Research Progress of Antimicrobial Peptides and the Potential Applications of *Bacillus subtilis*

Lu CHEN*, Yingbing HE, Zengli WANG, Dongxu WU, Quan SHI

Shanghai Tongguang Shanliang Health Technology Co., Ltd., Shanghai Dongli Health Research Institute Co., Ltd., Shanghai 200063, China

Abstract This paper presents a comprehensive account of antimicrobial peptides (AMPs) derived from various sources, including animal, plant, and microbial origins, along with an examination of their structural characteristics and biological activities. Specifically, the potential of *Bacillus subtilis* as a safe and effective host for the production of AMPs is discussed. *B. subtilis* exhibits a notable capacity for protein secretion and is also capable of efficiently producing AMPs without the presence of endotoxin contamination. The research indicates that the production efficiency of AMPs derived from *B. subtilis* can be significantly enhanced through the application of genetic engineering and synthetic biology techniques. This advancement holds considerable potential for applications in food preservation, agriculture, medicine, and various other fields. The paper additionally investigates the stability of AMPs under diverse conditions of temperature, pH, and enzymatic treatment, and highlights the necessity for further research to facilitate the advancement of these AMPs for practical applications.

Key words Antimicrobial peptides (AMPs), Animal-derived AMPs, Plant-derived AMPs, Microbial-derived AMPs, *Bacillus subtilis*

1 Introduction

Antimicrobial peptides (AMPs) are recognized as potent agents against a diverse array of pathogens, including bacteria, fungi, viruses, tumor cells, and protozoa, due to their broad spectrum of nonspecific defensive mechanisms[1-2]. AMPs, which are small molecular peptides, play a crucial role in defending against external microbial invasions and in the elimination of abnormal cells. They are fundamental components of an organism's intrinsic immune system[3]. AMPs typically consist of more than ten to several dozen amino acids, exhibiting distinct molecular structures and biological activities. AMPs are a class of defense peptides synthesized by the body to combat the invasion of pathogenic microorganisms, typically averaging 18 amino acids in length. In the primary structure of proteins, the N-terminal region is characterized by a high concentration of hydrophilic amino acid residues, whereas the C-terminal region is predominantly composed of hydrophobic amino acid residues. This structural characteristic renders AMPs both water-soluble and antibacterial. The secondary structures of AMPs can be categorized into four distinct types: α-helix, β-sheet, lamellar structure, and cyclic structure[4-5].

2 AMPs derived from various sources

2.1 Animal-derived AMPs Zasloff *et al.*[6] isolated an AMP from the skin of *Xenopus* in 1987 and designated it as magainin. Since then, researchers have successfully isolated a diverse array of AMPs from the gastrointestinal mucosa of amphibians, as well as from the granular glands located within their skin[7]. Yu Shihong[8] successfully isolated AMPs from the skin of *Hylarana guentheri* using various isolation and purification techniques, including intermittent voltage-stabilized electrical stimulation. These peptides demonstrated a significant ability to inhibit the proliferation of *Staphylococcus aureus*, *Escherichia coli*, and several strains exhibiting high levels of drug resistance. Casteels *et al.*[9] successfully isolated the AMP abaecin from honey bees. This peptide exhibits broad-spectrum antimicrobial activity and is composed of 34 amino acids. Subrahmanyam *et al.*[10] identified the AMP lebocin in the body of *Lasioderma serricorne*, which exhibits activity against gram-negative bacteria. Li Zhongjie *et al.*[11] successfully isolated the peptide Hp1404 from the scorpion venom of *Heterometrus petersi*, demonstrating antimicrobial activity against gram-positive bacteria. This peptide not only circumvented the issue of resistance associated with *S. aureus*, but also exhibited low toxicity to mammalian cells. Mor *et al.*[12] isolated the AMP dermaseptin I from the skin of *Rhacophorus*. This peptide comprises 34 amino acids and demonstrates broad-spectrum activity against various microorganisms, including bacteria, yeasts, and fungi. The AMP PK-1, derived from rabbit kidney and identified by Andrew *et al.*[13], demonstrated targeted activity against *E. coli* at a pH of 7, characterized by a distinctive sequence that includes a single arginine residue. The AMP dermaseptin, isolated from frog skin by He Xiaoqin *et al.*[14], demonstrated a remarkable capacity to inhibit *S. aureus*.

