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Anti-Microbial Peptides: The Importance of Structure-Function Analysis in the Design of New AMPs

Awatef Ouertani, Amor Mosbah and Ameer Cherif

Abstract

In recent years the rapid emergence of drug resistant microorganisms has become a major health problem worldwide. The number of multidrug resistant (MDR) bacteria is in a rapid increase. Therefore, there is an urgent need to develop new antimicrobial agent that is active against MDR. Among the possible candidates, antimicrobial peptides (AMPs) represent a promising alternative. Many AMPs candidates were in clinical development and the Nisin was approved in many food products. Exact mechanism of AMPs action has not been fully elucidated. More comprehensive of the mechanism of action provide a path towards overcoming the toxicity limitation. This chapter is a review that provides an overview of bacterial AMPs named bacteriocin, focusing on their diverse mechanism of action. We develop here the structure–function relationship of many AMPs. A good understanding of AMPs structure–function relationship can help the scientific in the conception of new active AMPs by the evaluation of the role of each residue and the determination of the essential amino acids for activity. This feature helps the development of the second-generation AMPs with high potential antimicrobial activity and more.

Keywords: Multidrug resistant bacteria, Antimicrobial peptide, mechanism of action, peptide synthesis

1. Introduction

The routinely use of antibiotics decreased their efficiency and allowed bacteria to adapt to antibiotics, resulting in the emergence and rapid propagation of resistant bacterial strain [1]. This feature is a serious health and economic problem, leading to increased rates of morbidity and mortality associated with bacterial infections caused by multi resistant bacteria [2] such as Methicillin-Resistant *Staphylococcus aureus* (MRSA), Vancomycin-Resistant Enterococci (VRE) or MDR [3–5]. To fight against this health problem, it is imperative to find new alternatives to antibiotics [6]. Several resources were investigated, for their ability to provide antimicrobial agent as well as animals, plants derived compounds and microorganisms [7]. Among natural resources, bacteria are known to be a good producer of antimicrobial agents [8] such as lipopeptides, glycopeptides cyclic peptides and

natural peptides named as bacteriocins [9–11]. The latter are considered as the first line defense of bacteria and allows them to gain a competitive advantage and to thrive in complex ecosystems [11]. Bacteriocins received a great interest as potential antimicrobial agents with high activity against numerous bacterial, fungal, yeast and viral species [12–14]. Riley et al. have reported that all bacteria are able to produce bacteriocins [15]. A large variety of bacteriocins have been identified, and some bacteria can produce bacteriocins with activity against MDR bacteria [16]. This broad collection of antimicrobial molecules allows many biotechnological, industrial and pharmaceutical applications [17]. Moreover, their toxicity is a limiting factor. Nisin is the only bacteriocin that have been legally approved by the world health organization (WHO) and by the food and drug administration (FDA) for human use as a food preservative, and it has been given a generally-regarded-as-safe (GRAS) designation by the FDA [17]. It is safe for human consumption and is not toxic to animals. Thus, toxicology studies have demonstrated that nisin ingestion does not cause toxic effects to the human body, and LD50 reported was 6950 mg/kg when administered orally [18]. Nisin was shown to be effective against various Gram positive bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Enterococcus faecalis* [19]. In addition, Nisin derivatives are more active [17].

Bacteriocins possess a key traits that makes them a good alternative to antibiotics [20]:

- i. They are abundant with large diversity [20, 21].
- ii. Various bacteriocins such as nisin [22] have demonstrated distinct mode of action compared to conventional antibiotics.
- iii. The use of bacteriocin with narrow spectrum of inhibition preserves the natural healthy microbiota [23].
- iv. The long term bacteriocin exposure is safe with no side effects and do not lead to bacterial resistance [24, 25].

