Review

Understanding bacterial resistance to antimicrobial peptides: From the surface to deep inside☆

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ABSTRACT

Resistant bacterial infections are a major health problem in many parts of the world. The major commercial antibiotic classes often fail to combat common bacteria. Although antimicrobial peptides are able to control bacterial infections by interfering with microbial metabolism and physiological processes in several ways, a large number of cases of resistance to antibiotic peptide classes have also been reported. To gain a better understanding of the resistance process various technologies have been applied. Here we discuss multiple strategies by which bacteria could develop enhanced antimicrobial peptide resistance, focusing on sub-cellular regions from the surface to deep inside, evaluating bacterial membranes, cell walls and cytoplasmic metabolism. Moreover, some high-throughput methods for antimicrobial resistance detection and discrimination are also examined. This article is part of a Special Issue entitled: Bacterial Resistance to Antimicrobial Peptides.

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Contents

1. Introduction

In recent years, antibiotic resistance has increasingly become an uncontrollable health problem. Bacterial infections caused by resistant strains can be found in hospitals around the world, being extremely common in immune compromised patients [1]. Antibiotics are able to control bacterial infections, interfering with microbial metabolism and physiological processes, such as DNA replication and cell wall biosynthesis. Although multiple compounds are often used, cases of resistance to the majority of antibiotic classes used in hospitals have been reported [2].

The last report from the American Centers for Disease Control estimated that over two million illnesses and 23,000 deaths were caused by drug-resistant microbes in the USA in 2013 [3]. These numbers have encouraged health organizations to establish stricter policies for antibiotic use in order to curtail the emergence of resistance. These

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policies are unquestionably helping to protect patients in many countries. If, on the one hand, a reliable policy for the use of antibiotics is necessary, the development of new drugs with potential activity against these pathogens is also essential.

Antimicrobial peptides (AMPs) are effective antibiotic agents found in plants, animals and microorganisms. These molecules have a broad spectrum of action, often being active against bacteria, fungi and protozoans. The amphipathic structure, common to AMPs, facilitates their interactions and insertion into the anionic cell wall and phospholipid membranes of microorganisms [4]. Frequently, AMP activity results from the disturbance of cell membrane integrity. However, AMPs can act in different cell targets including DNA [5], RNA [6], regulatory enzymes [7] and other proteins [8], appearing as a promising alternative to classic antibiotics [9]. Nevertheless, once AMPs have been put into current clinical use, the development of AMP-resistant strains will be inevitable [10–12]. Thus, the understanding of bacterial resistance against these compounds is extremely necessary for a possible rational planning of the next antibiotic generation.

To shed some light on the bacterial resistance process, several technologies including mass spectrometry and high-throughput techniques have been applied to analyses of bacterial physiology in response to antibiotic stress [13]. In this review article we discuss different strategies by which bacteria can develop AMP resistance from the surface to deep inside, evaluating the bacterial resistance process layer by layer. Moreover, some technologies for detecting antimicrobial resistance are also discussed.

2. Conventional and high-throughput methods to discriminate bacterial resistance

Currently the increase in the development of bacterial resistance to available antimicrobial agents is a major health public problem in the 21st century. Therefore, it is necessary to investigate and monitor antibiotic resistance in order to discriminate the pattern of resistant bacterial strains and to propose the appropriate treatment. These efforts may be helpful to reduce medical expenses and treat patients effectively [14].

In order to measure the resistance of microorganisms to antimicrobial agents, a wide variety of different and conventional laboratory methods is available. Among these can be cited the disk diffusion assay, the broth dilution test and automated commercial systems based on classical biochemical analysis [14]. Either broth (macrodilution and microdilution) or agar dilution methods may be used to measure quantitatively the in vitro activity of an antimicrobial agent against a given bacterial isolate, reporting the minimal inhibitory concentration (MIC).

In order to perform these tests, a series of tubes or plates is prepared with a broth or agar medium, as appropriate to each test, in which various concentrations of the antimicrobial agents are added. The tubes or plates are then inoculated with a standardized suspension of the test organism and after incubation, the tests are examined and the MIC is determined [15]. In susceptibility testing methods using an agar-based medium, such as disk diffusion and Etest, the sizes of the zones of inhibition depend on many variables (i.e. the antimicrobial agent, disk content and inocula), which may represent a disadvantage of these methods [14,16]. These biases have been reduced using firmly established standardized interpretative breakpoints and automated systems.

