Biotechnology and Bioprocess Engineering 20: 708-713 (2015)
DOI 10.1007/s12257-015-0350-3

RESEARCH PAPER

Structural Characterization and Temperature-dependent Production of C_{17}-Fengycin B Derived from *Bacillus amyloliquefaciens* subsp. *plantarum* BC32-1

Jiyoung Nam, Min Young Jung, Pyoung Il Kim, Hyang Burm Lee, Si Wouk Kim, and Chul Won Lee

Received: 21 May 2015 / Revised: 10 July 2015 / Accepted: 3 August 2015

Abstract Cyclic lipopeptides were produced by *Bacillus amyloliquefaciens* subsp. *plantarum* strain BC32-1 that was isolated from yellow loess soil in the Jeonnam province of South Korea. Several lipopeptides were isolated from the bacteria using organic solvent extraction and reverse-phase high-performance liquid chromatography (RP-HPLC). Purified iturin-, surfactin-, and fengycin-type lipopeptides were identified using liquid chromatography-mass spectrometry (LC-MS) analysis. Among the lipopeptides, C_{17}-fengycin B showed strong antifungal activity against the phytopathogenic fungus, *Fusarium oxysporum* f. sp. *radicis-lycopersici,* and then the fengycin was further characterized by UV, Fourier transform-infrared spectroscopy (FT-IR), and LC-MS/MS analyses. C_{17}-fengycin B was highly produced at quantities of up to 15 μg/mL at 37°C, whereas little amount of the fengycin was produced at 25°C. Purified C_{17}-fengycin B inhibited mycelial growth of *F. oxysporum* with a minimal inhibitory concentration of 50 μg/mL. This study suggests that C_{17}-fengycin B is a major antifungal component produced by the BC32-1 strain that could be used as an environmentally friendly agent to control the phytopathogenic *F. oxysporum.*

Keywords: antifungal lipopeptide, *Bacillus amyloliquefaciens* subsp. *plantarum*, biocontrol, biosurfactant, fengycin

Jiyoung Nam, Chul Won Lee*
Department of Chemistry, Chonnam National University, Gwangju 500-757, Korea
Tel: +82-62-530-3374; Fax: +82-62-530-3389
E-mail: cwlee@jnu.ac.kr

Min Young Jung, Pyoung Il Kim
Bio Control Research Center, JBF, Jeonnam 516-944, Korea

Hyang Burm Lee
Division of Food Technology, Biotechnology & Agrochemistry, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 500-757, Korea

Si Wouk Kim*
Department of Environmental Engineering, Chosun University, Gwangju 501-759, Korea
Tel: +82-62-230-7154; Fax: +82-62-225-6040
E-mail: swkim@chosun.ac.kr

1. Introduction

The development of environmentally friendly antimicrobial agents would be beneficial to both agriculture and human health. Some microorganisms act as antimicrobial agents and can strongly suppress phytopathogens; this controls plant disease and promotes plant growth, in an eco-friendly manner [1]. Bacteria-derived cyclic lipopeptides are the key components of antifungal pesticides [2,3]. These lipopeptides are composed of a long hydrophobic alkyl chain linked to a cyclic polypeptide backbone [4]. The amino acids in the peptide backbone are frequently D-isomers and unnatural amino acids-presumably to resist the activity of proteases [5]. These lipopeptides directly interact with the plasma membrane of pathogens and disrupt their lipid bilayer.

It is well known that bacteria belonging to the genus *Bacillus* can produce a variety of biologically active metabolites with antifungal activities [6,7]. For instance, *Bacillus subtilis* strains produce several antifungal cyclic lipopeptides, including iturin-, fengycin-, and surfactin-type lipopeptides [2]. Iturin-type lipopeptides consist of a cyclic heptapeptide linked to a β-amino fatty acid from C_{14} to C_{17}. Iturin-type lipopeptides including iturin A, iturin C, iturin D, iturin E, bacillomycin D, bacillomycin F, bacillomycin L, bacillomycin Lc, and mycosubtilin, display