The study of bacteriocin structures and amino acids composition helps to understand their detailed mechanism of action. This feature is critical towards the development of bacteriocins as therapeutics and can also be used to prioritize hits in their genome mining studies [26]. Hence a library of synthetic bacteriocin variants served as a tool; to recognize key residues responsible for activity and could continue to inspire the development of new therapeutic agents [27].

2. Bacterial antimicrobial peptides bacteriocins

Bacteriocins are ribosomally synthesised peptides produced by Gram positive, Gram negative bacteria and fungi to kill or inhibit significant pathogenic bacteria [28]. Bacteriocin was discovered for the first time by A. Gratia in 1925 when he was involved in the method of searching for approaches to kill bacteria. The first bacteriocin that inhibited *E. coli* was named colicin [29]. This powerful biological arm allows microorganisms to compete for resources and space [11]. Bacteriocin can be incorporated in the food products as purified or semi purified form also the producer strain can be incorporated. Nisin is exploited in various commercial preparations, such as Nisaplin, Chrisin and DelvoNis. It is commonly used in dairy industries to control clostridia and post-processing contamination from *Listeria* strains [30].

Furthermore, nisin and further bacteriocins have been shown to inhibit several pathogens in many food matrixes. Many studies have demonstrated the anti-biofilm properties of nisin and the capacity to act synergistically in combination with conventional therapeutic drugs [31]. In addition, nisin could stimulate the adaptive immune response with an immunomodulatory function. Also, nisin can control the growth of tumors and exhibit selective cytotoxicity towards cancer cells [32]. Bacteriocins have also been identified as promising next generation antibiotics to combat MDR pathogens [33].

2.1 Classification

During the years of discovery, numerous approaches have been taken to classify bacteriocins into a number of groups. This includes the nature of the producing strains, the methods by which these molecules are produced, common resistance mechanisms and the peptides mechanism of actions [34]. The most useful classification established by Claenhamer et al. (1993) subdivides bacteriocin into four classes [35] as follow:

ClassI: small bacteriocin with a molecular weight less than 5 kDa, heat stable and harbor non-standard amino acids such as lanthionine, β methyllanthionine, dehydrobulurine, dehydroalanine and labyrinthine [36]. Class I is subdivided into:

- i. class Ia (lantibiotics): consists of flexible, elongated, positively charged, and hydrophobic peptides associated with a pore formation in bacterial membranes. Nisin is the most representative bacteriocin of this group.
- ii. class Ib (labyrinthopeptins): it regroups globular and inflexible bacteriocins that are negatively charged or with no net charge. These bacteriocins can inhibit catalytic enzymes crucial for the proliferation of susceptible bacteria [37].
- iii. class Ic (sanctibiotics) are sulfur-to- α -carbon-containing peptides [38]. Nisin is the most studied class I bacteriocin [39].

ClassII: Class II bacteriocin regroups peptides with a molecular weight less than 10 kDa, heat stable and with no modified amino acids. This class is subdivided into four sub-classes. Class IIa also named pediocin like bacteriocins [40]. It regroups bacteriocin typically comprised of 25–28 AA with a conserved amino acid sequence YGNGV on their *N*-terminal domains [41]. Class IIb or two peptides bacteriocin. This class regroups two different peptides, both are essential for the antimicrobial activity [37]. Class IIc: circular bacteriocins produced by Gram-positive bacteria represent a diverse class of antimicrobial peptides that are more stable compared to linear bacteriocins [42]. Class IId: linear non-pediocin-like one-peptide bacteriocins [43].

Class III: the large and heat labile bacteriocins. Colicin is one of the well-characterized Class III bacteriocin They have a bacteriolytic (IIIa) or nonlytic mechanism of action (class IIIb) [44].

Class IV: this class regroups complex protein associated with one or more chemical moieties either lipid or carbohydrate [45].

2.2 Biosynthesis

Bacteriocins are primary metabolites with simpler biosynthetic machinery [46]. Bacteriocin coding genes are generally in operon clusters with the minimum genetic machinery, composed of the structural gene and the associated immunity. The clusters harbored in the genome, plasmid or other mobile genetic elements [47].

