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Isolation and Identification of Lipopeptide Antibiotics from *Paenibacillus elgii* B69 with Inhibitory Activity Against Methicillin-Resistant *Staphylococcus aureus*

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Two lipopeptide antibiotics, pelgipeptins C and D, were isolated from *Paenibacillus elgii* B69 strain. The molecular masses of the two compounds were both determined to be 1,086 Da. Mass-spectrometry, amino acid analysis and NMR spectroscopy indicated that pelgipeptin C was the same compound as BMY-28160, while pelgipeptin D was identified as a new antibiotic of the polypeptin family. These two peptides were active against all the tested microorganisms, including antibiotic-resistant pathogenic bacterial strains such as methicillin-resistant *Staphylococcus aureus* (MRSA). Time-kill assays demonstrated that pelgipeptin D exhibited rapid and effective bactericidal action against MRSA at 4×MIC. Based on acute toxicity test, the intraperitoneal LD_{50} value of pelgipeptin D was slightly higher than that of the structurally related antimicrobial agent polymyxin B. Pelgipeptins are highly potent antibacterial and antifungal agents, particularly against MRSA, and warrant further investigation as possible therapeutic agents for bacteria infections resistant to currently available antibiotics.

***Keywords*:** *Paenibacillus elgii*, polypeptins, pelgipeptins, lipopeptide antibiotics

Overuse of antibiotics has led to a growing number of antibiotic-resistant pathogenic bacterial strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Muto *et al.*, 2003; Klevens *et al.*, 2007). The Centers for Disease Control and Prevention of USA estimated that MRSA was responsible for 94,360 serious infections and 18,650 nosocomial deaths in the United States in 2005, a mortality rate exceeding that caused by AIDS (Klevens *et al*., 2007). The glycopeptide antibiotic vancomycin has long been used to treated MRSA infections (Levine, 2006; Moellering, 2006), but vancomycin-resistant bacteria have also emerged (Weigel *et al.*, 2003). Thus, there is an urgent need for new potent antimicrobial agents, particularly against multidrug-resistant pathogens.

Lipopeptide antibiotics, generally consisting of a hydrophilic cyclic peptide attached to a fatty acid chain, represent a promising new class of antibiotics against multidrug-resistant pathogens. Lipopeptide antibiotics include the established polymyxins, but also new compounds such as the recently approved daptomycin (Pirri *et al.*, 2009). Endospore-forming bacteria like *Bacillus* and *Paenibacillus* are well known for their ability to produce lipopeptide antibiotics (Wang and Liu, 2008; Chen *et al.*, 2010). In recent years, a number of bioactive compounds with impressive potential applications have been identified. For instance, the potential biocontrol agents surfactant, bacillomycin, iturin A, and fengycin are isolated from *Bacillus* strains (Kim *et al.*, 2004, 2010), while the potent antimicrobials polymyxins and fusaricidins are produced by *Paenibacillus* strains (Li *et al.*, 2007; Wu *et al.*, 2011). *Paenibacillus* was first defined as a new genus in 1993 after detailed comparative analysis of 51 species of the genus *Bacillus*. At that time, the genus contained 11 species, including the prototype species *Paenibacillus polymyxa* (Ash *et al.*, 1993; Araújo da Silva *et al.*, 2003). The genus *Paenibacillus* has now expanded to include 120 recognized species (http://www.bacterio.cict.fr/p/paenibacillus.html), and these new *Paenibacillus* species may be a valuable source for many novel lipopeptide antibiotics.

Paenibacillus elgii B69, first isolated from mountain soil in Tianmu Mountain National Reserve in Hangzhou, China, possesses potent antimicrobial activity against a wide range of pathogens (Wu *et al.*, 2010). Two lipopeptide antibiotics, pelgipeptins A and B, were isolated from this organism previously. Through the optimization of fermentation conditions and chromatography during the separation of bioactive molecules, two additional compounds with potent activity against MRSA were obtained from this strain. This study describes the isolation, chemical characterization, antimicrobial efficacy, and toxicity of these two new antibiotics.

Materials and Methods

Strains

Paenibacillus elgii B69 (CCTCC M 2010234) was isolated from a soil sample collected from the Tianmu Mountain National Reserve (Hangzhou, China). The bacteria used in antimicrobial assays, including *Escherichia coli* Top 10, *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213 and ATCC 25923), MRSA ATCC 43300, *Staphylococcus epidermidis* (CMCC 26069), *Enterococcus faecalis* (ATCC 29212), *Psedomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (CMCC 49027), and *Bacillus subtilis* (CGMCC 1.1470) were routinely grown at 30°C on nutrient agar or broth. The pathogenic fungi *Candida albicans* (ATCC 10231) was cultivated on potato dex-

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trose agar at 28°C. ATCC 43300 was used as the indicator strain for bioactivity-guided fractionation and purification. For long-term storage, all the strains were stored in 20% (v/v) glycerol at -80°C.

Optimization of fermentation media

Several media compatible with *Paenibacillus elgii* B69 growth were tested for antimicrobial production, including SSP medium (0.3% sucrose, 0.3% soluble starch, 1% peptone, and 0.5% NaCl), nutrient broth (0.5% peptone, 3.0% beef extract, and 0.5% NaCl), LB medium, potato dextrose broth, and GS medium (2% glycerin, 1% $(NH_4)_2SO_4$, 0.2% $KH_2PO_4 \cdot 3H_2O$, and 0.04% $MgSO_4 \cdot 7H_2O$, at pH 7.2). In order to alter the production and secretion of bioactive agents, different amino acid supplements (each at 1.2 g/L) were tested, including the twenty common amino acids and one nonprotein amino acid (2,4-diaminobutyric acid, DAB). Precultures were grown at 30°C in 100 ml Erlenmeyer flasks containing 20 ml media on a rotary shaker (200 rpm) for 48 h. A sample of the preculture cell suspension (2.5 ml) was then used to inoculate 50 ml of GS media in a 250 ml Erlenmeyer flask. Bacteria were grown at 30°C and 200 rpm for 96 h.

