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Review

Overview of ribosomal and non-ribosomal antimicrobial peptides produced by Gram positive bacteria

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Abstract: The increasing incidence of antimicrobial resistance bacterial infection and decreasing effectiveness of conventional antibiotics to treatment have caused serious problems worldwide. The demand for new generationantibiotics to combat microbial pathogens is imperative. Cationic antimicrobial peptides (AMPs) with different sources from prokaryotic to complex eukaryotic organisms, with variable length, amino acid composition and secondary structure, have been considereddduring the past decades. The advantages of large number of AMPs are related to broad spectrum and morphogenetic activities, low resistance rate among microorganismswithout side effect on human cells, rapid killing of bacteria via membrane damage and intracellular targets,and their critical roles in anti-inflammatory. Ribosomal synthesized peptides of Gram positive bacteria with various post translational modificationsrepresent extended types of antimicrobial peptide with different structural and functional diversity. These types of peptides have been considered as new therapeutic agents for pharmaceutical development .In addition, non- ribosomal synthesized peptides are a wide range of peptides , an extremely extensive range of biological activities and pharmacological properties that are not synthesized by ribosomes, show interesting biological properties ranging from antibiotic to bio surfactants. This review focused on genetics, mechanism of action and modifications, resistance mode of Gram positive bacteria to AMPs and the biotechnological application of ribosomally and non-ribosomally synthesized peptides derived from Gram positive bacteria.

Key words: Antimicrobial peptide; Antimicrobial target; Bacterial resistance; Bacteriocin biosynthesis; Modified antimicrobial peptide.

Introduction

In recent years, much concern has been placed on antibiotic resistance which isa serious public-health problem. The increasing prevalence of multidrug resistant bacterial strains worldwide causes significant impact on healthcare systems (1, 2).

In 2014, the World Health Organization (WHO) presented their global report on surveillance of bacterial resistance in the world, with the theme "post-antibiotic era" when minor infections might lead to lethal outcome, and emphasized on the urgent need for the progress of alternative intervention antimicrobial agents against antimicrobial resistant bacteria(3) .

Despite the increasing spread of pathogenic bacterial resistance, the novel agents to inhibit the emerging problem are yet to be discovered (4-6). Most of antibiotic chemical agents introduced to general clinical use were discovered in golden age between 1930 and 1962 (7) . During 2000 to 2006, just a few new classes have been introduced to the market for human usage. Linezolid in 2000 and daptomycin in 2006 were applied for treatment of Gram positive bacteria and oritavancin was introduced in 2014 to for treatment of acute bacterial skin infections in adults (8, 9). The urgent need for the development of new antimicrobial agents is an emergent preference for pharmaceutical industries(10-12). One of the alternative compounds for antimicrobial agents are antimicrobial peptides (AMPs) which have been considered since 1980 (13).

Up to date, hundreds of peptides with different sources from single cell microorganisms to invertebrate, vertebrate and human, in addition to their role in the innate immune systems, have been characterized (14, 15).

Till date, more than 2000 AMPs have been submitted to the Antimicrobial peptide data base (http://aps.unmc.edu/AP/main.php).

AMPs are an important part of innate immune defense in organisms. They are generally small molecules (10-100 amino acids), with overall net positive charge (+2 to +9) and amphiphilic properties. Based on their target, they are categorized into antibacterial, antifungal, antiparasitic, and antiviral peptides (15, 16).

The most common AMPs correspond to cationic peptides with wide range of secondary structures including,α-helices, β-sheets with two or more disulfide bridges, loop with a single disulfide bond and extended structures which contain specific amino acids like proline, arginine, tryptophan and glycine (14, 17).

The other important structural properties such as size, charge, hydrophobicity, amphipathicityand solubility are essential for their antimicrobial activities and the mechanisms of action of AMPs to bind specifically to bacterial cell membrane (17, 18).

Although the exact mechanism of AMPs has not been defined, but it can be generally categorized in two types of actions: membrane acting (barrel stave, toroidal, carpet and micellar aggregate models) and non-mem-

brane acting (intracellular targets). In membrane acting mechanisms, peptides cause damage to cell membrane. Particularly, the permeability of the bacterial cell membrane was proposed for cationic AMPs operation. The accurate mode of action is based on electrostatic interaction between cationic peptide and negatively charged components on bacterial envelope surface such as phosphate group in lipopolysaccharides (LPS) or lipoteichoic acid in Gram negative and Gram positive bacteria, respectively (19, 20). AMPs also interact with intracellular targets which result to cell damages by inhibition of cell wall or nucleic acids and/or protein synthesis (7).

AMPs exhibit different potential activities for therapeutic applications. Their broad spectrum ability as anti-infective agents against most Gram-negative and Gram-positive bacteria in humans and animals, cancer treatment, immune modulatory by enhancing innate immunity and releasing prostaglandin, septic endotoxin neutralizing of LPS, inducing wound healing are considered as the basic elements for the development of new generations of anti-microbial agents (21-23).

Till date, more than fifty of the AMPs produced by bacteria, also known as bacteriocins, have been characterized and isolated from various Gram-positive and Gram negative bacteria. The important role of these peptidesis related to sustaining bacterial in a community of bacterial cells. They generally exhibit effective antimicrobial activities on the other bacteria having similar or different genera (24) .

Most of the AMPs produced by bacteria are divided into two categories: ribosomally and non-ribosomally synthesizedpeptides.

This article aims to review different types of Gram positive antimicrobial peptides, classification according to their structure and composition, modes of action and the strategies of Gram positive bacteriato obtain resistance to AMPs, briefly.

Ribosomally synthesized peptide of Gram positive bacteria

Ribosomally synthesized antimicrobial peptides are a diverse group of biologically active bacterial molecules which are characterized as important defense against other micro-organisms. They exhibit variations in primary structure but their cationic and amphiphilic properties makes them to attack target cells by permeabilizing the cell membrane (25).

Structure and classification of ribosomal synthesized peptide

Ribosomally synthesized peptides of Gram positive bacteria are a large group of natural products which exhibit extended activities such as antifungal, antibacterial, and antiviral properties (25).

