B in red using standard single-letter amino acid abbreviations.

Conclusion

In conclusion, the current study showed that *B. amyloliquefaciens* subsp. *plantarum* BC32-1 produced three major lipopeptides. Although fengycins exhibited a higher antifungal activity against *F. oxysporum* f. sp. *radicis-lycopersici*, iturins and surfactins may play a synergistic role in the antifungal activity in combination with fengycins. From these results, we suggest that strain BC32-1 may have high potential as a new biocontrol agent in agricultural and other industrial applications.

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Table 2. Summary of proton chemical shifts of fengycin A and B recorded in DMSO-d_6 at 298 K.

Peptide	Sequence	HN	Hα	Hβ	Others
Fengycin A	Glu1	8.1	4.32	1.81, 1.94	Hγ: 2.34, 2.28
	Orn2	8.28	4.36	1.59	Hγ: 1.79; Hδ: 2.82, Hε: 7.69
	Tyr3	8.18	4.5	2.89, 2.94	Hδ: 7.22, Hε: 6.99
	aThr4	7.79	3.99	3.55	Hγ2: 0.81
	Glu5	7.94	4.01	1.87, 2.07	Hγ: 2.4, 2.24
	Ala6	8.17	4.64	1.17	
	Pro7		4.3	2.13	Hγ: 1.78, 1.83; Hδ: 3.50
	Gln8	8.03	4.24	1.76	Hγ: 2.22; Hε2: 7.34, 6.84
	Tyr9	8.05	4.76	2.84	Hδ: 7.05, Hε: 6.69
	Ile10	8.65	4.12	1.84	Hγ12: 1.37; Hγ13: 1.12; Hγ21: 0.9; Hδ11: 0.86
Fengycin B	Glu1	8.13	4.3	1.79, 1.94	Hγ: 2.27, 2.34
	Orn2	8.31	4.38	1.6	Hγ: 1.8; Hδ: 2.84; Hε: 7.69
	Tyr3	8.17	4.51	2.87, 3.02	Hδ: 7.22; Hε: 6.99
	aThr4	7.78	3.99	3.55	Hγ2: 0.8
	Glu5	7.69	4.18	1.96	Hγ: 2.28
	Val6	8.3	4.5	2.1	Hγ: 0.91
	Pro7		4.3	2.13	Hγ: 1.78, 1.83; Hδ: 3.50
	Gln8	7.94	4.2	1.76	Hγ: 2.24; Hε2: 7.34, 6.84
	Tyr9	7.95	4.71	2.87	Hδ: 7.05, Hε: 6.69
	Ile10	8.76	4.05	1.84	Hγ21: 0.94; Hδ11: 0.86

References

1. M. Ongena, P. Jacques, *Trends Microbiol.* **2008**, *16*, 115.
2. T. Schneider, A. Muller, H. Miess, H. Gross, *Int. J. Med. Microbiol.* **2014**, *304*, 37.
3. E. Katz, A. L. Demain, *Bacteriol. Rev.* **1977**, *41*, 449.
4. L. Delcambe, F. Peypoux, F. Besson, M. Guinand, G. Michel, *Biochem. Soc. Trans.* **1977**, *5*, 1122.
5. F. Peypoux, J. M. Bonmatin, J. Wallach, *Appl. Microbiol. Biotechnol.* **1999**, *51*, 553.
6. J. M. Raaijmakers, B. I. De, O. Nybroe, M. Ongena, *FEMS Microbiol. Rev.* **2010**, *34*, 1037.
7. A. Romano, D. Vitullo, A. Di Pietro, G. Lima, V. Lanzotti, *J. Nat. Prod.* **2011**, *74*, 145.
8. J. Chun, K. S. Bae, *Anton. Leeuw.* **2000**, *78*, 123.
9. M. A. Larkin, G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, D. G. Higgins, *Bioinformatics* **2007**, *23*, 2947.
10. K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, *Mol. Biol. Evol.* **2013**, *30*, 2725.
11. M. Rance, O. W. Sørensen, G. Bodenhausen, G. Wagner, R. R. Ernst, K. Wüthrich, *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479.
12. A. Bax, D. G. Davis, *J. Magn. Reson.* **1985**, *65*, 355.
13. J. Jeener, B. H. Meier, P. Bachmann, R. R. Ernst, *J. Chem. Phys.* **1979**, *71*, 4546.
14. S. Macura, Y. Huang, D. Suter, R. R. Ernst, *J. Magn. Reson.* **1981**, *43*, 259.
15. F. Delaglio, S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer, A. Bax, *J. Biomol. NMR* **1995**, *6*, 277.
16. B. A. Johnson, R. A. Blevins, *J. Biomol. NMR* **1994**, *5*, 603.
17. Y. Pecci, F. Rivardo, M. G. Martinotti, G. Allegrone, *J. Mass Spectrom.* **2010**, *45*, 772.