Production and purification of antagonistic compounds

Strain B69 was cultivated in 4 L GS medium supplemented with 1.2 g/L L-Val at 30°C for 96 h in a 7.5-L NBS Bioflo 110 Fermentor (New Brunswick Scientific, USA). A total of 10 L of the cultivation media was used to isolate bioactive compounds. The broth was centrifuged at 6,000×g for 30 min. The cell-free supernatant (CFS) was collected and extracted twice with n-butanol:water at a 1:1 ratio (v/v) and the organic solvent was dried with a rotary vacuum evaporator. The remaining material was dissolved in distilled water and then run on an MCI GEL CHP20P (75-150 μm) column. The column was washed extensively with 45% aqueous methanol and then eluted with 85% aqueous methanol. Each fraction was collected and tested for antibacterial activity using the agar diffusion method (Wu *et al.*, 2010). The active fractions eluted with 85% methanol in water were combined, and then purified on a reversed-phase HPLC system (Dalian Elite, China) equipped with a YMC-pack DOS-A C_{18} column (5 μm, 250×20 mm). The mobile phases were Milli-Q water with 0.2% formic acid (solution A) and acetonitrile (solution B). A linear gradient of 28-42% solvent B (60 min) was used for elution at a flow rate of 8 ml/min. The elution was monitored at 210 nm. All isolatable peaks were collected and assessed for antibacterial activity. Active fractions were evaporated and stored at -20°C until further analysis.

Structure analysis

Electrospray ionization (ESI) and collision-induced dissociation (CID) mass spectrometry analysis were performed on a Thermo Finnigan LCQ Advantage system (Thermo Electron Corporation, USA). The electrospray source was operated at a capillary voltage of 46 V, a source voltage of 4.5 kV, and a capillary temperature of 300°C. For the CID experiment, helium was used as the collision gas and the collision energy was set at 25%. The amino acid analyses were carried out using the advanced Marfey's method with LC/MS. The FDLA derivatives of the purified antibiotics were obtained as described (Fujii *et al.*, 1999). Then, L-type and D-type derivatives were loaded on a ZORBAX SB-C_{18} analytical column (3.5 μm, 150×2.1 mm). The mobile phases were 20 mM ammonium acetate adjusted to pH 2.0 with formic acid (solution A) and acetonitrile (solution B). The following HPLC program was used: 10-20% B (5 min), 20-50% B (35 min), and 50-90% B (5 min). The flow rate was 0.2 ml/min. Elution was monitored by 340 nm absorbance. For nuclear magnetic resonance spectroscopy, the sample was dissolved in deuterated pyridine, or 90% H_2O and 10% D_2O solution, and the data were obtained using an AVANCE DMX-600 spectrometer (Bruker) at 27°C.

Biological assay

The minimum inhibitory concentrations (MICs) of the purified compounds against microorganisms were determined using a microbroth dilution method in 96 multi-well microtiter plates as reported previously (McVay and Rolfe, 2000). Methanol (10 μl) and Mueller-Hinton broth (0.2% meat extracts, 1.75% acid digest of casein, and 0.15% starch) alone were used as negative controls. Vancomycin and polymyxin B, freshly dissolved in sterile-distilled water, were used as positive controls. Overnight cultures were used to prepare inoculants of 10^6 CFU/ml strain. The plates were incubated at 35°C, and visible growth and optical density (OD) at 630 nm were recorded after 18 h of incubation and read with a Multi-scan MK3 UV microtitre reader (Thermo Electron, China). The MIC was defined as the lowest concentration that completely prevented visible growth after incubation at 35°C for 18 h.

Time-kill assays

Time-kill assays were performed to further evaluate the antibacterial activity of isolated bioactive compound against MRSA ATCC 43300 according to the procedure described previously (McKay *et al.*, 2009). Overnight culture was used to inoculate fresh Mueller-Hinton broth containing different concentrations pelgipeptin D with 10^6 CFU/ml MRSA ATCC 43300. The culture was incubated with shaking (120 rpm) at 37°C for 24 h. Surviving bacteria were counted after 0, 3, 6, and 24 h of incubation by sub culturing 100 μl serial dilutions of samples in 0.9% sodium chloride on Mueller-Hinton plates. All tests were carried out in triplicate. A bactericidal effect was defined as a ≥3 log10 CFU/ml decrease compared to the initial inoculums after 24 h of incubation.

Toxicity of pelgipeptin D in mice

Juvenile KM mice, 6 to 8 weeks old and weighing 17 to 22 g, were used to test the toxicity of the newly isolated antimicrobials (Slaccas Laboratory Animal Co., Ltd., China). Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996). Moribund mice were humanely euthanatized by cervical dislocation when it was determined that death was imminent. Each test group consisted of 20 mice (10 male and 10 female). For the intraperitoneal toxicity test, mice were intraperitoneally injected with 0.2 ml of pelgipeptin D dissolved in 0.5% CMC-Na solution at doses of 9.4, 18.7, 29.2, 36.7, 45.4, and 55.0 mg/kg. The number of dead mice at each dose was counted daily for 14 days. Medial lethal dose (LD_{50}) was calculated by probit analysis with 95% confidence intervals from these data. For the oral toxicity test, 0.2 ml of pelgipeptin D dissolved in 0.5% CMC-Na solution was orally administered with to mice at 50, 100, 200, 400, and 560 mg/kg. Mice of the control group were administered 0.2 ml 0.5% CMC-Na solution. The mice were observed for 14 days and reweighed on day 14. The difference between the test group and the control group body weight after treatment was evaluated by a paired, two-tailed Student's t-test using SPSS software (version 16.0).