AMPs of Gram positive bacteria are classified based on their chemical structure, modified amino acids, enzymatic sensivity, size, thermo-stability and mechanisms of actions. They are categorized into three classes. Class 1 includes lanthipeptides are identified as small (<5 kDa) ribosomally encoded peptides, consisting of 19-38 and unusual amino acids lanthionine (Lan) with diverse posttranslational modification to acquire active forms. Lanthipeptides utilize their effect on bacterial cell envelop. The posttranslational peptide process is regularly based on dehydration reactions of three amino acids; serine, threonine and the sulfhydryl analog cysteine. In few cases, modification of Lys, Asp, and Ile residues have been discovered. The final results of dehydration reaction are didehydro alanine)Dha) from serine and didehydro aminobutyric acid (Dhb) from threonine in unsaturated forms (26-28).

The modified amino acids have electrophilic centers which are targeted to neighboring nucleophile groups. The thio-ether bond is formed when the double bond in these amino acids reacts with the thiol (–SH) group of neighbouring cysteine residue, resulting in formation of lanthionine (in Dha) and ß-methyl-lanthionine (in Dhb). This mechanism finally leads to the formation of globular lanthipeptides structures (26). Lanthipeptides are divided into four different classes depending on the posttranslational modifications (PTMs) undergone (29).

Type-A of lanthipeptides are extended, cationic peptides with up to 34 residues in length that are characterized by similarities in the arrangement of Lan bridges in their structures. The mechanism of these peptides is identified by the disruption of the membrane of target bacterial cells. Lantipeptides type-B are globular, with up to 19 residues in length with ability to kill bacteria by disrupting of enzyme functions. Type C and D of lanthipeptides lack of significant antibiotic activity. Overall the modified peptides are classified into linaridins, proteusins, head-to-tail cyclized peptides, sactipeptides, peptides, auto-inducing peptides, which are well described by Arnison et.al.(30) .Recently, modified peptides of Gram positive bacteria are characterized extensively according to their biosynthetic and structural features. The common peptides of Gram positive genera are shown in Table 1.

Class II bacteriocins are characterized as small unmodified peptides (less than 10 kDa) with 30-60 amino acid residues, one or more disulfide bonds in their structure, heat-stable, non-Lan-containing and act by pore formation in target membrane(32). This class is subdivided into IIa, IIb, IIc and IId subclasses. Subclass IIa (pediocin-like) with the N-terminal motif, YGNGV, is the largest group which usually has effective impacts on Listeria monocytogenes. Subclass IIb (two component) bacteriocins require two combined peptide action for effective activity. AMPs in subclass IIc (circular) have a cyclic structure which is formed by covalent bonding of carboxyl and amino groups at terminal residues, while the sub class IId (miscellaneous) is composed of different types of linear peptides (33, 34).Class III are large (>30 kDa) heat-labile proteins with active –SH group and class IV are complex proteins containing lipid or carbohydrate moieties which require more biochemical investigation. Class V peptides currently are subjected as a new class that are serine-rich at the carboxyl-terminal region (29, 32).

Posttranslational modification enzymes

Most ribosomally synthesized antimicrobial peptides consist of inactive pre-peptides with an N-terminal

Table 1. Common ribosomally synthesized peptide of Gram positive bacteria and their potential application (30, 31).

Ribosomally synthesized Peptides	Critical features	Examples	Applications
Lanthipeptides (Lan)	Possess Lan or MeLan thioether linkage	Nisin A , Lacticin 3147,Subtilin,Mersacidin,Cinnamycin	Antibacterial agents ,Food industry , Blood pressure regulation , Bacterial mastitis , Oral hygiene
Linaridins	Contain thioether crosslinks in dehydroamino acids but lack of Lan/MeLan linkage in their linear structure	Cypemycin	Antimicrobial agents
Linear azol(in)e-containing peptides (LAPs)	Linear peptide with thiazole and /or (methyl) oxazole heterocyclic rings	Streptolysin S	Antimicrobial agents
Thiopeptides	A central six-membered nitrogen-ring (central pyridine, dehydropiperidine or piperidine) also heterocyclic PTMs	Thiomuracins	Antimicrobial agents Antimalarial agents Anti-cancer agents
Bottromycins	Possess macrocyclic amidine and a decarboxylated C-terminal thiazole with extended C –terminal follower peptide instead of N- terminal leader sequence	Bottromycin A2	Antimicrobial agents (against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE))
Lasso peptides	Contain an N-terminal macrolactam with the C-terminal tail threaded through the ring, a side-chain carboxylate of a glutamate or aspartate at position 8 or 9 at N-terminnal domain	Siamycin I, II	Antibacterial agents (extended activtity), Anti-HIV agent (like siamycin II), Anti-mycobacterial agents
Sactipeptides	Contain intramolecular thioether bonds between cysteine sulfur and the α-carbon of other amino acids	Subtilosin A	Antimicrobial agents (narrow activity)
Bacterial head-to-tail cyclized peptides	Have an intra-molecular peptide bond between the C and N terminal domain	Enterocin AS-48	Antibacterial agents
Glycocins	Glycosylated antimicrobial peptides	Sublancin 168, Glycocin F	Antimicrobial agents

leader peptide as a signal sequence, a pro fragment of cationic peptide and finally a C-terminal. Leader peptide plays an important role in the precursor peptides. The sequence is recognized by PTMs enzymes before export from cell. The mature lanthipeptides are formed from pre-lanthipeptide by removing signal peptide via proteolytic enzymes like serine-proteases. In some peptides, follower peptide sequence at C-terminal region is recognition sequences that are important for excision and cyclization(24, 30) . The schematic modification of lanthipeptides is presented in Fig 1.