a broad spectrum of antifungal activity [2,8]. The surfactin-type lipopeptides consist of a β-hydroxyl fatty acid linked to the N-terminal amino acid of a heptapeptide to form a cyclic lactone ring structure; they exhibit antibacterial, antiviral, and hemolytic activities [2,9]. Fengycin-type lipopeptides are decapeptides with an internal lactone ring in the peptidic moiety and a β-hydroxyl fatty acid chain [10,11]. Fengycins have a strong antifungal activity against filamentous fungi and much lower hemolytic activity than surfactins [12]. The strong broad-spectrum antimicrobial activity of *Bacillus*-derived cyclic polypeptides and their low toxicity compared to chemical pesticides has made them attractive as alternative agents against fungal phytopathogens [13]. The family of *Bacillus amyloliquefaciens* is one of the most promising microorganisms for biocontrol agent. The analysis of the plant-associated *B. amyloliquefaciens* FZB42 genome revealed a number of gene clusters involved in nonribosomal peptide synthesis and producing secondary metabolites to stimulate plant growth and control plant pathogens [14]. In addition, the genome of *B. amyloliquefaciens* GA1 contains the gene clusters for the synthesis of biocontrol agents which include various cyclic lipopeptides and antimicrobial compounds [15]. Several groups have recently explored the potential use of *B. amyloliquefaciens* as biocontrol agent. For example, *B. amyloliquefaciens* Q-426 that was isolated from compost samples showed an effective suppression of the growth of various pathogenic fungi by producing antifungal compounds such as bacillomycin D, fengycin A, and fengycin B, suggesting that the Q-426 strain can be used as a biocontrol agent [16].

In our previous study, *B. amyloliquefaciens* strain BC32-1 was isolated from yellow loess soil and displayed a strong antifungal activity against *Fusarium oxysporum* f. sp. *radicis-lycopersici*, a well-known, soil-borne fungus [17]. The objective of the present study was to determine the effective antifungal metabolites of the BC32-1 strain for *F. oxysporum* and to optimize the culture temperature for high production of the antifungal metabolites to utilize the BC32-1 strain as a biocontrol agent in the field. Here, we purified several lipopeptides from the cell-free supernatant of the BC32-1 culture media and identified that C_{17}-fengycin B is a major antifungal compound of the BC32-1 to control the mycelial growth of *F. oxysporum*. These studies indicate that the BC32-1 strain is a promising biocontrol agent for fungal plant pathogens and diseases.

2. Materials and Methods

2.1. Microbial strains and culture conditions

Bacillus amyloliquefaciens subsp. *plantarum* BC 32-1 was isolated from yellow loess soil in the Jeonnam province of South Korea and was cultured at 37°C [17]. The strain was stored in tryptic soy broth (TSB, BD) broth containing 20% glycerol at −70°C. The plant fungal pathogen *F. oxysporum* f. sp. *radicis-lycopersici* KACC 40031 was provided by the Korean Agricultural Culture Collection (KACC; Suwon, South Korea). The fungal pathogens obtained from KACC were subcultured twice on a potato dextrose agar (PDA, BD) plate and incubated at 26 ± 2°C for 3 ~ 5 days with the BC32-1 strain or antifungal lipopeptide samples.

2.2. Extraction and purification of lipopeptides

After culturing the cells in Luria-Bertani (LB, Bio Basic Inc.) at 37°C, the broth was centrifuged for 10 min at 8,000 × *g* and a cell-free supernatant was obtained. The lipopeptides were obtained by extracting the supernatant with ethyl acetate three times and removing the solvent under reduced pressure. The crude extract was dissolved in methanol and was applied to reverse-phase high-performance liquid chromatography (RP-HPLC) equipped with a C_{18} column (Waters μBondapak® C_{18}; 300 mm × 3.9 mm). The mobile phase components were 0.1% trifluoroacetic acid (TFA) in water (mobile phase A) and 0.1% TFA in acetonitrile (mobile phase B). The products were eluted using a linear gradient of mobile phase B (increasing from 5 to 95%) at a flow rate of 1 mL/min for 50 min, and monitored by measuring the absorbance at 230 nm. Fractions were collected and lyophilized to assess their antifungal activity against *F. oxysporum* f. sp. *radicis-lycopersici*.

2.3. Spectroscopy and mass spectrometry

The UV spectrum was recorded by a photodiode array detector (Shimadzu SPD-M10Avp, Shimadzu, Japan) from 190 to 330 nm, at a resolution of 1 nm. The IR sample was dissolved in methanol and the IR spectrum was measured using an FT-near infrared spectrometer (FT-NIR; Spotlight400™, Perkin Elmer, UK). The lipopeptides were characterized using liquid chromatography-mass spectrometry (LC-MS) analysis (API2000™, AB Sciex, USA) by elution with water (mobile phase A)/acetonitrile (mobile phase B) containing 0.1% formic acid. The products were eluted using a linear gradient of mobile phase B (increasing from 5 to 95%) at a flow rate of 1 mL/min for 50 min. MS was performed using a triple quadrupole equipped with an ESI source.