2.2 Plant-derived AMPs To safeguard against microbial threats, plants have evolved intricate defense mechanisms. These systems encompass a variety of active compounds, particularly those exhibiting antimicrobial properties. The primary classes of these compounds include alkaloids, coumarins, flavonoids and their derivatives, lipids, steroids, terpenes, and phenolic compounds. Plant-derived AMPs have the potential to augment the inherent resistance of plants to a variety of stressors[15]. For instance, the radish-derived AMP RSAFP demonstrates broad-spectrum activity against a diverse array of fungi, with particularly pronounced effects on filamentous fungi[16]. In 2005, Wong *et al.*[17] isolated the AMP vulgarinin from the seeds of *Phaseolus vulgaris*, and its biological activity had been demonstrated across various domains. Vulgarinin exhibited notable antifungal activity, demon-

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* Corresponding author. E-mail: c12345678v9@163.com

strating significant inhibitory effects against prevalent fungal species, including *Botrytis cinerea*. Further investigations demonstrated that this AMP was not solely restricted to the inhibition of fungal growth, and also exhibited a substantial inhibitory effect on the proliferation of cancer cell lines, including L1210 and MCF-7. Additionally, vulgarinin has been shown to effectively reduce the activity of HIV-1 reverse transcriptase.

2.3 Microbial-derived AMPs A diverse array of microorganisms, encompassing both bacteria and fungi, possess the ability to synthesize AMPs. These peptides, frequently referred to as bacteriocins, are generated during the metabolic processes of bacteria. They are primarily synthesized by bacterial ribosomes and subsequently secreted extracellularly to exert their antimicrobial effects. The functions of bacteriocins encompass the ability to kill or inhibit the growth of specific prokaryotic organisms, as well as to demonstrate antimicrobial properties against gram-positive bacteria and multidrug-resistant strains. Lantibiotics, which are significant constituents of the bacteriocin family, effectively inhibit the cell wall synthesis pathway by specifically binding to lipid II, a crucial precursor in the synthesis of bacterial cell walls. The binding effect results in the improper formation of the bacterial cell wall, thereby compromising the structural integrity and mechanical strength essential for bacterial survival, ultimately culminating in bacterial death[18]. Naoki *et al.*[19] isolated the AMP lactococcin Z from *Lactobacillus* species, which demonstrated superior activity against *L. lactis*. Huang *et al.*[20] successfully isolated the AMP GAFP from the leaves of *Reseda odorata*. The mechanism of action primarily involves the enhancement of cell membrane permeability in fungi, including *Fusarium graminearum* and *F. moniliforme*, thereby facilitating the formation of ion channels.

3 AMPs derived from *Bacillus subtilis*

3.1 Advantages of AMPs production by *B. subtilis* The prohibition of growth-promoting antibiotics in animal feed has intensified the need to identify alternatives to these antibiotics. Although researchers have explored various alternatives to antibiotics in animal production, including plant extracts, polysaccharides, and probiotics, the effectiveness of these replacements has been limited. AMPs possess not only broad-spectrum antimicrobial activity and immunomodulatory ability, but also the advantages of good safety and low propensity for the development of drug resistance. These attributes render them a promising alternative to traditional antibiotic products. The challenges associated with the isolation and purification of natural AMPs, coupled with the high costs associated with chemically synthesized AMPs, are insufficient to satisfy the increasing demand for scientific research and practical applications. Advances in genetic engineering and synthetic biology have enabled the production of recombinant AMPs using genetically modified bacteria. *E. coli* predominantly expresses AMPs in inclusion bodies, which become contaminated with cell wall endotoxins following cellular fragmentation. *B. subtilis* is acknowledged as a safe microorganism that possesses a significant ability to secrete proteins. AMPs exhibit host-killing properties and are typically obtained through a fusion expression strategy, whereby the fusion protein is isolated and purified at a subsequent stage and subsequently cleaved using specific enzymes. The steps involved in this strategy are complex and the associated costs are substantial, which hinders the process of industrialization.

B. subtilis is a member of the genus *Bacillus*, which is a group of aerobic, gram-positive bacteria capable of forming spores in response to adverse conditions. *B. subtilis* is extensively utilized in industrial applications and is recognized as one of the primary bacterial sources for enzyme production[21]. *B. subtilis* is acknowledged as a safe microorganism and is included in the most recent list of feed additives. Additionally, it is regarded as a biologically safe strain by the US Food and Drug Administration (FDA)[22]. In comparison to *E. coli* expression systems, *B. subtilis* offers several advantages, including the absence of endotoxin contamination risk, the capability to secrete target proteins into the extracellular environment, minimal codon preference, reduced requirements for medium conditions, and the potential for high-density fermentation. These factors contribute to improved control over production costs. *B. subtilis* possesses certain limitations, including the secretion of a substantial quantity of proteases alongside the target protein, which results in the degradation of the target protein. Additionally, the transmission of plasmids over more than 50 generations may result in plasmid loss and other associated complications[23]. To mitigate the risk of plasmid loss, one effective strategy is to integrate exogenous genes, along with signal peptide genes, into the genome of the expression host.