The generic nomenclature for lanthipeptides was introduced by Vos et al.(35). On the basis of this terminology, Lan was applied for proteins which are encoded by the *lan* gene clusters precursor peptides. The *lan* gene clusters play important roles in biosynthesis and translocation of lanthipeptides. The new classification was purposed in 2007 which considered the homology of the leader sequence, structure of the biosynthetic cluster and peptide activities (27). According to the current classification, Class I lanthipeptides are modified by two distinct enzymes: 1) dehydratase, named LanB, which dehydrates the hydroxyl group of serine and threonine residues by glutamylation and removes glutamate to form dehydroalanine and dehydrobutyrine, respectively and 2) Cyclase, LanC, which mediates cyclization and forms the thioether rings in lanthipeptides. LanT belongs to the group-A ABC-transporters (ATP-binding cassette) and is responsible for transporting active peptides from the cell to the extracellular environment by ATP hydrolysis. When the core peptide is modified as described before, LanP acts as a protease and cleaves to the leader peptides from the modified pre-peptide. Therefore, the active peptides are secreted out of the cell (36-38). Peptides of class II lanthipeptides are modified by a bi-functional LanM enzyme which exhibits dehydratase at N-terminal domain, without sequence homology to LanB , and cyclase activities at C-terminal domain which displays homology with LanC of class I lan-

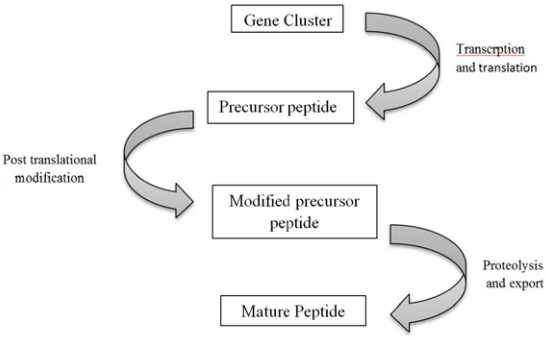

Figure 1. Ribosomally synthesized and post-translational modified peptides pathway. The gene cluster of lanthipeptides is transcripted and translated respectively to precursor peptide. The product converts to modified precursor peptide by post-translational modification process. Finally by removing leader peptide from N- terminus region through proteolysis and exporting peptide, the active mature peptide is formed(30, 31).

thipeptides. The secretion and removal of leader process is performed by a multifunctional protein. Class III and IV lanthipeptides are catalyzed by a family of enzymes (RamC/LanKC and LanL) which modify the dehydration and cyclization reactions (30, 37-39). It was recommended in venezuelin from Steptomyces venezuelae that LanL enzyme should be composed of phosphor-Serine and phosphor-Threonine lyase domain at N-terminal, Ser and Thr kinase at central domain and finally cyclase domain at C-terminal (40).The peptides belonging to classes III and IV may lack significant antibiotic activity(41). Additional Lan enzymes like LanEFG and LanI are determined as transporters which are involved in self-immunity. They form ABC-transporters that present immunity to the cells from synthesized peptides (34, 36). LanK/R are two-component response regulators that are characterized with histidine kinase and regulatory activities. LanO , LanX and LanJ enzymes are recommended for various oxidation activities, hydroxylase reaction and convert dehydroalanine to D-Ala, respectively (28, 34, 42, 43).

Molecular regulation of lanthipeptide synthesis

It was well defined that lanthipeptides biosynthetic genes are generally present in several gene clusters which can be encoded on transposal, chromosomal and plasmid gene. The lanthipeptides gene clusters contain several genes which contribute in the synthesis and regulation of peptides. The expression and product yields of lanthipeptides usually occur at late exponential or early stationary growth phase, depending on some environmental factors like pH , temperature, presence of other competing bacteria, exposure to air and quorum sensing mechanisms (auto inducer peptides, AIP) (27, 44).

In most cases, lanA plays a critical role as a structural gene which encodes other modification and transporter enzymes (lan, lanC, LanM, LanD, lanP , and LanT). Other enzymes like self-immunity and regulatory enzymes are closely related to structural gene(31) .

Quorum sensing is an auto inducer mechanism utilized by Gram positive bacteria.In this mechanism membrane-interacting peptide pheromones play critical roles as an induction factor (IF). A remarkable example of three-component system regulators is entrocin (Class IIa bacteriocins with a double glycine at leader sequence) of *E. faecium*. This system consists of a sensory histidine kinase (HK) on cytoplasmic membrane that acts as a receptor of extracellular signals forming histidine phosphorilated. For this reason, phosphate group is transferred to aspartic acid residue on intracellular response regulator (RR) protein which contributes to control biosynthesis and expression enterocin operons. The adaptive reaction of RR protein is related to activate signaling cascade which is induced by an IF. According to this triggered system, increasing concentration of IF during cell growth leads to induce HK signaling receptors and subsequently AMP gene is transcribed.By the ABC transporter LanT, a pre-protein is transported outside (31, 45-47). This type of regulatory system is involved in the production of enterocin A, B and avicin A (45,48, 49).

In the cases of class IIa, IIb, and partly IId, lanthipeptides are followed by two operons; genes encoding the bacteriocin are contiguous to each other in a single operon (which involves all relevant genes)or three clustered operons. In class IIb, genes are located at the same operon and are adjacent to encoding the structural and immunity genes (Lan I). They response to expresses a pheromone peptide and protect the bacteriocin-producing bacteria from their own destroying bacteriocin. ABC transporter / protease is used for exporting the bacteriocin out of the bacteriocin-producing bacteria .An additional not fully known protein suggested for secretion of the some bacteriocin like lactococcin G(produced by lactic acid bacteria)(50, 51). Three-operon bacteriocins consist of the structural and immunity genes, the ABC transporter/protease with the accessory proteins determinants and finally bacteriocin –produced regulatory genes. Sakacin P (class IIa) is an example which belongs to the recent genetic organization (52, 53).

Two signal transduction regulatory systems contribute to lanthipeptide synthesis by LanRK. Subtilin from *B. subtilis* and nisin from *L.lactis* are mediated by two-component system. They act as signal molecules or auto inducers for their own synthesis through two-component system (TCS). This system is also composed of HK and RR (54, 55).

B.subtilis utilizes a SpaRK TCS which induces the operons of subtilin lanthipeptide structural genes and exhibits self-immunity to subtilin while in the case of nisin, the NisRK TCS induces the *nisABTC* and *nisI-FEG* operons (54).