Measurement of hemolytic activity

To measure hemolytic activity, freshly collected human red blood cells with heparin were centrifuged to remove the buffy coat, and the erythrocytes obtained were washed three times in 0.9% saline. The red

blood cells were then incubated for 4 h in 0.9% saline with pelgipeptin D at 50, 100, 150, 200, and 250 μg/ml. The hemoglobin released in the supernatant was measured at 570 nm (OD_{570}) on a microplate reader (Zhao *et al*., 2009).

Results and Discussion

Production and isolation of antibiotics

Two antibiotics, pelgipeptins A and B, with broad antimicrobial activity were isolated from the culture medium of *P. elgii* B69 in our previous work (Wu *et al*., 2010). Further study indicated that two additional bioactive analogs, named pelgipeptins C and D, were presented in the fermentation supernatants. In order to increase the production of these two unidentified antimicrobial compounds, strain B69 was cultivated in five different media. Results indicated that the highest total antibacterial activity was obtained after 96 h at 30°C in GS medium. Under this condition, however, pelgipeptin B was the major antibiotic released during fermentation, along with only minor amounts of pelgipeptins C and D (Fig. 1A).

Supplementing the basic growth medium with different precursors can alter the relative yields of various bacterial products (Thiericke and Rohr, 1993). In an attempt to increase the relative amounts of pelgipeptins C and D, we cultured *P. elgii*

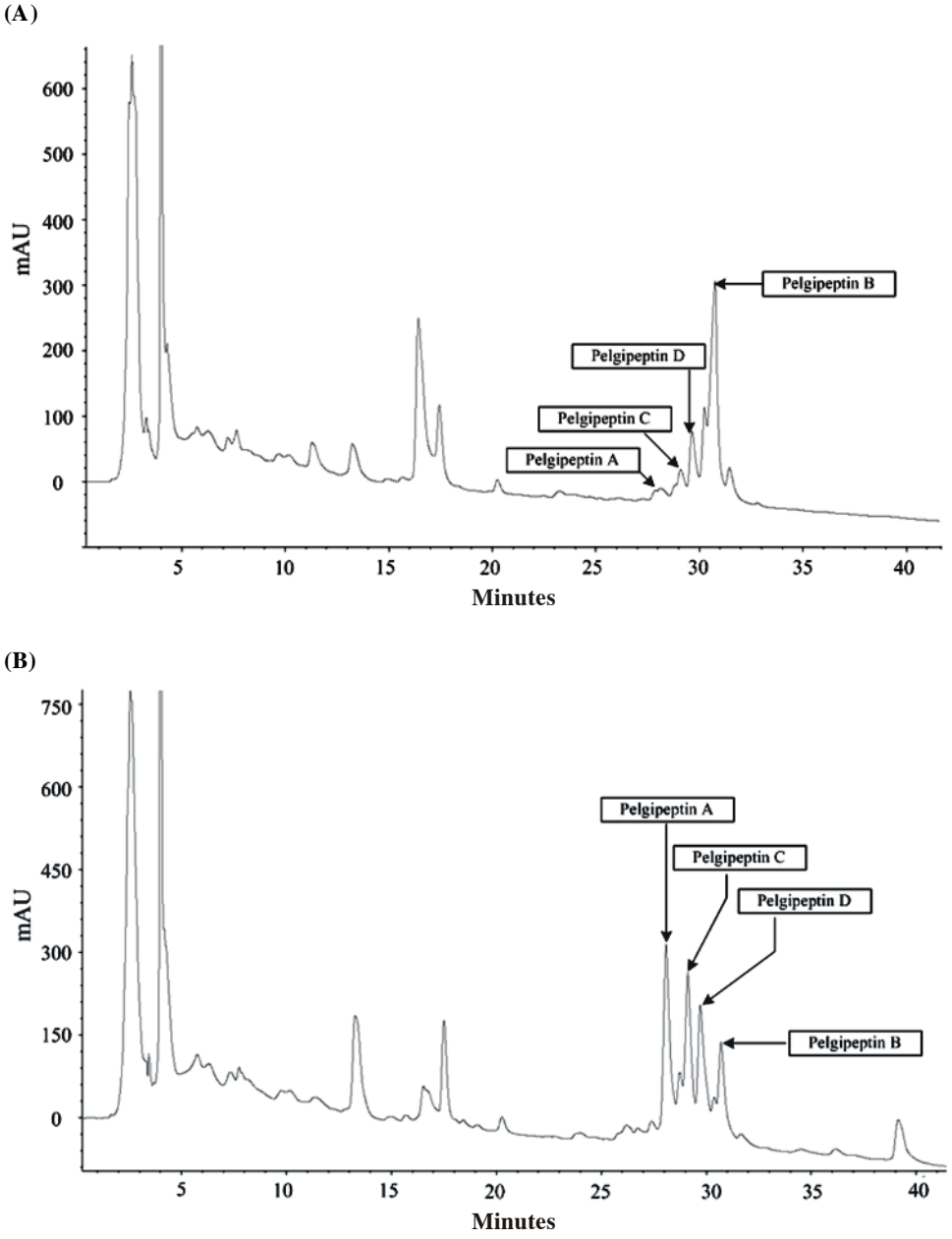

Fig. 1. Effect of amino acids supplementation to GS media on pelgipeptin production. (A) The control fermentation. (B) The fermentation added with L-Val (1.2 g/L).