2.4. Determination of minimal inhibitory concentration (MIC)

The antifungal activity of purified C_{17}-fengycin B against *F. oxysporum* was tested in sterile 96-well plates. Aliquots (100 μL) of the *F. oxysporum* suspension at 1 × 10^4 cell/mL in molten PDA (counted by hemocytometer) were added to

sample solutions (100 μL). After incubation for 3 days at 27°C, the MIC was determined by visual examination based on the lowest concentration of sample solution needed to ensure that no fungal growth was observed. All independent experiments were repeated three times. We also performed disc diffusion assay with various concentrations of fengycin to confirm the MIC value.

2.5. Bacterial growth curve of the BC32-1 strain

Growth curve characteristics of the bacteria were studied by inoculating overnight grown culture in LB broth and incubating at 25 and 37°C. Population was determined at intervals of 2 h, for 24 h, by measuring absorbance at 600 nm.

2.6. Quantification of C_{17}-fengycin B concentration

C_{17}-fengycin B lipopeptide was dissolved in acetonitrile and diluted to obtain different concentrations (2, 5, 10, 15, and 20 μg/mL) as standard solutions. Standard solution (50 μL) was injected into the RP-HPLC sequentially using the same chromatographic conditions described above, and the linear regression equation was obtained. A calibration curve was constructed as a function of the concentrations of standard analytes (*x*) *vs.* their peak areas (*y*). Culture broth was collected every 2 h and used to determine the quantities of the lipopeptide produced.

3. Results and Discussion

3.1. Screening and identification of antifungal bacteria

The inhibition of *F. oxysporum* f. sp. *radices-lycopersici* KACC 40031 by bacterial isolates was tested in PDA medium. Suppression of mycelial growth was estimated after further incubation for more than 3 days. The BC32-1 strain exhibited a strong antifungal activity and was selected for further experiments (Fig. 1A).

3.2. Identification and purification of lipopeptides

The lipopeptides in the culture supernatant of the BC32-1 strain were identified by LC-MS analysis, which revealed iturin-, surfactin-, and fengycin-type lipopeptides (Fig. 2). Several lipopeptides were obtained from RP-HPLC purifications, and each fraction was lyophilized for use in antifungal experiments. After fractionation, the active fractions were identified by monitoring their antifungal activity against *F. oxysporum* f. sp. *radicis-lycopersici*. Among them, the fengycin-type lipopeptides showed effective antifungal activities against *F. oxysporum* (Fig. 1B). In particular, the fengycin with *m/z* 1506 Da showed higher growth-inhibitory activity with an MIC of 50 μg/mL against *F. oxysporum* (supplementary Fig. S1) than fengycin A lipopeptide (MIC of 200 μg/mL) [17].

3.3. Structural analysis of C_{17}-fengycin B

The BC32-1 strain produced several types of lipopeptide. Since the fengycin with *m/z* 1506 Da showed the highest antifungal activity against the *F. oxysporum*, we investigated the structural characteristics of the fengycin using UV and FT-IR spectroscopy. The UV spectrum of the fengycin was examined at wavelengths of 190 ~ 330 nm. The lipopeptide displayed maximal absorbance between 220 and 240 nm, which is characteristic of proteins and peptides (Fig. 3A). The lipopeptide nature of the fengycin was further confirmed using IR spectroscopy (Fig. 3B). Characteristic absorbances at around 3,370, 1,670, and 1,540/cm indicated that the compound contained peptide bonds. Absorbance bands were also detected at 2,930 and 2,860/cm, which indicated the presence of aliphatic chains. The absorbances at 1,190 and 1,140/cm may have been associated with the