3.2 Recent advances in AMPs production by *B. subtilis* Piyush *et al.*[24] conducted a detailed analysis of two AMPs isolated from SK. DU. 4, a salt-tolerant strain of *B. subtilis*, examining their properties comprehensively. The study commenced with the preliminary characterization of AMPs utilizing matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The molecular weights of the AMPs were ascertained by dissolving the freeze-dried peptides in methanol, followed by analysis with a MALDI mass spectrometer. To evaluate the bacteriostatic properties of AMPs, this study determined the minimum inhibitory concentration (MIC) of these peptides through a microtiter plate dilution assay. The findings indicated that the AMPs demonstrated substantial inhibitory effects against various pathogenic bacteria, including *S. aureus*. The study also assessed the stability of the AMPs under varying conditions of temperature, pH, and enzyme treatment. The findings indicated that the antimicrobial activity of the AMPs was altered following treatment at temperatures of 60, 80, 100, and 121 ℃ for a duration of 15 min, respectively. The AMPs exhibited varying degrees of stability across a pH range of 2.0 to 12.0. In addition, the stability of APMs was evaluated in the presence of various proteolytic enzymes, including pepsin, trypsin, chymotrypsin, protease K, and protease E. The lipopeptide was isolated from the bacterial culture medium through acid and solvent extraction method. The culture broth was subjected to centrifugation, followed by acidification, precipitation, re-centrifugation, and extraction with methanol to isolate the purified

AMP. This method guaranteed a high level of purity for AMPs, facilitating subsequent analysis and application. This study illustrates the efficacy of AMPs derived from salt-tolerant *B. subtilis* in combating bacterial pathogens. The material's excellent thermal and pH stability provide them with a broad spectrum of applications in food preservation, medicine, and agriculture. The findings indicate that the two AMPs extracted from the *B. subtilis* strain SK. DU. 4 exhibit substantial antimicrobial activity and demonstrate high stability, suggesting a broad range of potential applications. Further research is essential for the advancement of practical applications of these AMPs in the fields of food, agriculture, and medicine.

Lettieri *et al.* [25] have effectively characterized the AMPs produced by *B. subtilis* subsp. *subtilis* and have demonstrated their inhibitory effects on a diverse array of pathogenic bacteria. The AMPs were subjected to isolation, purification and feature analysis using various methodologies. The findings indicated that *B. subtilis* synthesized bacteriostatic substances during its exponential growth phase. Among the various enriched media tested, including BHI, LB, and LN, Landy's medium demonstrated superior yields at lower cell concentrations. This finding indicates that Landy's medium is particularly suitable for the production of AMPs. The supplementation of histidine and lactose effectively enhanced the production of AMPs without a corresponding significant increase in cell growth. The AMPs exhibited a significant inhibitory effect on a diverse array of gram-positive bacteria, including *S. aureus* and *Streptococcus* sp., through testing against various pathogenic bacterial strains. These AMPs retained their efficacy against certain clinical isolates that displayed resistance to conventional antibiotics. The stability of the AMPs was assessed under varying temperature and pH conditions. The AMPs exhibited stability at 80 ℃ for a duration of 30 min. However, its activity diminished to 75.6% when exposed to 100 ℃, and it became entirely inactive at 121 ℃ after 15 min of exposure. The AMPs exhibited stability within a pH range of 4 to 9, but their activity diminished considerably at extreme pH levels. The AMPs were further purified using thin-layer chromatography (TLC) and reversed-phase high-performance liquid chromatography (HPLC). Its molecular weight was determined to be 2 158 Da through MALDI-TOF MS. The proteinaceous nature of the AMPs was substantiated by their resistance to various enzymes, such as proteinase K, indicating that they are classified as a non-ribosomal synthetic peptide. This study elucidates the potential of AMPs synthesized by *B. subtilis* subspecies in combating bacterial pathogens, with particular emphasis on their applications in food preservation and pharmaceuticals. These AMPs not only demonstrate significant antimicrobial activity but also exhibit enhanced thermal and pH stability, thereby providing a crucial theoretical foundation for their development in practical applications. The findings indicate that the AMPs synthesized by *B. subtilis* subspecies exhibit significant bacteriostatic effects, demonstrate good stability, and possess a broad range of potential applications. Further research is necessary to advance the applications of these AMPs in the fields of food, agriculture, and medicine.