Bacteriocins are synthesized as inactive precursor peptides composed of an N-terminal leader peptide close to the C-terminal pro-peptide. The leader peptide serves as a recognition site for the biosynthetic enzymes implicated in the maturation process and its transport outside of the producer strain [2]. Bacteriocins are transported and cleaved to generate the mature form through enzymatic processes [48]. Recently, various leaderless bacteriocin has been reported [49, 50] with no common biosynthesis and regulation mechanism [51]. Leaderless bacteriocins do not undergo post translational modification and become active after [51]. Coelho et al. (2016) have reported that a complex mechanism involving a protein with a helix-turn-helix (HTH) AurR, an alternative transcription factor σ^B , and a phage regulator $\phi 11$, regulates the production of aureocin A70 [52]. An ABC-type multi-drug resistance transporter protein, LmrB, has been reported to be implicated in secretion and immunity of the LsbB, leaderless bacteriocin [53].

2.3 Mode of action

Usually, Gram-negative bacteria are naturally resistant to the bacteriocins, due to their outer membrane, which acts as an effective barrier [54, 55]. Microcin B17 (MccB17) is an antibacterial peptide produced by strains of *Escherichia coli* harboring the plasmid-borne mcb17 operon [56]. This bacteriocin passes through the outer membrane via the porin OmpF and is transferred across the inner membrane in a manner that is dependent on the inner-membrane peptide transporter SbmA. The bacteriocin then acts by inhibiting DNA gyrase-mediated DNA supercoiling, thus interfering with DNA replication [20].

The bacteriocin MccJ25 is recognized by the iron siderophore receptor FhuA at the outer membrane and requires TonB and SbmA at the inner membrane to go through the cell. After entering the cell, MccJ25 block the secondary channel of RNA polymerase resulting on the transcription inhibition [57]. MccC7-C51, passes through the inner layer of the *E. coli* cell wall via the YejABEF transporter [58], after which the bacteriocin is processed by one of the many broad-specificity cytoplasmic aminopeptidases of the bacterium [59] to generate a modified aspartyl-adenylate. This, in turn, inhibits aspartyl-tRNA synthetase, thus blocking mRNA synthesis (Figure 1) [20].

Figure 1. Illustration of mechanism of action of representative bacteriocins that inhibit gram-positive (a) and gram-negative bacteria [20].

The general cationic nature of bacteriocins plays a key role in their initial interaction with the cell membrane of the target strains. The negative charge of bacterial cell membranes and the positive charge of bacteriocin generate an electrostatic interaction between them, thus facilitating the approach of the molecules to the membranes [60]. Lantibiotic such as nisin have dual killing mechanism that require its interaction with lipid II receptor leading to i) pore forming that induces the dissipation of the membrane potential and the efflux of small metabolites such as ions, amino acids, nucleotides and other cytoplasmic solutes, resulting in the execution of all biosynthetic processes and the cell death. ii) prevention of peptidoglycan, the main component of the bacterial cell wall, synthesis, causing cell death [61]. Whereas, Members of class IIa bacteriocins have been shown to bind to mannose phosphotransferase system (Man-PTS) proteins, the sugar-uptake system of target bacteria, to exhibit their antimicrobial activity [62]. Their anti listerial activity is due to the conserved N-terminal YGNGV motif, while the less conserved C-terminal domain is responsible for their antimicrobial activity against other strains [41]. Circular bacteriocin such as enterocin AS-48, gassericin A, subtilisin A, and carnocyclin do not require a receptor molecule for their activity. Their basic amino acid residues patch on the surface of their compact hydrophobic globular structure was responsible for the electrostatic interaction between the bacteriocin and the surface membrane of the target cell [63]. However, garvicin ML, a new member of circular bacteriocins, exhibits its activity through binding to a maltose ABC-transporter protein as a target receptor of garvicin ML, which facilitates the efflux of intracellular solutes resulting to the cell death [42].