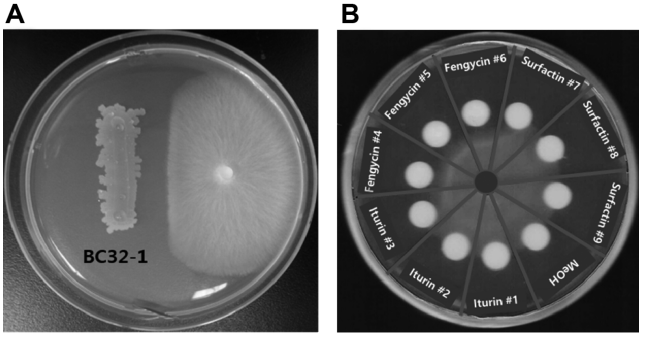

Fig. 1. Inhibition of growth of *Fusarium oxysporum* f. sp. *radices-lycopersici* KACC 40031 on PDA plates. (A) BC32-1 strain showed a strong antifungal activity. (B) BC32-1–derived lipopeptide fractions purified using RP-HPLC. Fengycin-type lipopeptides exhibited higher growth-inhibitory activity than iturins and surfactins. The lipopeptides of 1 mg/mL concentration were used for Fig. 1B.

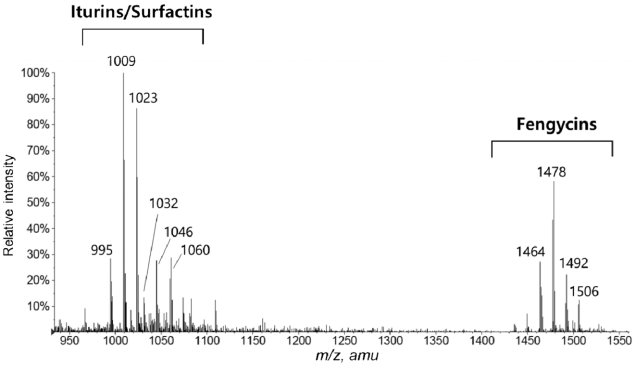

Fig. 2. Positive ESI-MS spectrum of crude extract of BC32-1 strain. Clusters of iturin (*m/z* 1032, 1046, and 1060), surfactin (*m/z* 995, 1009, and 1023), and fengycin (*m/z* 1464, 1478, 1492, and 1506) molecular ion species are labeled.

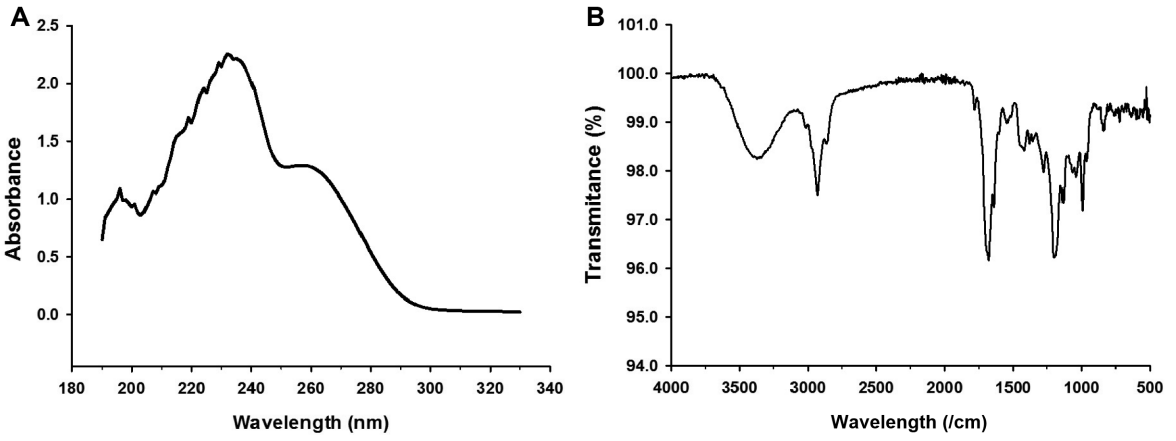

Fig. 3. (A) UV and (B) FT-IR spectra of the C_{17}-fengycin B lipopeptide (*m/z* 1506) produced by *Bacillus amyloliquefaciens* BC32-1.

