A Novel Broad-spectrum Lipopeptide Antimicrobial Agent, Paenibacterin, against Drug-resistant Bacteria: Structural Elucidation, Biosynthesis, and mechanisms of action

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Abstract

The ongoing explosion of infections caused by antibiotic-resistant bacteria continues to challenge the global public health. However, the discovery and development of novel antibacterial drugs are on the decline in the past decades. Therefore, it is urgent to develop potent and safe antimicrobial agents. Here we report the chemical structure, biosynthesis and modes of action of a novel antimicrobial agent, paenibacterin, from a strain of *Paenibacillus thiaminolyticus* OSY-SE. The producer microorganism was isolated from a soil sample collected in Columbus, OH in 2011. The potent antimicrobial agent was extracted by acetonitrile from the producer cells and purified using HPLC. The core peptide structure of paenibacterin was elucidated by mass-spectrometry (MS) and nuclear magnetic resonance (NMR) while the lipid tail of compound was determined by GC-MS. In order to identify the biosynthetic pathway of the compound, the whole genome of the producer strain OSY-SE was sequenced using the high throughput Illumina sequencing technology. The paenibacterin gene cluster (*pbt*) was further identified by bioinformatic analyses and confirmed by *in vitro* protein functional analyses. Furthermore, the mechanisms of action of paenibacterin were studied by fluorescence microscopy, membrane integrity assays, and hydroxyl radical production assays. Paenibacterin is a broad-spectrum antimicrobial agent with potent activity against foodborne pathogens and clinical drug-resistant isolates. Paenibacterin generally yielded a minimum inhibitory concentration (MIC) at 2-8 μg/ml against Gram-negative strains, and 8-64 μg/ml

against Gram-positive strains. Paenibacterin is a cyclic lipopeptide, consisting of 13 amino acids and a C_{15} fatty acid side chain. Among the amino acids, some are D-amino acids and non-proteinogenic amino acid (ornithine). Paenibacterin is biosynthesized by the producer strain through non-ribosomal peptide synthetases (NRPS). The biosynthetic gene cluster was identified within 52-kb region, encoding three NRPSs (PbtA, PbtB and PbtC) and two ABC-transporters (PbtD and PbtE). Paenibacterin damages bacterial cell membrane, resulting in cytoplasmic membrane deporlarization and potassium ions release. Furthermore, paenibacterin triggers radical production via Fenton reaction and subsequently leads to cell death. This study reported a unique and potent antimicrobial agent with activity against drug-resistant bacteria. The structure of the compound could serve a scaffold for designing even more potent compounds by chemical synthesis. In addition, the elucidation of the biosynthetic pathway expedites the effort to produce paenibacterin derivatives by genetic engineering. This new and potent compound is a very promising candidate for agricultural and clinical application.

Introduction

There is a constant need for novel, safe and effective antimicrobial agents because bacterial can evolve rapidly and acquire resistance to antibiotics in use today. Bacterial natural products remain the important source of novel antibiotics with new scaffold and mechanism of action (Clardy, Fischbach, & Walsh, 2006). Since thousands of antibiotic compounds have been discovered from soil microorganisms (Clardy et al., 2006), new strategies should be used to combat the rediscovery of old antibiotics (Clardy et al., 2006; Fischbach & Walsh, 2009). In this study, we used two non-conventional media, soil-extract agar (Hamaki et al., 2005) and

dilute nutrient agar (Janssen, Yates, Grinton, Taylor, & Sait, 2002), to isolate new bacteria strains producing antibacterial agents. Using these new culturing media, a new isolate, designated as *Paenibacillus thiaminolyticus* OSY-SE, was found from a soil sample producing novel antimicrobial agent. The new compound, paenibacterin, exhibits antimicrobial activities against both Gram-positive and Gram-negative bacteria.

Paenibacillus was originally classified under *Bacillus* and was reclassified as a new genus (Ash, Priest, & Collins, 1993). *Paenibacillus* spp. are spore-forming bacteria that are widely distributed in environments such as soil and plant root. Some species promote plant growth and nitrogen fixation (Khianngam, Akaracharanya, Tanasupawat, Lee, & Lee, 2009; McSpadden Gardener, 2004; Timmusk & Wagner, 1999; von der Weid, Duarte, van Elsas, & Seldin, 2002). A wide variety of antimicrobial agents, including lantibiotics (He et al., 2007), lipopeptides (Martin et al., 2003), and macrolide antibiotic (Wu et al., 2010) are produced by strains of *Paenibacillus* species.

Structural Elucidation of Paenibacterin

The antimicrobial agent was extracted from bacterial cells with acetonitrile, and purified using liquid chromatography. After analyses by mass spectrometry (MS) and nuclear magnetic resonance (NMR), the antimicrobial compound was determined to be a cyclic lipopeptide consisting of a C_{15} fatty acyl (FA) chain and thirteen amino acids. The deduced sequence is: FA-Orn-Val-Thr-Orn-Ser-Val-Lys-Ser-Ile-Pro-Val-Lys-Ile. The carboxyl terminal Ile is connected with Thr by an ester linkage (**Figure 1**). The new compound, designated as paenibacterin, showed activity against Gram-positive and Gram-negative bacteria, including *Listeria monocytogenes*, methicillin-resistant *Staphylococcus aureus*, *Escherichia coli* O157:

H7, and *Salmonella enterica* serovar Typhimurium. Paenibacterin is resistant to trypsin, lipase, α-glucosidase and lysozyme. But the compound is sensitive to pronase and polymyxin acylase. Paenibacterin is readily soluble in water, and fairly stable to exposure to heat and a wide range of pH values.