Leaderless bacteriocins have been shown to not involve a receptor molecule to exhibit their antimicrobial activity [51]. Fujita et al. [64] characterized the mode of action of Lacticin Q, leaderless bacteriocins produced by *L. lactis* QU 5. It demonstrates antimicrobial activity against various Gram-positive bacteria such as *Bacillus* sp., *Lactobacillus* sp., *Enterococcus* sp., *Lactococcus* sp., and *Staphylococcus aureus* [64]. Lacticin Q has been shown to form toroidal pore (HTP) causing depletion of intracellular components such as proteins, ions and ATP, leading to cell death. The HTP formation mechanism initiates with the electrostatic interaction of the cationic lacticin Q and the negatively charged membranes. These binding results in the formation of HTPs associated with lipid flip-flop [51]. In addition, the leaderless aureocin A53 produced by *S. aureus* A53 was shown to permeate the membranes of the bacteria without forming pores [65]. It demonstrates stronger interaction with

Figure 2.
 Illustration of different mechanism of action of three classes' bacteriocins [37].

neutral membrane rather than negatively charged lipids. Studies on the leaderless bacteriocin, LsbB, isolated from *L. lactis subsp. lactis* BGMN1–5, shed light on a zinc-dependent membrane metallopeptidase, YvjB, as its receptor molecule [66]. The C-terminal end of LsbB harbors the receptor binding domain [67] that interacts with the highly conserved Tyr356 and Ala353 residues located at the transmembrane domain of YvjB [68] (**Figures 1 and 2**).

2.4 Structural analysis: amino acids and activity

NMR resolution structure of circular bacteriocins such as; enterocin AS-48, car-nocyclin A, enterocin NKR 5-3B and acidocin B demonstrates a conserved structural motif consisting of four to five α -helices surrounding a hydrophobic core, with the C-terminus and N-terminus ligation occurring within an helix secondary structure [63, 69–72] (**Figure 3**). Various studies suggested that the circularization is not essential for antimicrobial activity but more important for stabilization of the three-dimensional structure of the bacteriocin [73–76]. Jimenez et al. (2005) demonstrated that a fragment of enterocin AS-48 harboring the cationic puta-tive membrane interacting region exhibited competitive membrane binding with no antibacterial activity. This result suggests that the cationic surface patches are involved in an initial electrostatic interaction between the peptide and the negatively charged phospholipids bilayer of target cell membrane. Furthermore, other physico-chemical properties of the bacteriocins may be required for antimicrobial action [42, 51, 77]. Additionally, mutation of aromatic residues in AS-48 reduced activity which shed light on the role of aromatic amino acids on antimicrobial activity.

Furthermore, the crystal structure and site directed mutagenesis of plantacyclin B21AG reveals that Phe8, Trp45 and Lys19 are essential for antimicrobial activ-ity and a significant reduction in activity was observed with Alanine substitution mutagenesis supporting the notion of a similar role of these residues [78] (**Figure 4**). Moreover, many Trp rich AMPs (TrAMPs) has shown interesting antifungal activity such as synthetic peptides PW2 [79], PAF2 [80] and PEP6 [81]. Also, Blondelle et al. have synthesized Combi-1, Combi-2 and Cyclo-Combi, three TrAMPs with high antimicrobial activity against *E.coli*, *S.aureus*, *S.sanguis* and *C.albicans* [82].

Figure 3.
Crystal structure of AS-48 (PDB 1O82) [26].

Figure 4.
 Crystal structure of B21AG showing amino acids essential for activity [78].

Trp residues are always associated with Arg residues which have a positively charged guanidium group located at the end of the side chain. This group ensures i) the attraction of the TrAMPs to target membranes by forming hydrogen bonds with the negatively charged membrane component. ii) Electrostatic and H-bonding with anionic and polar molecule resulting on the cell penetrating peptides. These distinctive properties are crucial for the highly activity of the Arg and Trp –rich peptides even at very short peptide lengths [83].