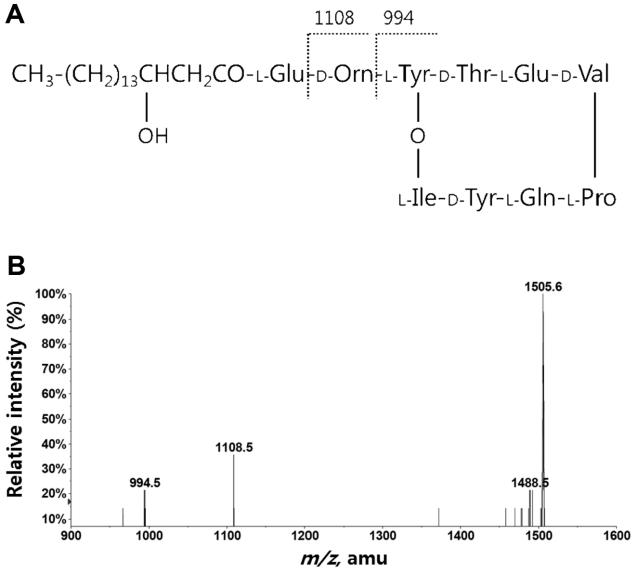

Fig. 4. Structure and mass spectrum of C_{17}-fengycin B. (A) Cyclic structure and proposed fragmentations of C_{17}-fengycin B (product ions *m/z* 1108 and 994). (B) Mass spectrum showing fragments peaks $[M + H]^+$ of C_{17}-fengycin B at *m/z* 1506.

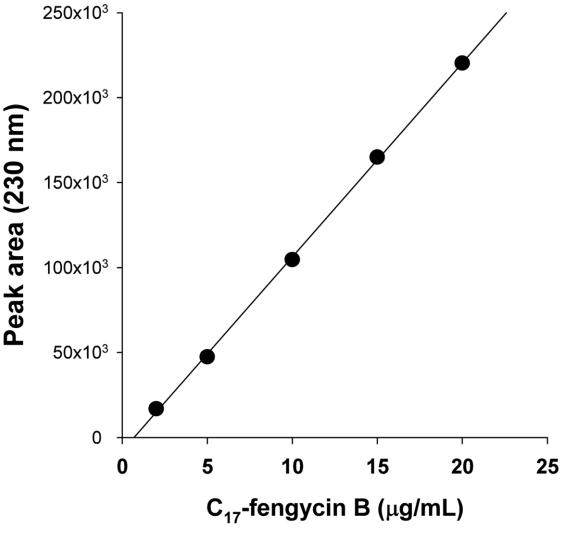

Fig. 5. Calibration curves exhibited good linear regression ($Y = 11413x$ 7823, $r^2 = 0.999$).

C-O-C vibrations of esters. Together, the IR spectra were consistent with those of previously reported for fengycin-type lipopeptides [18].

The chemical structure of the fengycin with *m/z* 1506 was further analyzed using LC-MS/MS using *m/z* 1506 as the precursor ion (Fig. 4) [19]. The data showed the appearance of product ions at *m/z* 1108 and 994 from precursor ions at *m/z* 1506 (Fig. 4). This confirmed that the fengycin at *m/z* 1506 was fengycin B containing a C_{17} lipid chain (referred to as C_{17}-fengycin B).

3.4. Temperature-dependent production of C_{17}-fengycin B

We measured the amount of C_{17}-fengycin B produced at 25 and 37°C. Standard calibration curves were prepared with purified C_{17}-fengycin B (Fig. 5). The growth rate of the bacteria was slower at 25°C than at 37°C (Fig. 6A). The bacterial growth at 37°C reached the stationary phase in 16 h, while the optical density at 600 nm (OD_{600}) continually increased until 24 h at 25°C. To determine the amount of C_{17}-fengycin B that was produced by the BC32-1 strain, the amount of peptide in the crude extract was quantified using RP-HPLC analysis. The amount of C_{17}-fengycin B continuously increased during incubation at 37°C. At temperature 25°C, the amount of C_{17}-fengycin B remained low for 24 h (Fig. 6B). These results suggest that production of C_{17}-fengycin B is highly dependent on the culture temperature and that BC32-1 strain should be cultured at around 37°C to effectively produce fengycin-type lipopeptides.