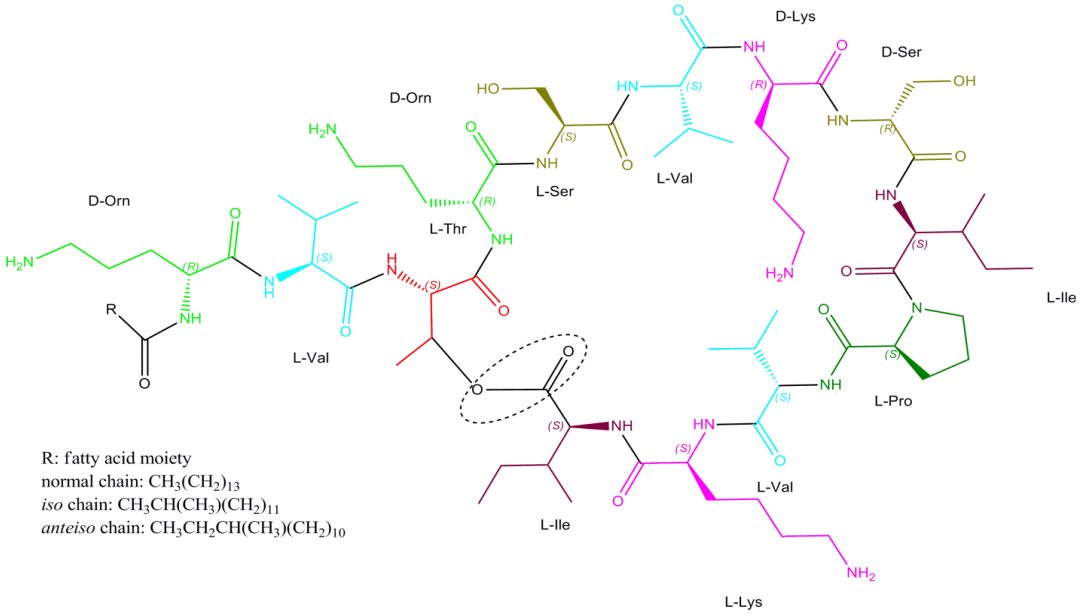
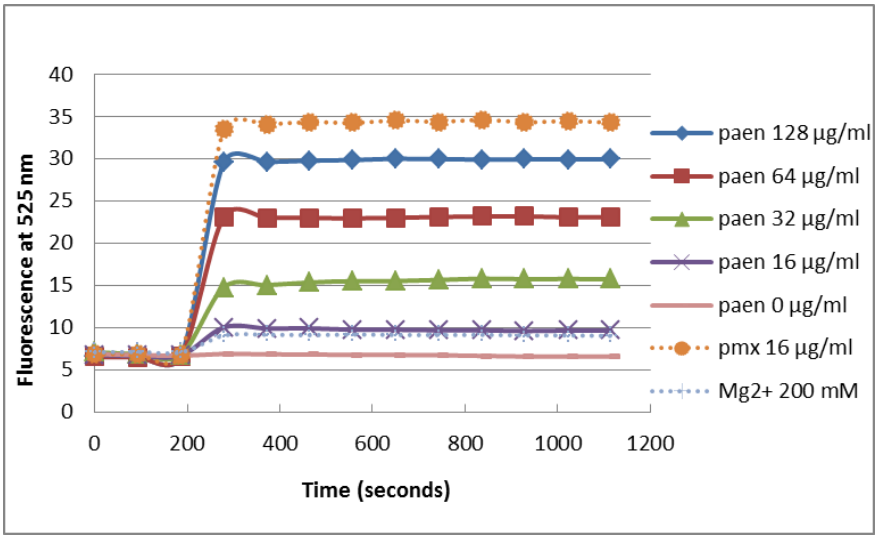


Figure 1 The chemical structure of paenibacterin.

Mechanism of action of Paenibacterin

This study explored the mechanisms of action of paenibacterin against both Gram-positive and Gram-negative bacteria. Some Gram-negative bacteria are resistant to many antibiotics due to the permeability barrier of outer membrane (Vaara, 1992). The molecular basis of permeability lies in the lipopolysaccharide networks that are electrostatically linked by divalent cations (Mg^{2+} and Ca^{2+}). Cationic agents and ion chelators are well-known permeabilizers that disorganize the outer membrane (Vaara, 1992). Cationic peptides, such as polymyxins, enter bacterial cells by a self-promoted uptake pathway (Hancock, 1997). A fluorescent-labeled polymyxin B (BODIPY FL conjugate, Invitrogen, Carlsbad, CA) was used to measure the binding affinity of paenibacterin to purified LPS from

E. coli O111: B4 and to the intact bacterial cells of *E. coli* ATCC 25922. In this study, we found that paenibacterin had high affinity to lipopolysaccharides. The interaction with lipopolysaccharides may displace the Mg^{2+} and Ca^{2+} and promote the uptake of paenibacterin. The LPS binding ability of paenibacterin also suggested that paenibacterin can neutralize LPS and prevent endotoxinamia during antibiotic treatment (Hancock, 1997). As an outer membrane permeabilizer, the combination of paenibacterin with other antibiotics may show synergistic interaction against drug-resistant pathogens.