Non ribosomal bacteriocin and polyketides

Non-ribisomal peptides(NRPs) and polyketides (PKs) are a vast group of natural products with significant structure (linear, cyclic and branched structures),a wide-range of biological activities and pharmacological properties, such as anticancer(calicheamicin and bleomycin), immunosuppression (rapamycin), and antibacterial)erythromycin and vancomycin) (56). They are non-ribosomal synthesized compounds which are synthesized by thio-templates on large multi-modular enzyme complex as catalytic units. The multi subunit enzymes consist of sizes ranging from 100 to >1600 kDa. The precursors including pseudo, non-proteinogenic amino acids, fatty acids, hydroxyl acids, and N-methylated residues are accumulated and comprised of various structures of lipopeptides, depsipeptides, and peptidolactones with modular arrangement. It is possible that these peptides are modified by N-methylation, acylation, glycosylation, or heterocyclic ring formations. It was reported that prevalence of NRPs enzymes is over a wide variety of bacteria and fungi in different environmental situation (57, 58).

Synthesis of NRPs and PKs

NRPsis composed of modules domain which is defined as a catalytic response to incorporate certain amino acid monomers into final peptides. The structural and functional properties were well described by Amoutzias et. al previously (59, 60). The modules of an enzyme consist of catalytic and carrier response domains. For this reason, related amino acids or hydroxyl acid are recognized and activated by ATP hydrolysis to orga-

nize unstable and intermediate adenylate analogues in adenylation domain (A). This structure is transferred to the chemistry site of the same module (T) in order to extend the peptide chain by phosphopantetheinyl bonds (thiolation or peptidyl carrier protein (PCP) domain), condensation domain (C) which comprises development of peptides by linkage between the extended amino acid chains and the activated amino acids (C-N bound).Finally the thioesterase (TE) domain at the termination of module releases the final product from synthetase by hydrolysis and/or cyclization. In some cases, C domain exhibited both condensation and cyclization properties(57, 58, 60).

The group of PKs are synthesized on enzyme complex and classified into three types based on their differentiation of catalytic domains. PKs-I are large multidomain enzymes which consist of three domains; acyl transferase (AT), ketosynthase (KS) and acyl carrier protein (ACP) which present similar function to A, C and PCP domains of NRPs, respectively. The AT domains include malonyl or methylmalonyl-CoA, the KS domains are responsible for C-C bond formation and ACP domain correspond to the PCP domain of NRPs(56, 57, 59). PKs- II applies mono functional proteins which exhibit independent operation whereas PKs III consist of chalcone synthase which is able to produce variety range of aromatic peptides from acyl-coenzyme A (CoA) substrates (61, 62).

Post translational modifications of NRPs

The post translational modification is defined as the required process to achieve active forms of NRPs. This reaction is catalyzed by external PPTase a 4'-phosphopantetheinyl transferase (4'-PP) cofactor which is sited at T domain of peptide synthetase.

4'-PP moiety of coenzyme A is transferred to a side chain of conserved serine residue of the peptide synthetase pro-peptide and is converted into an active holopeptide (57, 60).

The NRPsare created based on regular peptide synthesis process in N- to C- terminal. A domain is involved in the first step of peptide synthesis by recognizing the substrate which can be D or L or non proteinogenic forms of amino acids. In the primary steps of peptide synthesis, aminoacyl-tRNA synthetase interacts with A domain, resulting in an aminoacyl adenylated intermediate. In the second step which is known as elongation process by PCP domain, activated amino acids connect to 4'-PP by covalanet thioester bond. In this stage, the thiol-activated substrates can undergo further modifications like epimerization.

Thioester-activated carboxyl group of activated peptide residue is conveyed to the contiguous amino group of the next amino acid and leads to peptide polymerization of N to C terminus effect. Next, the produced peptide is transferred to C domain where peptide condensation step occurs and then the assembled peptide is released by TE domain (63, 64).

Additionally, some characteristic domain in catalytic modules were defined which are associated in NRPs for further modification in order to achieve mature forms (60, 64). Tailoring enzymes, like epimerisation (E) that changes an L-amino acid into a D-amino acid, (E/C) which is responsible for both epimerized and condensed activities, methylation (M), oxidation (Ox) which is found at either downstream of the PCP-domain or in the C-terminus of the A-domain, Reduction (R), Formylation (F), glycosylation (G),and heterocyclisation of cysteine, serine, and threonine residues(Cy) are critical processes. These diversity of features make the characteristic NRPspossess extended applications (57, 58).

In the case of PKs, modules are composed of core domains, AT domain is responsible for selecting suitable residue which is activated and transferred to ACP domain which is connected to the thioester bond and finally the KS domain which is incorporated for condensation between the activated residue and intermediated polyketide available on contiguous ACP domains.

Further domains like ketoreductase(KR), dehydrase (DH) and enoyl reductase (ER) are included for modification activities(59, 65). Some of the natural products are a complex compound which is encoded on hybrid gene clusters and consist of NRPs and PKs proteins together. NRPs-PKs systems are categorized into two classes; (I) consist of natural products produced by NRPs and PKs independently and finally joined together to create a hybrid product and (II) the NRPs and PKs enzymes which associated result in a hybrid metabolite (66, 67).

Genes associated in NRPs synthesis

The regulation gene synthesis of NRPs is well known in *Bacillus* spp. Most of the reports presented the associated genes to NRPs synthesis like srfA, bac, and bmy which encodes surfactin, bacilysin and bacillomycin, respectively.

In the case of surfactin, the ComP–ComA two-component system is stimulated by cell density signals (ComX pheromone activated). The initial transcription process is induced by autophosphorylation of ComP. In the next step, the phosphate group is transferred to ComA resulting in formation of phosphorylated ComA. This molecule is an active form of the regulator thatables to bind to the promoter region of the srfA and regulates the expression of srfA (68, 69) .

Bacillomycin D, a member of iturin family of lipopeptides, is a cyclic heptapeptide with a β-amino fatty acid moiety. The significant fashioned structure of iturin is tyrosine in the D -form at the second amino acid position and other two D -amino acids at positions 3 and 6 (68) . The expression of bacillomycin is related to bmy operon (37.2 kb) which leads the biosynthesis of bacillomycin D. This operon consists of four genes (bmyD, bmyA, bmyB, and bmyC). The bmyD is a starter of operons and encodes a putative malonyl coenzyme A transacylase. BmyA, BmyB, and BmyC are encoded sequentially and associated in condensation, adenylation and thiolation . It was illustrated that by deletion in bmyD promoter site, activated sigmaA factor responded to the expression. For this reason, DegU directly bind to two distinct sites at upstream of promoter and regulate the posttranscriptional expression of bacillomycin D (68, 70).