3.5. Significance of BC32-1 strain as a biocontrol agent

The pathogenic *F. oxysporum* f. sp. *radicis-lycopersici*

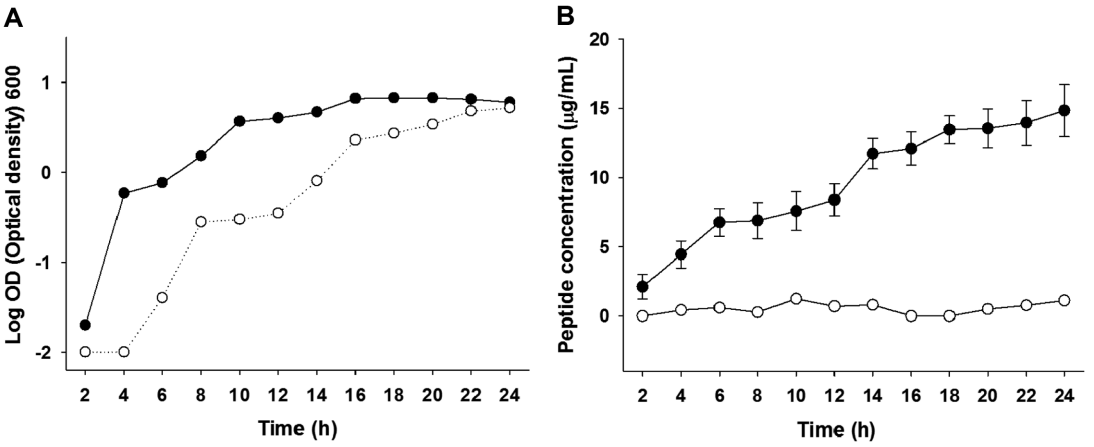

Fig. 6. (A) Growth curves of *B. amyloliquefaciens* BC32-1 in LB media at 37°C (filled circle) and 25°C (open circle). (B) Amount of C_{17}-fengycin B produced by *B. amyloliquefaciens* BC32-1 at 37°C (filled circles) and 25°C (open circles). Data show means and standard deviation of three independent experiments.

infects a broad range of host species, causing wilt or rot disease in significant agricultural plants [20]. For example, tomato foot and root rot (TFRR) is a disease caused by *F. oxysporum*. Because TFRR cannot be efficiently treated by chemical pesticides, the use of bacterial biocontrol agents is a promising alternative to prevent the infection [21]. The use of rhizosphere-isolated bacteria as biocontrol agents, such as *Bacillus* species, may provide advantages for control of microbial plant disease, mainly because of their ability to form endospores, which are highly resistant to harsh environmental conditions [16]. *B. amyloliquefaciens*, which is closely related to *B. subtilis*, produces structurally diverse antimicrobial compounds containing cyclic lipopeptides belonging to families such as iturin, fengycin, and surfactin, which confer strong antifungal activities. In particular, the fengycin family has well-known potential uses in biotechnology owing to its excellent antifungal and antibacterial properties. Here, we studied antifungal lipopeptides produced by *B. amyloliquefaciens* subsp. *plantarum* BC 32-1. Among them, C_{17}-fengycin B was produced efficiently by the bacterium, and showed strong antifungal activity against *F. oxysporum* f. sp. *radicis-lycopersici* and other phytopathogenic fungi (supplementary Fig. S2). The results suggest that the BC 32-1 strain is a promising biocontrol agent in preventing plant diseases caused by *F. oxysporum* f. sp. *radicis-lycopersici*. We are currently studying the bacterium in field experiments, for use as a biocontrol agent against various fungal plant diseases.

4. Conclusion

We characterized and investigated the structure and production of C_{17}-fengycin B from *B. amyloliquefaciens* BC32-1 that was isolated from yellow loess soil. C_{17}-fengycin B was identified using UV, FT-IR, and LC-MS/MS analyses and was produced in a temperature-dependent manner with production being higher at a higher temperature. C_{17}-fengycin B showed strong antifungal activity with an MIC of 50 μg/mL. Our data suggest that C_{17}-fengycin B is a major antifungal lipopeptide produced by the BC32-1 strain that could be used as an antifungal agent against the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici.*

Acknowledgements

This study was supported by the Bio-industry Technology Development Program 2012, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea (Grant No. 112003-3).

References

1. Compant, S., B. Duffy, J. Nowak, C. Clement, and E. A. Barka (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol*. 71: 4951-4959.
2. Ongena, M. and P. Jacques (2008) *Bacillus* lipopeptides: Versatile weapons for plant disease biocontrol. *Trends Microbiol*. 16: 115-125.
3. Raaijmakers, J. M., I. de Bruijn, and M. J. de Kock (2006) Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: Diversity, activity, biosynthesis, and regulation. *Mol. Plant Microbe Interact.* 19: 699-710.
4. Katz, E. and A. L. Demain (1977) The peptide antibiotics of *Bacillus*: Chemistry, biogenesis, and possible functions. *Bacteriol. Rev.* 41: 449-474.
5. Schneider, T., A. Muller, H. Miess, and H. Gross (2014) Cyclic