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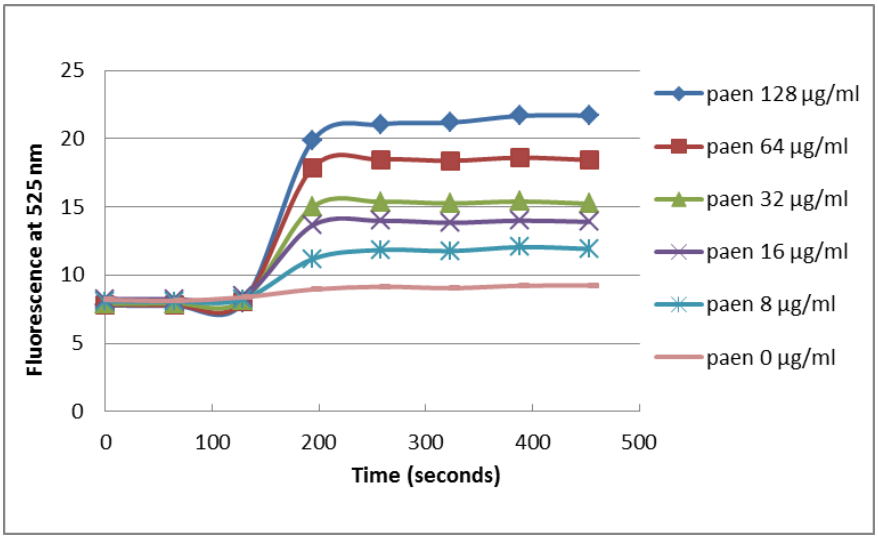
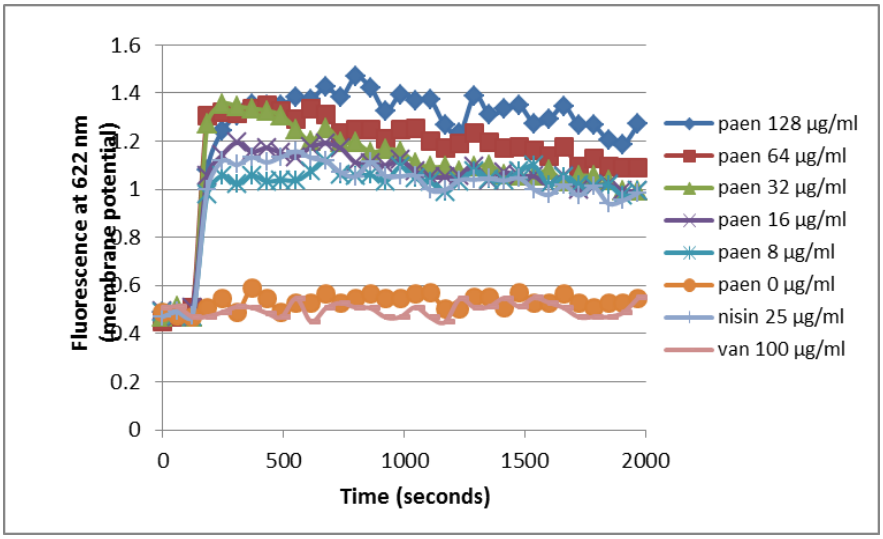


Figure 2 BODIPY FL-labeled polymyxin B displacement assays.(A) *in vitro* polymyxin B-LPS displacement; (B) *in vivo* polymyxin B-cells displacement assay using *E. coli*.

(A)