Bacilysin (L-alanyl-(2,3-epoxycyclohexanone-4)-L-alanine), a dipeptide AMP consists of L –alanine residue

at the N-terminal region and a non-proteinogenic L-anticapsin(71).

Bacilysin biosynthesis is comprised of the bac ywfB-CDEFGH operon. bacA and bacB are responsible for changing prephenate into dihydro-4-hydroxyphenylpyruvate (H2HPP4) and dihydro-5-hydroxyphenylpyruvate (H2HPP5). Following this, H2HPP5 is converted to H4HPP6 by ywfh. The *bacD* and *bacE* gene products incorporate in amino acid ligation and bacilysin self-protection respectively. BacC was interpreted as a putative dioxygenase. The bacilysin operon is regulated by guanonsine 5'-diphosphate 3'-diphosphate (ppGp) and a quorum-sensing mechanism.

In this operon, bacA is a promoter of gene cluster which is related to ComA and DegU products. DegU, global regulator protein, bind to the bacA at three sites. The expression of bacA promoter depends on sigA which is activated by interaction with DegU at the final stage of vegetative growth (72, 73).

AMPs mode of actions from Gram positive bacteria

The classical mechanism of AMPs actions involve the ability of electrostatic interaction between AMPs and cell membrane of microorganisms and are responsible for the disruption of bacterial membrane. The positive amino acid residues from AMPs and a net negative charge of bacterial cell surface (Teichoic and lipoteichoic acids from the wall in case of Gram positive bacteria) respond to this reaction. Bacteriocins are introduced as effective bactericidal agents. Previous studies reported the narrow spectrum activities of bacteriocins on closely related species. While some other bacteriocin like nisin are effective against a broad range of bacteria. Different modes of actions have been demonstrated for lanthipeptide. Disruption of cell membrane by pore formation, inhibition of cell wall synthesis and disordering enzymes activities are the critical identified mechanisms of lanthipeptides(18). In Figure 2 the schematic of lanthipeptides actions are shown.

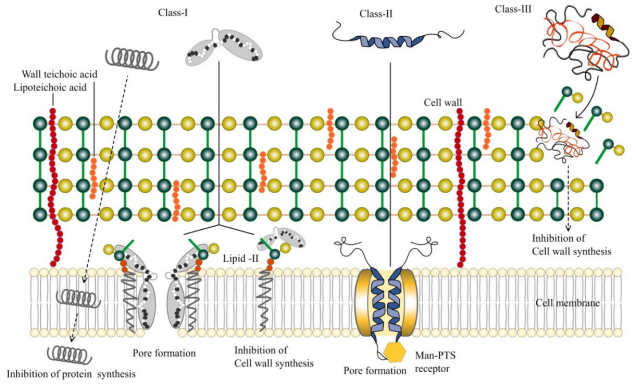

Figure 2. Modes of actions of different types of lanthipeptides effects on Gram positive bacteria (modified from (29)). The most of lanthipeptides inhibit the functional mechanisms in Gram positive bacteria through pore formation in cell membrane (class I and class II lanthipepdides), inhibit of peptidoglycan synthesis (Class I and class III lanthipeptides) and inhibit protein synthesis (thiopeptides). In the cases of nisin (class I) and some class I of lanthipeptides possess a dual mechanism of action ; (a)the ability to bind to lipid II (transporter of peptidoglycan subunits from the cytoplasm to the cell wall) leads to prevent cell wall synthesis and causes cell death and (b) the ability to insert to cell membrane and induce pore formation by lipid II as a docking molecule and finally leads to target cells death.

Pore formation mechanism

Several lanthipeptides type A, like nisin and some class II bacteriocins are associated with cell membrane disruption mechanism. They exhibit bactericidal effect through pores formation and cause disintegration of proton motive force (PMF) in cytoplasmic membrane. By disrupting the PMF, the energy requiring reactions processes of macromolecules such as DNA, RNA, proteins and polysaccharides are inhibited and result in death of cells by releasing intracellular small biological molecules like ions, ATP and amino acids. These actions are independent to specific target receptor binding while pore formation is an energy dependent process (74, 75).

So pore formation is a considered as a principal mode of nisin actions. Other mechanism of pore formation is identified as the nisin–membrane interaction. For this reason, nisin proposes tight connection to lipid bilayers via electrostatic attraction with the phospholipid head groups. As it was shown earlier, the C terminal domain of nisin contains residues with positive charge which interact with anionic phospholipids. Following the electrostatic interaction, translocation of C-terminal domain across the membrane is facilitated by binding to negatively charged carboxy-fluorescein inside the cell membrane, and this result in the formation of a wedge-like transient pore. After releasing anion molecules, free nisin returns to the surface and bind to another molecule(33, 76).

Class IIa of bacteriocin (pediocin-like bacteriocins) with conserved positively charged N-terminal region forms a three-stranded antiparallel β-sheet by disulfide bond which interact with the cell membrane. This region is followed by a central region to an α-helix and a C-terminal tail which forms hairpin structure by changing fold. When AMP electrostatically binds to the cell membrane, the hydrophobic C-terminal domain is inserting into the hydrophobic cell membrane region, consequently leading to pore formation (77, 78). Class IIb bacteriocins acts based on complementary action of two peptides and pore formation in the cytoplasmic membrane to cause cell death. The pore formation is related to the presence of GxxxG-motifs and helical structure in this class. Subsequently, bacteriocins form membrane-penetrating through helix–helix interaction involving their GxxxG-motifs (79, 80).

It was identified that the bacteriocins belong to class IIb form relative pores which act significantly as a transporter of molecules indeed induce membrane leakage or disruption (53). Lactococcin G has potential to form specific K^+ pores which induce the target cell membrane permeability for monovalent cations, such as Na^+, K^+, Li^+, Cs^+, Rb^+ and choline, but not for H+, divalent cations or anions. Ultimately, by increasing ATP hydrolysis due to ATP-driven K^+ uptake, cell death occurs(50). Lactacin F induces membranes permeable to K^+ and phosphate independent to PMF (53, 81). Permeabilization of plantaricin E/F and plantaricin J/K are in contrast to lactococcin G. They cause permeability of membranes for monovalent ions, including H^+ but not

for divalent ions such as phosphate and Mg^{2+}(53, 77).