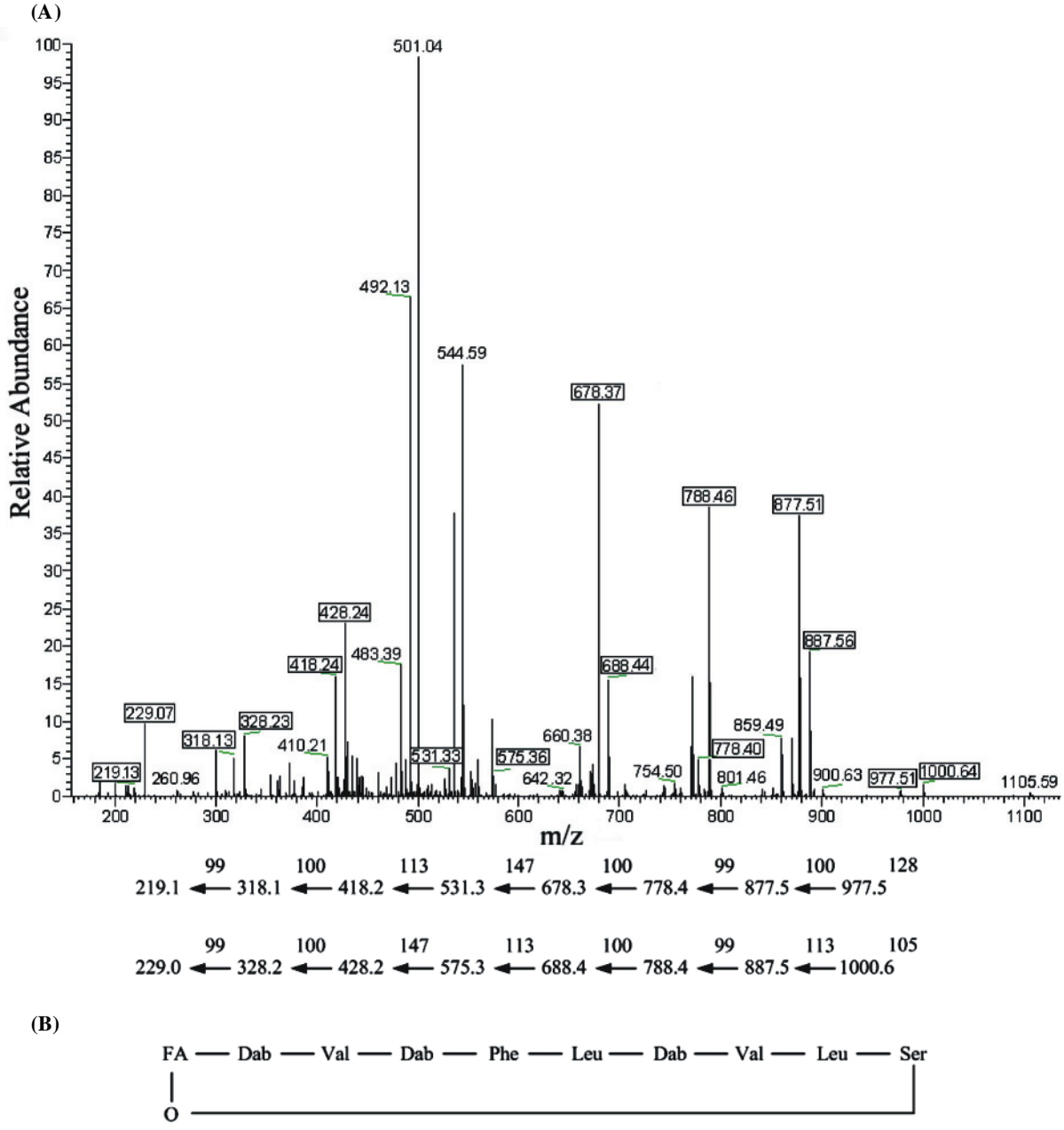

Fig. 2. (A) MS/MS spectrum of the doubly charged precursor ion at m/z 553.2 of the hydrolyzed pelgipeptin C. (B) Proposed structure of pelgipeptin C. Val, valine; Phe, phenylalanine; Leu, leucine; Ser, serine; FA, 3-hydroxy-4-methylhexanoic acid.

B69 in GS media supplemented with different amino acids at 1.2 g/L. Although the addition of tested amino acids generally improved the total antimicrobial activity of culture supernatants, HPLC profiles indicated that the major product in most cases was also pelgipeptin B. However, with the addition of L-Val to the medium, pelgipeptin B production was reduced approximately 4-fold, while recoveries of pelgipeptins C and D increased approximately 10-fold and 2-fold, respectively (Fig. 1B). Hence, a large-scale cultivation of *P. elgii* B69 in GS medium with 1.2 g/L L-Val was performed, followed by extraction and purification of pelgipeptins C and D by HPLC. The final yield was approximately 10.3 mg/L pelgipeptin C and 8.7 mg/L for pelgipeptin D.