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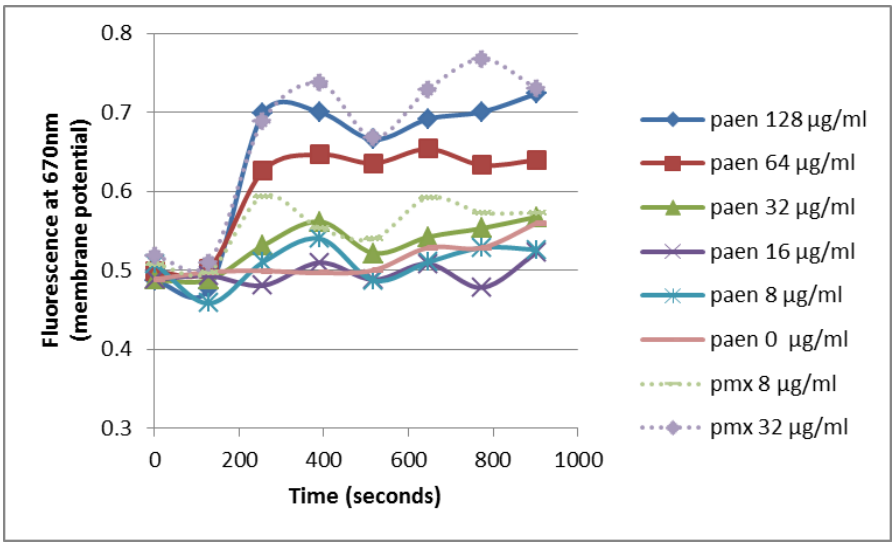
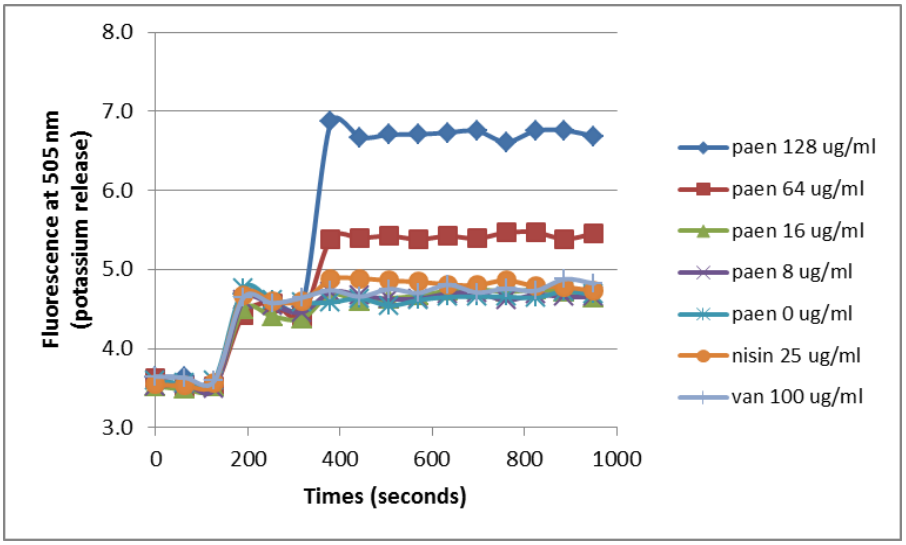


Figure 3 Effect of paenibacterin (paen), nisin vancomycin (van) and polymyxin E (pmx) on bacterial membrane potential. (A) *S. aureus* ATCC 6538 (B) *E. coli* ATCC 25922.

The bacterial cytoplasmic membrane is the direct target of paenibacterin for Gram-positive and Gram-negative bacteria. The membrane potential assays were performed using a fluorescence probe, 3,3'-Dipropylthiadicarbocyanine Iodide ($DiSC_3(5)$) (Invitrogen, Carlsbad, CA) according to previously reported method (Zhang, Dhillon, Yan, Farmer, & Hancock, 2000). Potassium ion release assays were performed using the cell impermeable K^+-sensitive probe (PBFI, Invitrogen) (Silverman, Perlmutter, & Shapiro, 2003). Paenibacterin

depolarized cell membrane (**Figure 3**), triggered K^+ release (**Figure 4**) and increased the uptake of propidium iodide (**Figure 5**). The cell membrane is essential to bacterial life as it contains one-third of proteins in a cell and is the place for crucial processes (Hurdle et al., 2010). Therefore, by damaging the cell membrane, paenibacterin may interfere with numerous cellular functions such as function of electrical transport chain.

(A)



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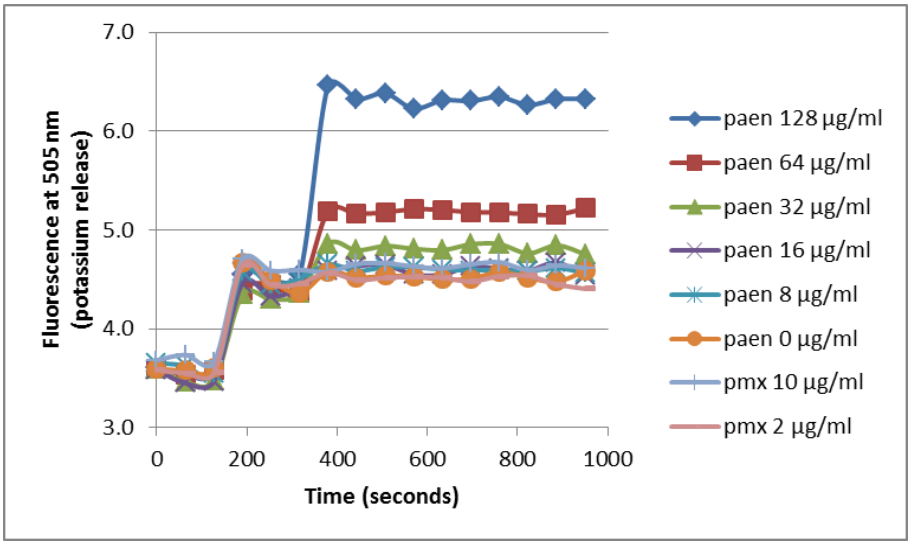
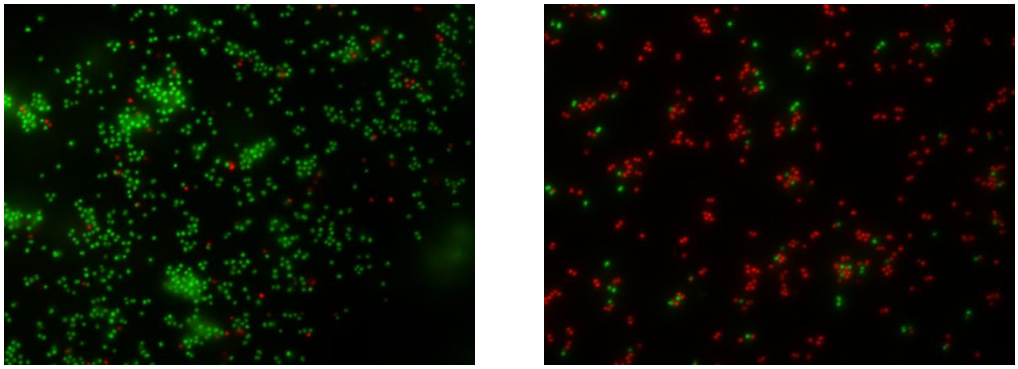