Bacteriocins of class IIc, like circular enterocin AS-48, exhibit a compact globular structure and arrange into 5 α-helixes in which their conformations change in hydrophobic environments. The peptides are inserted into bacterial membranes and induce permeable membrane which ultimately cause cell death (82)

The prediction of pore formation has been discussed based on a 'barrel-stave. In this model, cationic peptide accumulates at the membrane surface via electrostatically interaction and in parallel orientation. The presence of peptides makes the surface rigid and thin and result to displacement of phospholipids. After insertion of the peptides into membrane, cell interaction between hydrophobic faces to lipid bilayer and alignment of hydrophilic side chains in direction of pore center is deduced and result in pore formation (13, 15).

Inhibition of cell wall synthesis

On the basis of the results achieved by modeling of membrane systems, nisin displays strong *in vivo* potency in nanomolar concentration in comparison to its effectiveness in micromolar concentration in membrane model systems. The findings supported that additional activities or specific targets may be involved. The other inhibitory actions of nisin are related to inhibition of peptidoglycan biosynthesis through binding to an essential precursor of cell wall called lipid II.

lipid II (undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc) is an important docking molecule which may be associated to pore formation (83).

The mechanism of nisin action for cell wall inhibition was identified. The N-terminal domain of nisin plays an important role in binding to cell wall precursor, lipid II. Following the electrostatic interaction between positive charge of nisin and negative charge of cell wall component leads to nisin binds to lipid II. Subsequently, by changing the orientation of the membrane, C- terminal domain is trans-located through the membrane. By introducing additional peptides at insertion site, the trans- membrane pore is formed (23, 34, 83)

Subtilin and streptococcin SA-FF22 with similar modes of action, form larger pores than nisin. It was reported that pores diameter of subtilin are up to 2 nm with lifetimes of up to 10 s while SA-FF22 pores are about 0.5–0.6 nm in diameter and with lifetimes of only short seconds (84).

Mersacidin, another type B lanthipeptide, is effective against methicillin-resistant *S. aureus* (MRSA). The peptides utilizes antibacterial action by inhibition of peptidoglycan biosynthesis through binding to the sugar residue of lipid II . For this reason, the peptide prevents the incorporation of glucose and D-alanine into cell wall material.

Disarrangement in membrane associated at the level of trans-glycolisation reaction leads to monomeric precursor of lipid II being converted into polymeric nascent peptidoglycan (17, 81).

Two-peptide family of lanthipeptides are composed of two independent transcribed peptides. It has been demonstrated that one of the subunit of peptides binds to lipid II and the other forms trans-membrane pores (85). The maltose ABC transporter of the circular peptide and membrane metallo-peptidase are involved in cell wall synthesis inhibition (86).

It has been demonstrated that nisin and the related lanthipeptides exhibited induced lytic enzymes to degrade cell wall. Induced N-acetylmuramoyl-Lalanine amidase and an N-acetyl glucosaminidase with cationic structures bind to negative charged cell wall. Cationic peptides activate enzymes by replacing them through cationic exchange-like process. Ultimately, combination of peptide's activities such as increased osmotic pressure via pore formation and a destabilized cell wall causes cell lysis (85).

Non-ribosomally synthesized peptides like vancomycin and daptomycin act via cell wall inhibitory synthesis by targeting lipid II pathways. For this reason, vancomycin binds to

the D-Ala–D-Ala dipeptide in the cell wall-cross-linking bridge and blocks the cell wall synthesis by inhibition of trans peptidation reaction (85).

Inhibition of gene expression and protein synthesis

Some AMPs can transfer across the bacterial membranes, and target intracellular molecules such as nucleic acids and proteins to inhibit their functional activities.

Nocathiacins, thiostrepton, thiazomycin and several other thiopeptides bind to the 23SrRNA ribosomal subunit and make its function inactive. Other mechanisms like inhibition of the enzyme activity have been reported. The cinnamycin-like type-B lanthipeptides displays anti-bactericidal activity via increased membrane permeability, resulting in reduced ATP-dependent calcium uptake, ATP-dependent protein transport and calcium uptake. Duramycin and cinnamycin exhibit hemolysis effects of red blood cells and are identified to inhibit the enzyme phospholipase A2, which interferes in the synthesis of prostaglandins and leukotrienes in the human immune system (74, 87).

Resistance mechanisms of Gram-positive bacteria to AMPs

Bacterial resistant mechanisms to bacteriocins are categorized into two groups: I) naturally (intrinsic) resistant and (II) acquired resistance. Several genetic loci are incorporated into resistance mechanisms. Most of the findings of this field refer to the investigation of specific AMPs like nisin and class IIa members (31). By applying biotechnology methods and genetic engineering mutations generated by knockouts or deletions genes, overexpression of bacteriocin resistance genes were analyzed (88).

Gram positive bacteria show different strategies of natural and acquired resistance to AMPs. The most common resistance mechanisms include change in bacterial cell surface and blocking of the AMPs to access their targets.

One of the bacterial resistance mechanisms against bacteriocins is degradation of AMPs by secreting proteases. The extracellular proteolytic enzyme's interaction with AMPs is one of the natural resistant mechanisms which seem to be specific for bacteriocins. Most of the Gram positive bacteria like *Enterococcus faecalis*, *S. aureus* and *S.epidermidis* represent various pro-

teases with broad substrate specificity (89-91).