Structure analysis of antibiotics

ESI-MS was chosen initially to determine the molecular masses. The $[M+H]^+$ ions of both pelgipeptins C and D were 1,087 Da. Thus, the molecular masses of pelgipeptins A to D were 1072, 1100, 1086, and 1086 Da, respectively. The molecular masses differed by only 14 Da, suggesting that they were homologous molecules and were all members of the polypeptin family (Wu *et al*., 2010). The new isolated compounds with

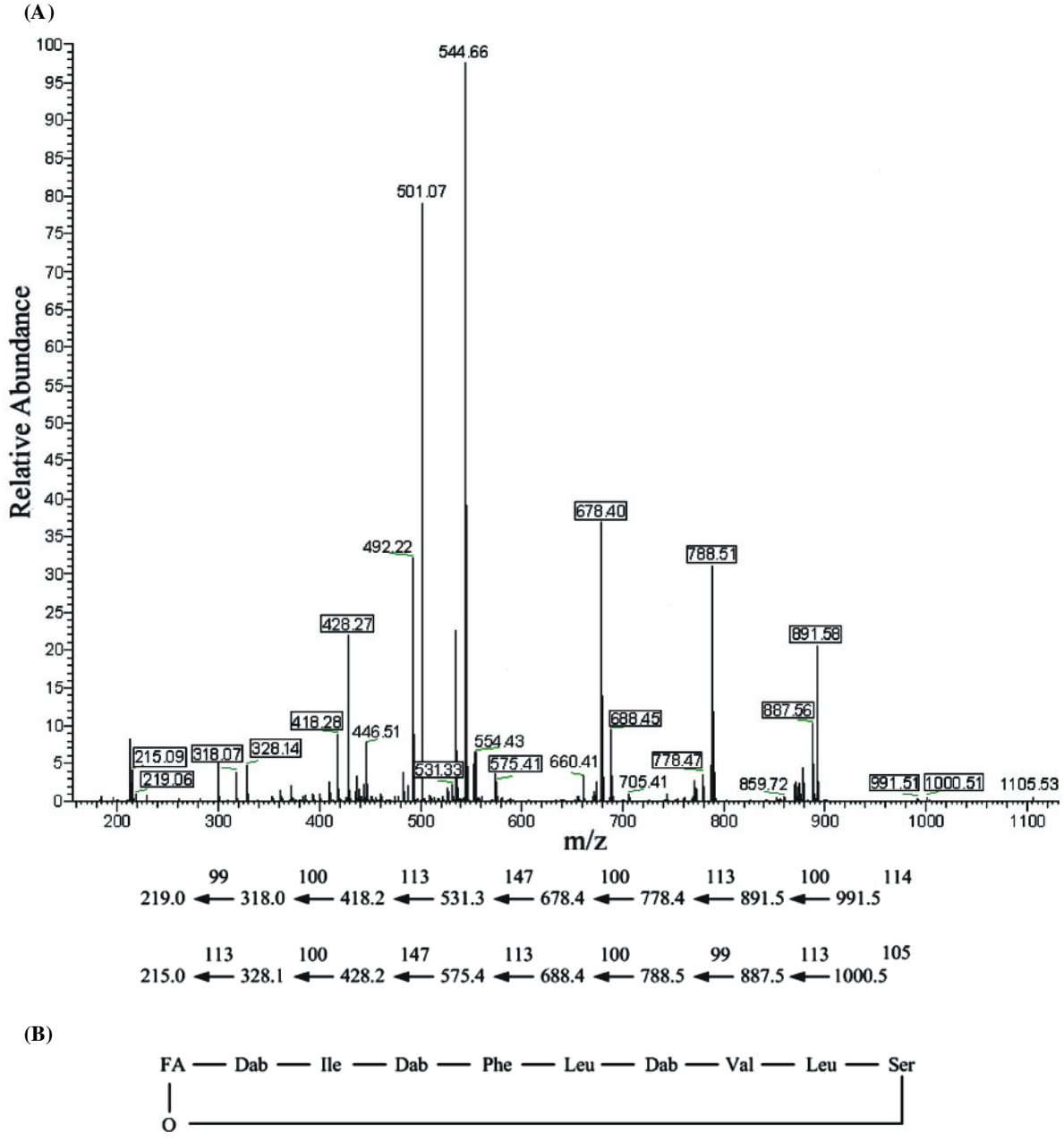

Fig. 3. (A) MS/MS spectrum of the doubly charged precursor ion at m/z 553.3 of the hydrolyzed pelgipeptin D. (B) Proposed structure of pelgipeptin D. Val, valine; Phe, phenylalanine; Leu, leucine; Ile, isoleucine; Ser, serine; FA, fatty acid.

protonated molecular masses of 1,087 Da were alkaline hydrolyzed in 1 M KOH solution overnight at room temperature. Each molecule yielded a new product with a MH^+ of 1,105 Da. The mass gain of 18 Da observed for each product was attributed to the hydrolysis of a lactone. The result indicated that each compound contained a cyclic domain, analogous to previously characterized lipopeptides of the polypeptin family (Sogn, 1976; Takeuchi *et al*., 1979; Sugawara *et al.*, 1984). The molecular masses of pelgipeptins C and D were identical to that of BMY-28160, suggesting that they were probably the same compound or homologous molecules.

To determine the amino acid sequences, collision-induced dissociation (CID) was applied to the purified pelgipeptin C before and after the lactone ring was opened, because more information about the amino acid sequence was obtained from the spectrum of the open form. The MS-MS spectrum of the doubly charged precursor ion at m/z 553.2 of the hydrolyzed pelgipeptin C is shown in Fig. 2A. Successive fragmentations from the two termini of the ring-opened lipopeptide resulted in b-type ions at m/z 1000.6, 887.5, 788.4, 688.4, 575.3, 428.2, 328.2, and 229.0, along with corresponding y-type ions detected at m/z 977.5, 877.5, 778.4, 678.3, 531.3,