Figure 4 Effect of paenibacterin (paen), nisin, vancomycin (van) and polymyxin E (pmx) on K^+ release. (A) *S. aureus* ATCC 6538 (B) *E. coli* ATCC 25922.

(A) (B)



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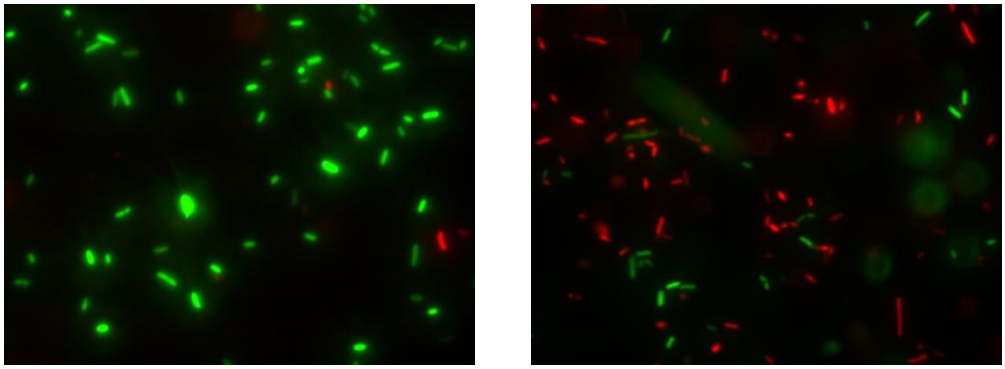


Figure 5 Effect of paenibacterin on bacterial cell membrane permeability. (A) Untreated cells of *S. aureus* ATCC 6538 (B) *S. aureus* ATCC 6538 treated with 80 μg/ml paenibacterin for 1hr; (C) untreated *E. coli* ATCC 25922 (D) *E. coli* ATCC 25922 treated with 64 μg/ml paenibacterin for 1 hr.

Reactive oxygen species (ROS), such as superoxide and hydroxyl radicals, are highly deleterious to bacterial cells by oxidizing macromolecules such as DNA (Imlay, 2002; Imlay, 2003). The hydroxyl radical levels were measured by the hydroxyl radical indicator, hydroxyphenyl fluorescein (HPF, Invitrogen) (Sampson et al., 2012). In addition to membrane damage, paenibacterin led to production of hydroxyl radicals in *E. coli* cells (**Figure 6**). Supporting the oxidative damage notion, we found that the radical scavenger, thiourea, partially protected cells from killing by paenibacterin. Also, pretreatment with the iron chelator dipyridyl reduced the killing effect of paenibacterin against *E. coli* (**Figure 7**). Kohanski et al.

(2010) pointed out that the drug-target interaction resulted in hyperactivation of electron transport chain and generation of superoxides (O_2^-) formation. Superoxide in turn damages the iron-sulfur cluster and thus releases ferrous irons, which stimulated the production of hydroxyl radical through Fenton reaction (Imlay, 2003; Kohanski et al., 2010).