Various studies have illuminated the picture of structural and functional relationships in nisin. Thus, the specific mutation shed light on the key regions essential for antimicrobial activity. N-terminal was found to be crucial for nisin binding to the lipidII pyrophosphate region [84]. Therefore, the first two residues IL1 and Dhb2 associated with N-ter lanthionine ring A and methyl lanthionine ringB forms a pocket that encloses the lipidII. This interaction is ensured by the hydrogen binding between the pyrophosphate group of lipidII and the backbone amides [26]

Figure 5.
 NMR structure (PDB: 1WCO) of nisin bound to a lipid II analog. Nisin is shown as a space-filling model and the lipid II analog is shown as sticks. The N-terminal (methyl)lanthionine rings (space-filling tan) of nisin envelop the pyrophosphate moiety (magenta and cyan sticks) of lipid II [26].

Figure 6.

The crystal structure of subtilisin A (PDB:1PXQ) illustrates a rigid hairpin-like structure. Acidic (D16, D21, E23) and basic (K2) amino acids are localized at different ends of the folded peptide. Residues participating in thioether linkages are shown as gold sticks [26].

(Figure 5). Wiedemann et al. have demonstrated that C-terminal region is crucial for binding to membrane with a negative surface charge. However, aa33 and aa34 did not play any role in vivo activity [85]. Also, the substitution of Val32 by Lys or Glu residue results on the drastic decrease of activity associated with a significant reduction on K^+ release. This feature demonstrated that the presence of a charged residue in the central segment of the molecule is not tolerable and affect the pore forming process [85]. The C and N-terminal regions are separated by a few residues that proceed as a flexible pivot around which the C-terminal of nisin can rotate and insert vertically into the phospholipids bilayer [26].

Subtilisin A a bacteriocin produced by *B.subtilis* was also characterized. It is composed of three cyclic segments. The intramolecular linkages are ensured by three thioether bridges connecting C4 with F31, C7 with T28, and C13 with F22, while an amide bond between N1 and G35 links the N- and C-terminus of the peptide (Figure 6). This feature contributes to the semi-rigid, cyclic nature of subtilisin A [86–88]. Subtilisin A contain many hydrophobic residues, but there are also three acidic residues, two Aspartate and one glutamate, that are localized on the loop end of the folded peptide and a basic residue, Lysine, present on the opposite end. This separation of charge confers a net anionic (-2). Thus, like bacteriocin, the model proposed for binding engages insertion of the loop-distal end of subtilisin A into the lipid bilayer first [88]. This end harbors the single basic residue, K2, which may interact with the negatively charged phosphate headgroup and a large, hydrophobic tryptophan residue, W34, which may disturb the lipid bilayer [88]. The insertion of subtilisin A causes an ATP efflux and a reduction of the transmembrane ion gradient [89].

3. Conclusion

In recent years, the increased number of MDR pathogens has become a serious problem, and developing a new generation of antimicrobial agents is becoming urgent.

Increased interest has been shown in bacteriocin, AMPs produced by bacteria, particularly the one produced by lactic acid bacteria (LAB) [90]. Numerous bacteriocins have been shown to be effective against many pathogenic bacteria [24]; however, Nisin is the only bacteriocin legally approved by the WHO and by FDA for use in the food, medicine and veterinary industry [91]. Many, derivatives of nisin have been developed and used in various applications.

Bacteriocins are diverse with different mechanism of action. A deepest comprehensive of mechanism of action and the identification of key amino acids and receptor crucial for activity helps to understand the detailed mechanism of action. This feature leads to the development of new antibiotics effective against MDR bacteria and to solve the problem of bacterial resistance.

To address this issue, in this chapter, we have compiled available data to shed light on the structural function relationship of various bacteriocins.

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