Table 1. *In vitro* activities of pelgipeptins against tested strains

Indicator strain	MIC (μg/ml)			
	Pelgipeptin C (1086 Da)	Pelgipeptin D (1086 Da)	Polymyxin B	Vancomycin
Gram-positive				
B. subtilis CGMCC 1.1470	3.12	1.56	25	ND[a]
E. faecalis ATCC 29212	12.5	12.5	100	ND[a]
S. epidermidis CMCC 26069	1.56	1.56	6.25	ND[a]
S. aureus ATCC 25923	12.5	12.5	100	1
S. aureus ATCC 29213	25	12.5	>100	2
MRSA ATCC 43300	12.5	12.5	100	1
Gram-negative				
E. coli Top 10	6.25	6.25	0.78	ND[a]
P. vulgaris CMCC 49027	>100	100	>100	ND[a]
P. aeruginosa ATCC27853	50	25	3.12	ND[a]
Fungi				
C. albicans ATCC 10231	25	6.25	100	ND[a]

[a] ND, not detected

418.2, 318.1, and 219.1. These fragment ions allowed for assignment of the following sequence: Dab1-Val2-Dab3-Phe4-Leu5/Ile5-Dab6-Val7-Leu8/Ile8-Ser9-OH. The b-type ions at m/z 229.0 corresponded to fatty acid (FA) - Dab1, indicated the fatty acyl moiety had the elemental composition $C_7H_{12}O_2$. These results demonstrated a close structural homology to the lipopeptide BMY-28160. In addition to signals arising from amino acid moieties, two methyls (δ_H 0.94 3H, t, J=6.5, δ_C 10.9; δ_H 1.00 3H, d, J=6.5, δ_C 15.5), an oxygenated methane (δ_H 5.31 1H, m, δ_C 76.4), and a carbonyl (δ_C 174.2) were observed in NMR spectra of pelgipeptin C, which indicated the presence of the same fatty acid in this compound as in BMY-28160. Detailed analysis of NMR spectra of pelgipeptin C revealed no Ile in this compound, indicating that both amino acids at positions 5 and 8 were Leu in pelgipeptin C. Amino acid analysis demonstrated the presence of L-Ser, L-Dab, D-Phe, L-Leu, D-Val, and L-Val in this molecule, further confirming that pelgipeptin C had the same amino acid composition and sequence as the lipopeptide BMY-28160 in Fig. 2B.

Structural elucidation of pelgipeptin D was performed using similar chemical tests. The doubly charged precursor ion at m/z 553.3 of the hydrolyzed pelgipeptin D was selected for CID (Fig. 3A). The fragment ion peak at m/z 1000.5 was explained by the loss of 105.1 Da, corresponding to Ser and water from a protonated molecular ion. The fragment ion peaks at m/z 1000.5 and 887.6 revealed the loss of Leu/Ile. Fragment ions at m/z 991.5, 891.5, 778.4, 678.4, 531.3, 418.2, 318.0, and 219.0 allowed the assignment of the following sequence: Dab1-Leu/Ile2-Dab3-Phe4-Leu/Ile5-Dab6-Val7. Thus, the CID mass spectrum revealed replacement of the Val at the second amino acid in BMY-28160 to Ile/Leu in pelgipeptin D (Fig. 2B). The ion at m/z 215.0 corresponded to FA-Dab1, indicated the fatty acyl moiety had the elemental composition $C_6H_{10}O_2$. In NMR spectra, two characteristic methyls (δ_H 1.04 3H, t, J=7.0, δ_C 11.9; δ_H 1.38 3H, d, J=7.0, δ_C 16.4) indicated the presence of Ile in this compound. As a consequence, there were two molecules of Leu in pelgipeptin D (Fig. 3B). Compared with the published Dab-containing antibiotics, the amino acid compositions of pelgipeptin D and permetin A were the same, but they differed from each other by 14 Da (-CH_2) in FA moieties (Takeuchi *et al.*, 1979). Pelgipeptin D, although having the same mass as BMY-28160, had an Ile at position 2 in place of Val and was unequivocally characterized as a new antibiotic of the polypeptin family.

Polypeptin (previously circulin) is one group of lipopeptide antibiotics first isolated from *Bacillus circulan* in 1948 (Sogn, 1976). It is grouped together with polymyxins (Choi *et al.*, 2009) because they are all cyclic polycationic peptides containing a high percentage of diaminobutyric acid and a fatty acid attached through an amide linkage (Storm *et al.*, 1977). To date, only a few members of this family have been described in detail, including polypeptin A and B (Sogn, 1976), permetin A (Takeuchi *et al*., 1979), and BMY-28160 (Sugawara *et al.*, 1984), which are all produced by *Bacillus circulans.* These antibiotics possess similar chemical structures, but differ in one or two amino acid residues or in the nature of the fatty acid. In our study, four members (pelgipeptins A to D) of this family were derived from *P. elgii* B69, highlighting *P. elgii* as an alternative source for these antibiotics.

The MICs of pelgipeptins

To determine the antimicrobial activities of these compounds, we measured the MICs against a number of gram-positive and gram-negative bacteria and against the pathogenic fungus *Candida albicans* using microdilution methods. As shown in Table 1, both pelgipeptins C and D exhibited inhibitory activity against all the tested strains, and the activities of these two compounds were almost the same. Although polymyxin B showed more potent activity than pelgipeptins C and D against gram-negative bacteria strains, pelgipeptins C and D were approximately four to eight times more active against gram-positive bacteria than polymyxin B, with the MIC values of 12.5-25 μg/ml for pathogenic *S. aureus* strains, including a MRSA strain. Interestingly, *S. epidermidis*, an important cause of medical-device-related infection, was highly susceptible to pelgipeptins C and D (MIC value of 1.56 μg/ml for both). Apart from bacteria, the pathogenic fungus *Candida albicans* was also acutely sensitive to pelgipeptins, especially to pelgipeptin D with the MIC value of 6.25 μg/ml. The activ-