Zhang *et al.* [26] conducted a detailed characterization of two AMPs isolated from *B. subtilis* strains. The study commenced with the initial purification of AMPs utilizing Sephadex G50 gel chromatography. Several peaks exhibiting antimicrobial activity were isolated based on the 280 nm UV absorption peak. Further purification utilizing reversed HPLC resulted in the identification of two predominant AMP peaks, designated as peptide-I and peptide-II, respectively. The molecular weights and amino acid sequences of the AMPs were analyzed utilizing liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) technique. Peptide-I exhibited a molecular weight of 988.5706 Da and comprised 8 amino acids with the sequence VFLENVLR. In contrast, Peptide-II demonstrated a molecular weight of 1 286.6255 Da and consisted of 13 amino acids with the sequence FSGSGSGTAFTLR. The amino acid sequences of both peptides were compared with those in the Antimicrobial Peptide Database, revealing low homology levels of 44.44% and 43.75%, respectively. The study assessed the antimicrobial efficacy of the AMPs by measuring the MIC. The findings indicated that the MICs of peptide-I against *S. aureus*, *B. cereus*, and *Salmonella enterica* were 64, 32, and 8 μg/mL, respectively. In contrast, the MICs of peptide-II against the same bacterial strains were determined to be 16, 64, and 16 μg/mL, respectively. This indicated that peptide-I exhibited the most potent inhibitory effect against *S. enterica*, whereas peptide-II demonstrated a superior inhibitory effect on *S. aureus*. The stability of AMPs was evaluated under varying temperature and pH conditions. Peptide-I demonstrated considerable stability at elevated temperatures (*e. g.*, 121 ℃) and across a broad pH range (2.0 to 10.0), with no significant drop in its antimicrobial activity. The activity of peptide-II was significantly diminished at elevated temperatures, specifically at 100 ℃ and 121 ℃, while it exhibited peak activity at a neutral pH of 7.0. Peptide-I demonstrated a higher tolerance to proteinase K, trypsin, and pepsin, while peptide-II exhibited a lower tolerance to proteinase K. The in-depth study of these two AMPs establishes a theoretical foundation for their practical production and application. The material's favorable thermal and pH stability provides them with extensive potential applications in food preservation, biomedicine, and agriculture. This study illustrates the remarkable efficacy of two novel AMPs derived from *B. subtilis*, specifically regarding their bacteriostatic properties and stability. Furthermore, it establishes a scientific foundation for the continued development and application of these natural antimicrobial agents.

4 Conclusions

B. subtilis, characterized by its exceptional protein secretion capacity, absence of endotoxin contamination risk, and ability to undergo high-density fermentation, serves as an optimal host for the production of AMPs. The advancements in genetic engineering and synthetic biology have facilitated the production of recombinant AMPs using genetically modified bacteria. This progress has significantly enhanced both the research and application of AMPs.

Over the past five years, researchers have successfully isolated a range of novel AMPs from *B. subtilis*. These AMPs exhibit diversity in terms of molecular weight, amino acid sequence, and biological activity. For instance, the AMPs extracted from the *B. subtilis* strain SK. DU. 4 demonstrated considerable stability across various temperature and pH conditions, and exhibited a significant inhibitory effect on pathogenic bacteria, including *S. aureus*. AMPs possess a diverse array of applications across food preservation, medicine, and agriculture. Their notable thermal and pH stability renders them valuable in the food industry for prolonging the shelf life of products. In the medical field, they are utilized for antibacterial therapies and anti-tumor research. Additionally, in agriculture, AMPs serve as biopesticides, offering a sustainable alternative to conventional chemical pesticides. Recent studies have demonstrated that the yield of AMPs synthesized by *B. subtilis* can be substantially enhanced through the optimization of culture media and the implementation of genetic engineering modifications. Nevertheless, several challenges persist, including the stability of AMPs under high-temperature and extreme pH conditions, as well as their susceptibility to degradation by proteases during the production process. Future research should prioritize addressing the technical challenges associated with the production of AMPs by *B. subtilis*. Additionally, efforts should be directed towards further optimizing the production process to enhance both the yield and purity of these AMPs. Concurrently, it is essential to investigate the application of AMPs across a broader spectrum of fields and to facilitate their industrialization.

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