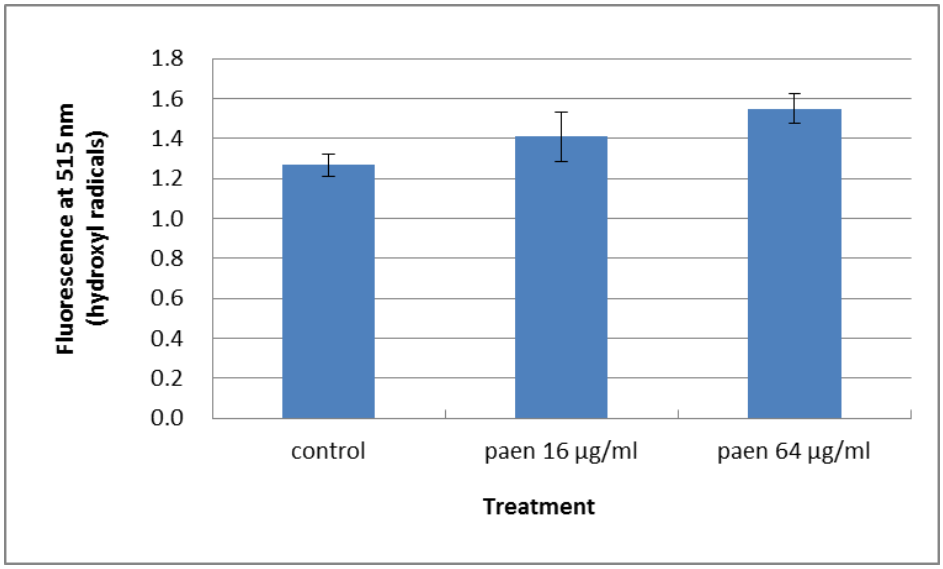
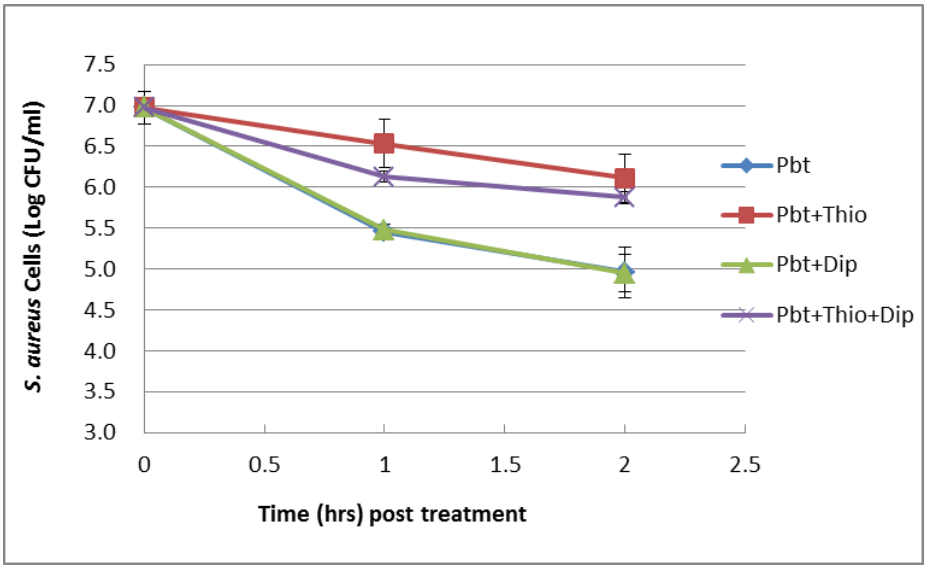


Figure 6 Hydroxyl radical production in paenibacterin treated cells of *E. coli* ATCC 25922.

In this study, we presented the dual mechanisms of action of paenibacterin, namely direct cell membrane damage and indirect oxidative cellular damages due to hydroxyl radical formation. Unlike other conventional antibiotics, bacteria are more difficult to acquire resistance to membrane-active peptides (Hancock & Sahl, 2006; Hurdle et al., 2010).

(A)



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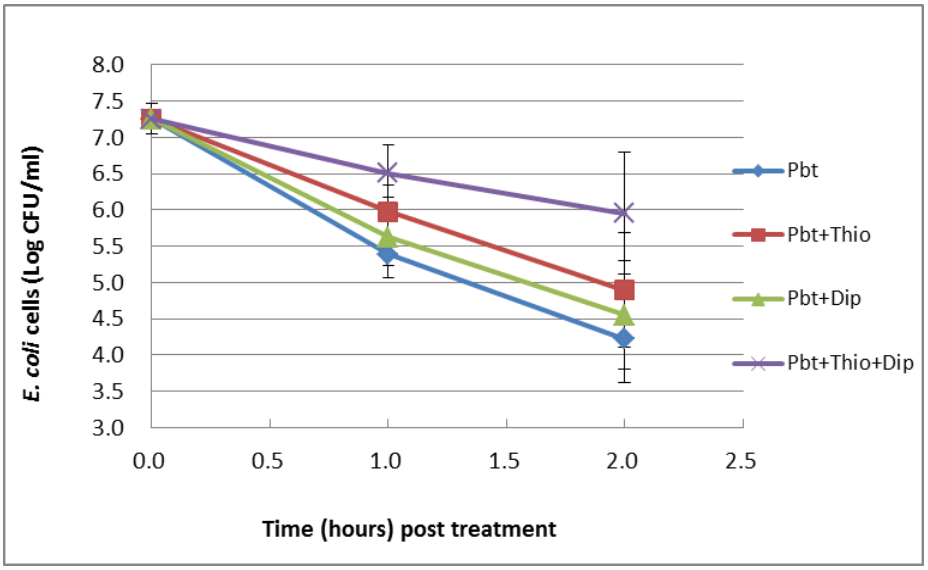


Figure 7 Effect of dipyridyl (Dip, 450 μM) and thiourea (Thio, 100 mM) on the killing efficacy of paenibacterin (Pbt). (A) *S. aureus* ATCC 6538, Pbt at 96 μg/ml (B) *E. coli* ATCC 25922, Pbt at 64 μg/ml