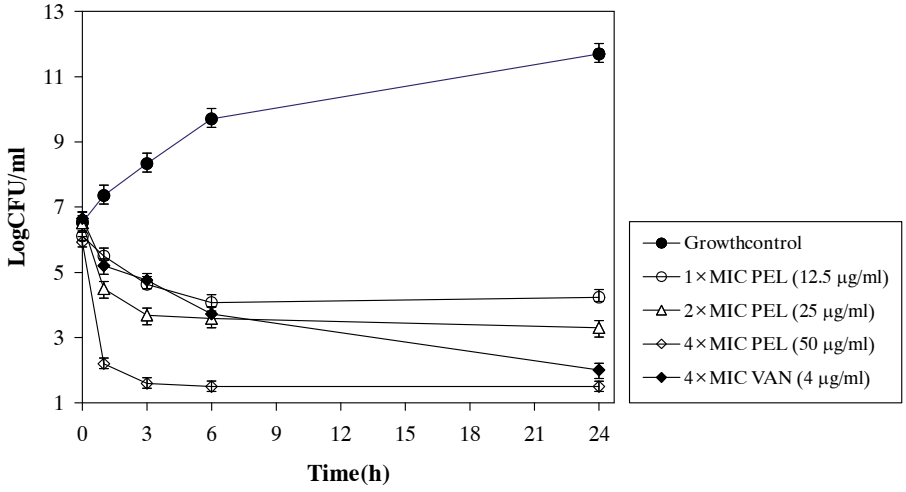

Fig. 4. Time-kill curves of pelgipeptin D against MRSA ATCC 43300 (PEL: pelgipeptin D; VAN: vancomycin).

ities of pelgipeptins C and D were consistent with the broad inhibitory spectrum of polypeptins towards gram-positive and gram-negative bacteria as well as fungi (Storm *et al*., 1977).

In vitro time kill assays

To further evaluate pelgipeptins' antimicrobial activity against MRSA, time-kill assays of pelgipeptin D were conducted. As shown in Fig. 4, pelgipeptin D at 1-2× MIC produced modest bacterial killing (≤2.5 log kill at any time point), but bacterial counts remained stable after 6 h. At 4× MIC, however, pelgipeptin D was bactericidal (≥3 log kill) against MRSA strain ATCC 43300, and yielded almost undetectable bacterial counts (i.e., <100 CFU/ml) by 6 h. Although vancomycin (4 μg/ml) was able to continually reduce the number of viable bacteria below the threshold of detection of this assay by 24 h, pelgipeptin D at 4× MIC killed ATCC 43300 more rapidly than vancomycin during the first 6 h. Collectively, this novel antibiotic compound displayed progressively greater activity as its concentration increased from 1× to 2× to 4× MIC, a feature consistent with a concentration-dependent inhibitory mechanism, and was a more rapid and effective bactericidal agent than vancomycin at high concentrations.

Although polypeptins were identified more than three decades ago, their antibiotic properties had not been characterized in detail. In this study we demonstrated that pelgipeptin D, a newly isolated polypeptin, exhibited rapid and complete bactericidal action against a MRSA strain at high concentration while the kill effect was limited when the agent concentration decreased. Similar results were observed for lipopeptide polymyxins, which were characteristically biostatic at low concentrations and biocidal at high concentrations (Storm *et al*., 1977).

Toxicity

In order to test the potential hazards and safety concerns associated with pelgipeptin D, the toxicity was evaluated in KM mice by intraperitoneal and oral administration. In the intraperitoneal toxicity test, all mice treated with 55.0 mg/kg died within 24 h, while no deaths were observed at 36.7 mg/kg. From the mortality rates, the intraperitoneal LD_{50} values for male and female mice were calculated to be 42.0 mg/kg and 38.5 mg/kg, respectively. These intraperitoneal LD_{50} values were slightly higher than that of the homolog permetin A (36 mg/kg) (Takeuchi *et al*., 1979) and polymyxin B (24 mg/kg for both male and female mice) (Gangolli *et al.*, 1970).

In the oral toxicity test, pelgipeptin D did not cause any death or toxic symptoms in mice even at a maximum dose of 560 mg/kg. The oral LD_{50} was not determined due to the limited amount of this peptide available. Student's t-test indicated that there were no significant differences in body weight between the test group treated with 560 mg/kg pelgipeptin D and the saline control group for both male and female mice, suggesting oral administration of pelgipeptin D at 560 mg/kg had little effect on the growth or health of mice.

The cytotoxicity of pelgipeptin D against mammalian cells was tested in a hemolysis assay. When the concentration of pelgipeptin D reached 100 μg/ml, the hemolytic activity against human erythrocytes was lower than 50%. At this concentration, pelgipeptin D effectively inhibited the growth of almost all tested bacteria, including MRSA.

Conclusion

In this report, two lipopeptide antibiotics, pelgipeptins C and D, were isolated from *P. elgii* B69. Structural analysis indicated that they were both members of the polypeptin family, and pelgipeptin D was distinct from any previously described polypeptin. The biological activity and toxicity analysis indicated that pelgipeptins are potential therapeutic agents for management of infections caused by antibiotic-resistant bacteria, including MRSA.

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References

Araújo da Silva, K., J. Salles, L. Seldin, and J. van Elsas. 2003. Application of a novel *Paenibacillus*-specific PCR-DGGE method and sequence analysis to assess the diversity of *Paenibacillus* spp. In the maize rhizosphere. *J. Microbiol. Methods* 54, 213-231.

Ash, C., F. Priest, and M. Collins. 1993. Molecular identification of rRNA group 3 *Bacilli* (ash, farrow, wallbanks and collins) using a PCR probe test. *Antonie van Leeuwenhoek.* 64, 253-260.