Aureolysin and SepA belong to the metallo-proteases group and serine endopeptidases like V8 protease from *S.aureus*, can hydrolyze cationic AMPs such as human cathelicidin LL-37(89). *Streptococcus* and *E.faecalis* produce SpeB cysteine protease and Gelatinase, respectively. *S. pyogenes* expresses the G-related alpha2M-binding (GRAB) protein which is able to attach to the surface and bind to protease inhibitor α2-macroglobulin. The of GRAB-α2-macroglobulin complex trap SpeB at the bacterial surface. SpeB possess proteolytic activitytowards LL-37(92). SpeB showed proteoglycan activity by realizing dermatan sulfate that degrade human α- defensin (93). Some strains of *Bacillusspp*. are nisin resistant via producing nisinase, which degrades nisin during sporulation. The enzyme breaks the C-terminal lanthionine ring. Nisin resistance has also been reported in some non-nisin-producing *Lactococcus spp*. In a recent case, nisin degrades by nisin resistance protein

(NSR). NSR-mediated nisin resistance occurred via proteolytic degradation of nisin . The protease located on the cell wall, and removes the C- terminal domain and makes the nisin incative(90, 94).

Sequestration protein-mediated resistance

Some Gram positive bacteria display another extracellular mechanism of protein mediated resistance to AMPs by sequestration method. In this mechanism, extracellular proteins are attached to AMPs and inhibit the binding to the bacterial cell membrane. The significant examples have been identified in *S.aureus*, *S.pyogenes* and *Lactococcus lactis*. Staphylokinase produced by *S. aureus* is an extracellular AMP sequestering molecule (95) . This molecule is encoded by *sak* gene and sequesters α-defensins (HNP-1and HNP-2)to reduce their bactericidal effects. By binding to cathelicidin, LL-37 leads to activation of the transformation of plasminogen to the host extracellular degrading enzyme. In the case of *Streptococcus Spp,* inducing of streptococcal inhibitor of complement (SIC) and streptokinase (Ska) leads to secretion of protein sequestrate AMPs which prevents them from reaching cell-surface targets and also affects activation of plasminogen, respectively. Both mechanisms in *streptococcus* protect the bacteria by binding to LL-37 and defending and neutralizing AMPs. Proteins attached to the cellular surface like M1 protein in *S. pyogenes* was described to bind to LL-37 and prevent AMP from attaching to cell membrane. Other structures are pilus subunit, PilB in *S. agalactiae* binds to cathelicidins (LL-37 and CRAMP) and associated to streptococcal AMP resistance (92, 96).

As described before, LanI as an immunty protein of bacteriocin, plays an important role by binding to AMPs or cellular target of AMPs to protect bacteria against bacteriocins activities. LanI and LanEFG (transporter) are often exhibited together to inactivate AMPs (36).

Inhibition of AMP activity by biofilm formation

Bacterial biofilm has been defined as a structured consortium of surface –attached manner to an aggregate of bacteria stuck and enclosed to each other in a matrix composed of proteins, extracellular DNA and polysaccharides. Experiments showed that bacteria in biofilms surface usually demonstrate much higher resistance (approximately 10–1000 times) to antibiotic agents than the planktonic bacterial cells due to increased AMPs penetration through the matrix (97).

The composition of intracellular polysaccharides varies greatly depending on their chemical structures, type of microorganisms and age of the biofilm generated in different conditions.

Intercellular polysaccharides adhesion, like poly-N-acetylglucosamine, contribute to biofilm formation of *S. aureus* and *S. epidermidis*. They are responsible for reduced bacterial cell death by human defensin hBD-3, cathelicidin (LL-37), and the anionic AMP dermcidin (97).

Bacterial capsular polysaccharides (CPSs) are created on the cell surface of various types of bacterial species. The CPSs are tightly associated with the bacterial cell surface via covalent attachments to either phospholipid or lipid-A molecules. CPSs are highly hydrated molecules composed of repetitive monosaccharaides linked by glycosidic bonds.

They are usually observed in Gram negative bacteria but the significant capsule exists in *S. pneumoniae* which protect bacterial membraneby binding to or rejecting AMPs. Hyaluronic acid capsules like M protein of group A *streptococci* induce resistance to LL-37. The other mechanism which is identified only in Gram-positive bacteria is related to poly-gamma-glutamic acid (PGA). PGA is involved in forming extracellular capsule which protects bacteria from phagocytosis, LL-37, HBD-3 and dermcidin (97, 98).

Modifying cell envelope surface

As earlier defined, the electrostatic interaction between AMPs and bacterial cell envelope (cytoplasmic membrane and cell wall) is an initial step to represent antibacterial activity. The Gram-positive bacterial cell wall consists of a thick peptidoglycan layer and polysaccharides. Anionic teichoic acid (TA) polymers are the key molecules to interact with cationic AMPs. Therefore the structural cell walls of Gram positive bacteria are protected from antimicrobial agents. The anionic polysaccharides remain in two position; binding to cytoplasmic membrane like lipoteichoic acid (LTA) and lipoglycans (LG) and attaching to peptidoglycan layer like wall teichoic acid (WTA) and TA (99). One of the protected bacterial mechanisms for contacting to AMPs is related to dlt ABCD operon. The operon promotes D-alanylation of TA and LTA, resulting in reduced anionic surface charges and consequently lower attraction between positively charged antimicrobials and bacterial cell envelope. This mechanism was detected in most of the Gram positive genera including *Staphylococcus, Enterococcus, Bacillus*, *Streptococcus, and Lactobacillus.* In the cases of*S. aureus* and *Staphylococcus xylosus* which possess many copies of dlt operon, resistance to defensin, protegrins, tachyplesins, magainin II, gallidermin, nisin and a gramicidin D was reported (18, 90).

Multipeptide resistance factor protein, MprF, is an integral membrane protein responsible for amino-acylation of phosphatidyl glycerol with lysine, resulting in modified phosphatidyl glycerol by the enzymatic trans-

fer of L-lysine. Following lysine transfer, the positive net charge of phosphatidyl glycerol will be increased(100). The bacterial peptidoglycan precursor, Lipid II, by replacing the terminal D-alanine is involved in bacterial resistance mechanisms (85). Lysozyme or N-acetylmuramide glycanhydrolase is an antimicrobial enzyme of the host innate immune defense. The cationic structure of lysozyme interacts with negatively charged bacterial surfaces. Next it hydrolyzes ß-1,4 linkages between N-acetylglucosamine and N-acetylmuramic acid of peptidogycan by muramidase domain. Lysis of the cell occur by breaking down the peptidoglycan subunits (101).