Biosynthesis of Paenibacterin

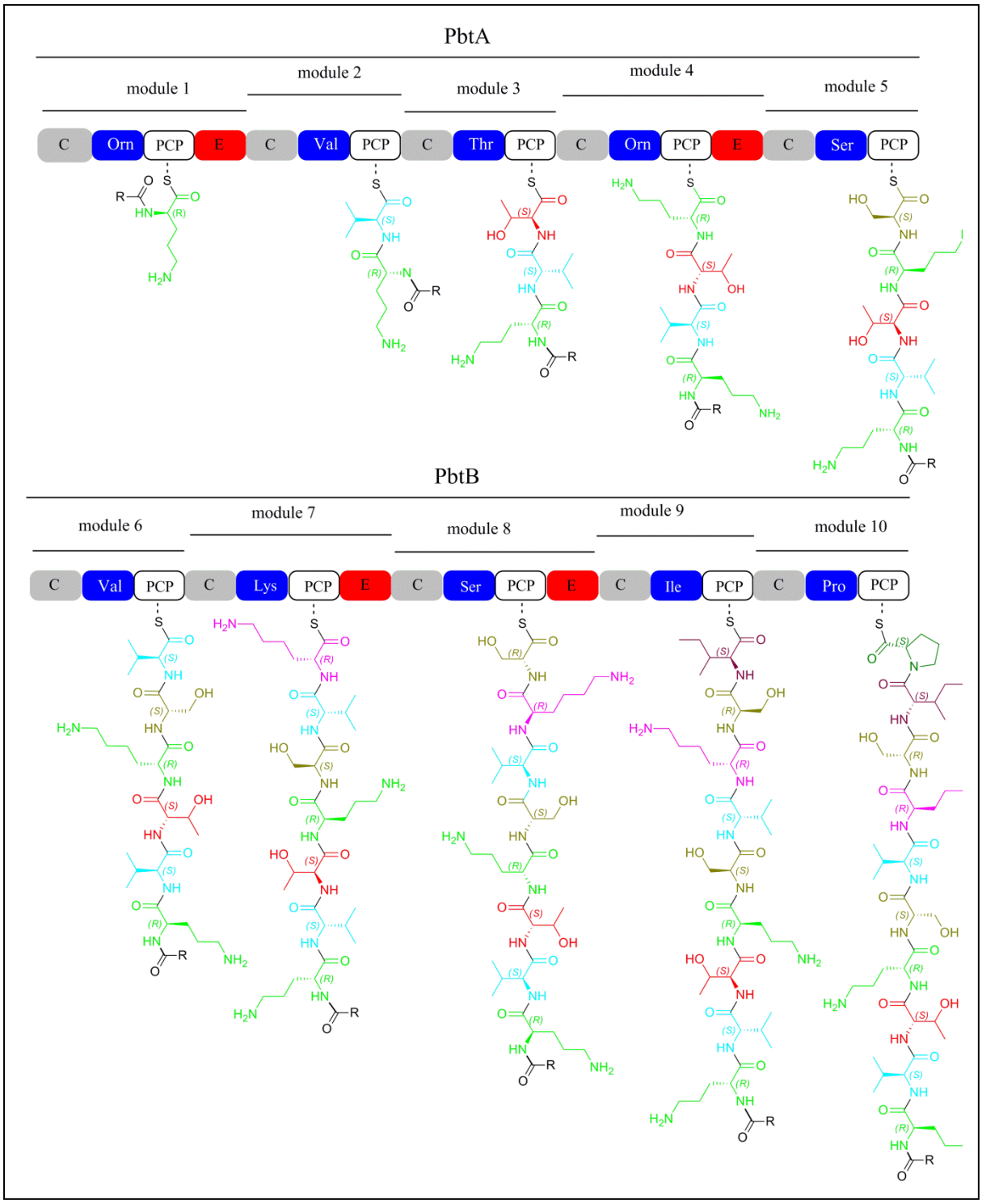
To elucidate the biosynthesis of paenibacterin in *Paenibacillus thiaminolyticus* OSY-SE, we determined the whole genome sequence of the producer strain. The gene cluster for paenibacterin biosynthesis was identified within 52-kb region, encoding three non-ribosomal peptide synthetases (PbtA, PbtB and PbtC) and two ABC-transporters (PbtD and PbtE) (**Table 1**). As deduced from the sequence data, each PbtA and PbtB enzyme consists

of five modules, whereas PbtC is composed of three modules. Each of the 13 modules assembles one amino acid into the paenibacterin peptide (**Figure 8**).