lipopeptides as antibacterial agents - potent antibiotic activity mediated by intriguing mode of actions. *Int. J. Med. Microbial*. 304: 37-43.
6. Mondol, M. A. M., H. J. Shin, and M. T. Islam (2013) Diversity of secondary metabolites from marine *Bacillus* Species: Chemistry and biological activity. *Mar. Drugs*. 11: 2846-2872.
7. Bernal, G., A. Illanes, and L. Ciampi (2002) Isolation and partial purification of a metabolite from a mutant strain of *Bacillus* sp with antibiotic activity against plant pathogenic agents. *Electron. J. Biotechnol*. 5: 12-20.
8. Delcambe, L., F. Peypoux, F. Besson, M. Guinand, and G. Michel (1977) Structure of iturin and iturin-like substances [proceedings]. *Biochem. Soc. T*. 5: 1122-1124.
9. Peypoux, F., J. M. Bonmatin, and J. Wallach (1999) Recent trends in the biochemistry of surfactin. *Appl. Microbiol. Biotechnol*. 51: 553-563.
10. Raaijmakers, J. M., I. De Bruijn, O. Nybroe, and M. Ongena (2010) Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: More than surfactants and antibiotics. *FEMS Microbial. Rev*. 34: 1037-1062.
11. Vanittanakom, N., W. Loeffler, U. Koch, and G. Jung (1986) Fengycin-a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. *J. Antibiot*. 39: 888-901.
12. Deleu, M., M. Paquot, and T. Nylander (2008) Effect of fengycin, a lipopeptide produced by *Bacillus subtilis*, on model biomembranes. *Biophys. J*. 94: 2667-2679.
13. Romano, A., D. Vitullo, A. Di Pietro, G. Lima, and V. Lanzotti (2011) Antifungal lipopeptides from *Bacillus amyloliquefaciens* strain BO7. *J. Nat. Prod*. 74: 145-151.
14. Chen, X. H., A. Koumoutsi, R. Scholz, A. Eisenreich, K. Schneider, I. Heinemeyer, B. Morgenstern, B. Voss, W. R. Hess, O. Reva, H. Junge, B. Voigt, P. R. Jungblut, J. Vater, R. Süssmuth, H. Liesegang, A. Strittmatter, G. Gottschalk, and R. Borriss (2007) Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nat. Biotechnol.* 25: 1007-1014.
15. Arguelles-Arias, A., M. Ongena, B. Halimi, Y. Lara, A. Brans, B. Joris, and P. Fickers (2009) *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. *Microb. Cell Fact.* 8: 63.
16. Zhao, P., C. Quan, Y. Wang, J. Wang, and S. Fan (2014) *Bacillus amyloliquefaciens* Q-426 as a potential biocontrol agent against *Fusarium oxysporum* f. sp. *Spinaciae*. *J. Basic. Microbiol.* 54: 448-456.
17. Nam, J., H. Yun, J. Kim, P. I. Kim, S. W. Kim, H. B. Lee, J. I. Kim, and C. W. Lee (2015) Isolation and NMR analysis of antifungal Fengycin A and B from *Bacillus amyloliquefaciens* subsp. *plantarum* BC32-1. *Bull. Kor. Chem. Soc*. 36: 1316-1321.
18. Benitez, L. B., R. V. Velho, M. P. Lisboa, L. F. Medina, and A. Brandelli (2010) Isolation and characterization of antifungal peptides produced by *Bacillus amyloliquefaciens* LBM5006. *J. Microbiol*. 48: 791-797.
19. Pecci, Y., F. Rivardo, M. G. Martinotti, and G. Allegrone (2010) LC/ESI-MS/MS characterisation of lipopeptide biosurfactants produced by the *Bacillus licheniformis* V9T14 strain. *J. Mass Spectrom.* 45: 772-778.
20. Szczechura, W., M. Staniaszek, and H. Habdas (2013) *Fusarium oxysporum* f. sp. *radicis-lycopersici* – the cause of *fusarium* crown and root rot in tomato cultivation. *J. Plant. Prot. Res.* 53: 172-176.
21. Kamilova, F., L. V. Kravchenko, A. I. Shaposhnikov, N. Makarova, and B. Lugtenberg (2006) Effects of the tomato pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici* and of the biocontrol bacterium *Pseudomonas fluorescens* WCS365 on the composition of organic acids and sugars in tomato root exudate. *Mol. Plant Microbe Interact*. 19: 1121-1126.