Efflux pumps

Efflux pumps are identified as a common energy-dependent mechanism of Gram-positive bacteria used to excrete toxic compounds and antimicrobials agent from cells to external environment. A major facilitator superfamily (MFS) efflux pumps have been reported for Gram positive bacteria. ATP-binding cassette (ABC) transporter is one of the AMP- efflux pumps. As described before, three-component, ABC transporters, or LanFEG systems, are found in AMP-producing bacteria and they protect bacteria against lanthipeptides self-producing (34).

Two-component ABC-transporters induce AMP resistance in non-AMP producing bacteria (50). Single membrane protein antimicrobial transporters create multi-drug resistance . ABC-transporters make bacterial resistance to peptide and non-peptide antibiotics. In *S. aureus* QacA transporter is involved in bacterial resistance to AMPs. This molecule belongs to the MFS of membrane transport proteins (102).

Biotechnological applications

By the potential usefulness of natural peptides which has been reported, bacteriocins AMPs exhibited broad-spectrum antimicrobial activity at low concentration against microorganisms, rapid bactericidal effects on pathogenic drug-resistant bacteria, non-cytotoxic to eukaryotic cells and low levels of induced resistance; these advantages make bacteriocin a good candidate of proposal for biotechnological applications in biomedical industry (31, 33).

By employing bio engineering methods, the discovery and production of natural and modified bacteriocins was developed. It is also facilitated the progress of production of large amounts of peptides and the design of recombinant peptide with higher effectiveness than wild type through the cloning and expression of vectors into host cells (103, 104).

The biotechnology technics provided the setting to survey the effects of mutations in improving structural and functional bacteriocins. By peptide engineering the stable structure of nisin (nisin Z) was produced. It has been reported that by further new posttranslational modifications and applying specific enzymes, the potent AMPs were produced. The recent method is useful for drug designs in pharmacology (105-107).

This new achievement of biotechnology method can be used to produce recombinant AMPs by fusing genes, encoding two types of AMPs,in order to increase the effective and broader antimicrobial spectrum (108).

The potential bacteriocins which showed effectiveness *in vitro*are nisin and lacticin 3147. They exhibited antimicrobial activity against *S.pneumoniae*, MRSA, vancomycin-resistant *enterococci* (VRE) and *Clostridium difficile*. It was shown that these two bacteriocin can be applied for bovine mastitis prevention in veterinary. By changing a single amino acid in nisin structure led to achieving nisin Z with higher solubility and effectiveness against Gram- negative bacteria (29, 31, 109, 110).

Application of bacteriocins in food products is another advantage of AMPs. Their ability to inhibit Gram-positive food –borne bacteria (like *L. monocytogenes*, *S. aureus*, *Bacillus cereus* and etc), susceptibility to digestive proteases; consistency in a wide range of temperature and pH beside no toxicity to eukaryotic cells, made them an alternative food additive to conserved and canned food products. Nisin and pediocin are licensed as food preservatives and are applied in varied food industries (33, 111-113).

The applications of NRPs are typically in human health and environmental products. Most of the NRPs products were extracted from fungi but some bacteria of various genera like Bacillus are able to induce NRPs(114, 115). Surfactants with microbial sources have been considered instead of chemical derived surfactant with petroleum sources. Application of bio surfactantis on the increase because of their potential activity, diversity, no side effects for environment and human health, high selectivity and specific activity at extreme temperatures. The critical Gram positive NRPs which are applied in human health is bacitracin from some species of *Bacillus licheniformis* and *Bacillus subtilis*(115-117).

In both cases of ribosomal synthesis peptides and NRPs, the obtained concentration during their production is still very poor. Industrial peptide production is commonly based on solid-phase synthesis, liquid-phase synthesis, and *in vivo* biotechnological recombinant technology which makes the production cost very high. Although industrial chemical synthesis has been set up for a few peptide but this method seems not to be cost-effective yet because of expensive materials and low income of purity products (37, 118, 119). Bioengineering bacteriocin products possess major advantages including high product concentrations, generation of the compounds in their right conformational forms and very few downstream processing steps. Conjugated AMPs with high potential activity is one of the major biotechnology strategy used to introduce efficient antimicrobial agents. Recently, STAMP (specifically targeted antimicrobial peptide) technology was introduced based on the structure of a fusion peptide. In this construction, two functionally independent components are joined together by a short flexible linker. One of them is responsible for attachment to the pathogen named targeting domain and the other is the AMP domain. Consulting of targeting domain is approved by specific determinants characteristics on target surface like charge, bacterial surface component and pheromone receptors. These signal leads to the accumulation and eventually increase in the concentration of AMP around the organisms (120).

It seems that by improving biotechnological knowledge and systems for generating AMPs, the biological process and production scale will be developed and become cost effective(106) .

Conclusion

Over the past decades, the increase in bacterial drug resistance has become an emergent health care problem, and thus one of the most promising agents which can serve as a next generation of antibiotics for combating MDR pathogenic bacteria.

Antimicrobial peptides present diverse structure and function are produced by all forms of life, possess broad spectrum of activity against microorganisms with combined strategies of eliminating them and exhibit no side–effects for human cell line. The AMPs generated by Gram positive bacteria are a wide antimicrobial substances including ribosomally and non-ribosomally synthesized peptides. Both kinds of peptides showed similarity in their post translational modifications with different structural synthetic routes. According to their varying structure, mode of actions and resistance mechanisms to AMPs, the commercial utilization of bacteriocins requires more improvement. Nowadays the applications of these biological compounds are more included in food industry than therapeutic usages. Though peptides derived Gram positive bacteria are good candidates for therapeutic applications as nanofilms or coating materials for surgical devices used to inhibit biofilm formations. Unlike the thousands of potential synthetic peptides that have been reported by academic studies, only a few peptides are available in the market, and most of them are in early or late clinical development phase. One of the best solutions to overcome the high cost of these products is to apply biological production techniques. By applying biotechnological methods like cloning and expression in host cells, the innovation to generate conjugated products with increased efficiency is accessible.

Therefore, AMPs derived from Gram positive bacteria are alternative candidates for conventional antibiotic agents against rapidly emerging bacterial resistance infections.

Conflict of interest

The authors declare that they have no conflict of interest.

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