Table 1 The biosynthetic cluster in *P. thiaminolyticus* OSY-SE responsible for paenibacterin synthesis

ORFs[a]	**Bases number**	**Amino acids number**	**Calculated molecular mass (Dalton)**	**Predicted function**
pbtA	19,818	6,605	745,827.1	Peptide synthetase
pbtB	19,251	6,416	723,020.7	Peptide synthetase
pbtC	9,513	3,170	356,884.2	Peptide synthetase
pbtD	1,713	570	63,374.5	ABC transporter
pbtE	1,749	582	64,584.7	ABC transporter

[a] ORFs open reading frames



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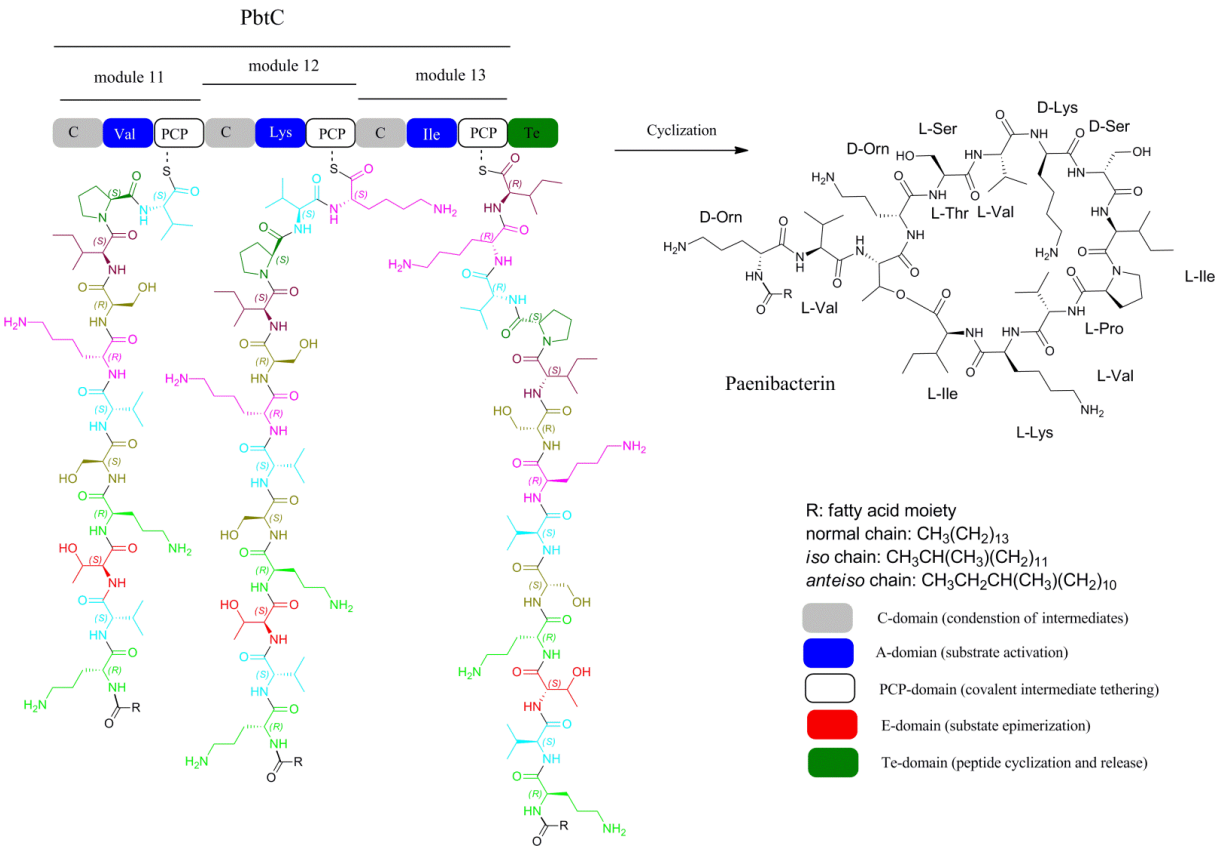

Figure 1 Schematic overview of paenibacterin biosynthesis in *P. thiaminolyticus* OSY-SE. The peptide core is assembled by three NRPSs PbtA, PbtB, PbtC.

Discussion

Paenibacterin is a promising antibiotic scaffold for developing new antibiotics targeting drug-resistant pathogens. The mechanisms of action of paenibacterin involve direct cell membrane damage and indirect oxidative cellular damage. Paenibacterin is a cationic lipopeptide with four positive charges. The electrostatic interaction between paenibacterin and LPS can displace the divalent cations on the LPS network and promote the uptake of paenibacterin. The cytoplasmic membrane is the direct target of paenibacterin. Paenibacterin depolarized cell membrane, triggered K^+ release and increased the uptake of hydrophobic nucleic acid stain, propidium iodide. In addition, paenibacterin led to production of hydroxyl

radicals in bacterial cells. The radical scavenger thiourea and the iron chelator dipyridyl reduced the killing effect of paenibacterin against *Escherichia coli* and *Staphylococcus aureus.*

The presence of non-proteinogeneic amino acids (ornithine) in the peptide sequence suggested that paenibacterin is synthesized by nonribosomal synthetases. In order to determine the genes for paenibacterin biosynthesis, we sequenced the whole genome of the producer strain using the next-generation sequencing technology. The gene cluster was identified within 52-kb region, encoding three non-ribosomal peptide synthetases (PbtA, PbtB and PbtC) and two ABC-transporters (PbtD and PbtE). As deduced from the sequence data, each PbtA and PbtB enzyme consists of five modules, whereas PbtC is composed of three modules. Each of the 13 modules assembles one amino acid into the paenibacterin peptide. Facing the challenges of antibiotic resistance, paenibacterin has the potential to become a new therapeutic approach against bacterial infections.

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