Chen, L., N. Wang, X. Wang, J. Hu, and S. Wang. 2010. Characterization of two anti-fungal lipopeptides produced by *Bacillus amyloliquefaciens* SH-B10. *Bioresour. Technol.* 101, 8822-8827.

Choi, S.K., S.Y. Park, R. Kim, S.B. Kim, C.H. Lee, J.F. Kim, and S.H. Park. 2009. Identification of a polymyxin synthetase gene cluster of *Paenibacillus polymyxa* and heterologous expression of the gene in *Bacillus subtilis*. *J. Bacteriol.* 191, 3350-3358.

Fujii, K., K. Sivonen, T. Kashiwagi, K. Hirayama, and K. Harada. 1999. Nostophycin, a novel cyclic peptide from the toxic *Cyanobacterium nostoc* sp. 152. *J. Org. Chem.* 64, 5777-5782.

Gangolli, S.D., P. Simson, M.T. Lis, B. Cheng, R.F. Crampton, and D.M. Matthews. 1970. Amino acid and peptide uptake in protein absorption. *Clin. Sci.* 39, 18.

Kim, P.I., H. Bai, D. Bai, H. Chae, S. Chung, Y. Kim, R. Park, and *et al.* 2004. Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. *J. Appl. Microbiol.* 97, 942-949.

Kim, P.I., J. Ryu, Y. Kim, and Y. Chi. 2010. Production of biosurfactant lipopeptides iturin a, fengycin, and surfactin a from *Bacillus subtilis* CMB32 for control of colletotrichum gloeosporioides. *J. Microbiol. Biotechnol.* 20, 138-145.

Klevens, R.M., M.A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L.H. Harrison, and *et al.* 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the united states. *JAMA.* 298, 1763-1771.

Levine, D. 2006. Vancomycin: A history. *Clin. Infect. Dis.* 42, S5-12.

Li, J., P.K. Beatty, S. Shah, and S.E. Jensen. 2007. Use of PCR-targeted mutagenesis to disrupt production of fusaricidin-type antifungal antibiotics in *Paenibacillus polymyxa*. *Appl. Environ. Microbiol.* 73, 3480-3489.

McKay, G.A., S. Beaulieu, F.F. Arhin, A. Belley, I. Sarmiento Jr, and G. Moeck. 2009. Time-kill kinetics of oritavancin and comparator agents against *Staphylococcus aureus*, *Eenterococcus faecalis* and *Enterococcus faecium*. *J. Antimicrob. Chemother.* 63, 1191-1199.

McVay, C.S. and R.D. Rolfe. 2000. *In vitro* and *in vivo* activities of nitazoxanide against *Clostridium difficile*. *Antimicrob. Agents Chemother.* 44, 2254-2258.

Moellering, R. Jr. 2006. Vancomycin: A 50-year reassessment. *Clin. Infect Dis.* 42, S3-4.

Muto, C., J. Jernigan, B. Ostrowsky, H. Richet, W. Jarvis, J. Boyce, and B. Farr. 2003. Shea guideline for preventing nosocomial transmission of multidrug-resistant strains of *Staphylococcus aureus* and *Enterococcus*. *Infect. Cont. Hosp. Ep.* 24, 362-386.

Pirri, G., A. Giuliani, S. Nicoletto, L. Pizzuto, and A. Rinaldi. 2009. Lipopeptides as anti-infectives: A practical perspective. *Cent. Eur. J. Biol.* 4, 258-273.

Sogn, J.A. 1976. Structure of the peptide antibiotic polypeptin. *J. Med. Chem.* 19, 1228-1231.

Storm, D.R., K.S. Rosenthal, and P.E. Swanson. 1977. Polymyxin and related peptide antibiotics. *Annu. Rev. Biochem.* 46, 723-763.

Sugawara, K., M. Konishi, and H. Kawaguchi. 1984. BMY-28160, a new peptide antibiotic. *J. Antibiot.* 37, 1257-1259.

Takeuchi, Y., A. Murai, Y. Takahara, and M. Kainosho. 1979. The structure of permetin a, a new polypeptin type antibiotic produced by *Bacillus circulans*. *J. Antibiot.* 32, 121-129.

Thiericke, R. and J. Rohr. 1993. Biological variation of microbial metabolites by precursor-directed biosynthesis. *Nat. Prod. Rep.* 10, 265-289.

Wang, Z. and X. Liu. 2008. Medium optimization for antifungal active substances production from a newly isolated *Paenibacillus* sp. Using response surface methodology. *Bioresour. Technol.* 99, 8245-8251.

Weigel, L., D. Clewell, S. Gill, N. Clark, L. McDougal, S. Flannagan, J. Kolonay, and *et al.* 2003. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science* 302, 1569.

Wu, X.C., X.B. Shen, R. Ding, C.D. Qian, H.H. Fang, and O. Li. 2010. Isolation and partial characterization of antibiotics produced by *Paenibacillus elgii* B69. *FEMS Microbiol. Lett.* 320, 32-38.

Wu, X.C., C.D. Qian, H.H. Fang, Y.P. Wen, J.Y. Zhou, Z.J. Zhan, R. Ding, and *et al.* 2011. Paenimacrolidin, a novel macrolide antibiotic from *Paenibacillus* sp. F6-b70 active against methicillin-resistant *Staphylococcus aureus*. *Microb. Biotechnol.* 4, 491-502.

Zhao, Z.H., Y.B. Ma, C. Dai, R.M. Zhao, S.R. Li, Y.L. Wu, Z.J. Cao, and W.X. Li. 2009. Imcroporin, a new cationic antimicrobial peptide from the venom of the scorpion *Isometrus maculates*. *Antimicrob. Agents Chemother*. 53, 3472-3477.