The commercial systems available are based primarily, or in part, on some of these standardized manual methods and may provide results essentially equivalent to these methods [15]. However, the use of automated or semi-automated systems, i.e., VITEK® 2, BD Phoenix® and MicroScan® WalkAway® [17] in microbiology labs, which also expose bacteria to graduated dilutions of antibiotic drugs, can give a result in fewer hours than the manual methods. They provide an advantage in detecting resistance, ordering fewer laboratory tests during the diagnostic process, completing the diagnostic workup using fewer sample collections, reducing laboratory costs and preventing resistant strains from spreading rapidly [18–20]. Automated systems performing

identification of reduced antimicrobial susceptibility strains are increasingly being used [15,20]. But in some cases a diversity of screening non-standardized methods plus confirmatory testing by more elaborate techniques have to be used to detect different levels of antimicrobial resistance between clinical isolates with a heterogeneous population of cells. This was observed in the GISA strain (GISA, glycopeptides intermediately susceptible to *Staphylococcus aureus*) and their heterogeneous variant hGISA (hGISA, heterogeneous glycopeptides intermediately susceptible to *S. aureus*), whose isolates probably represent the extremes of a common phenotype that confer a variable level of reduced susceptibility to glycopeptides [21,22]. In addition, Lo-Ten-Foe and co-workers [20] showed by comparison between testing different types of antimicrobial susceptibility that the automated system used was a reliable and easy-to-use tool to determine *Enterobacter cloacae* and *Acinetobacter baumannii* colistin resistance, but that it cannot detect antimicrobial resistance in hetero-resistant isolates [20].

Although resistance has usually been analyzed at the phenotypic level by monitoring bacterial growth in the presence of various antibiotics, molecular high-throughput subtyping methods are available and their use seems to be necessary in order to discriminate distinct levels of bacterial resistance and to overcome the difficulties encountered in conventional tests [23]. Moreover, in general, genotypic tests may be advantageous over phenotypic assays, being much faster and capable of circumventing problems associated with a sometimes low resistance phenotypic expression [24]. Hence, the high-throughput methods based on DNA-assays (genomic and transcriptomic tools) and on proteomic-assays have been used to discriminate bacterial resistance and to assist in the management of infections.

Studies carried out in pathogenic bacteria have revealed that genes across diverse functional categories participate in determining the level of intrinsic and acquired susceptibility/resistance to antibacterial agents, known as the resistome, and the ongoing delineation of this resistome may provide fundamental insights both into antimicrobials' mode of action and into the bacterial response to inhibition and resistance [14,25]. Thus, the use of these tools can be advantageous due to their sensitivity and rapid turnaround times, which may provide clinical benefits that offset the cost [26]. The molecular methods to detect antibiotic resistance based on genomic analysis, such as gene sequencing [25,27,28], have been increasingly implemented in clinical laboratories to complement diagnosis and treatment.

Some reports have described the development of variations on techniques as demonstrated by Zimenkov and co-workers [29], in a study which showed an uncomplicated and easily implemented microarray technique. This was capable of detecting mutations in the *gyrA* and *gyrB* genes responsible for fluoroquinolone resistance and mutations in the *rrs* gene and the *eis* promoter locus that are associated with the aminoglycosides and capreomycin resistance in *Mycobacterium tuberculosis*.

Another genomic approach, Scalar Analysis of Library Enrichments (SCALEs), was applied to map the effect of gene overexpression onto Bac8c (an 8 amino acid AMP) resistance in parallel for all genes and gene combination in the *Escherichia coli* genome, being capable of successfully identifying an elaborate network of genes for which overexpression leads to low-level resistance to this specific AMP [30]. In addition, a molecular test in association with conventional screening tests could provide valuable antibiotic resistance information to facilitate the management of patient therapy and the prevention of transmission [28].

Other DNA-based techniques, especially PCR, are often used to examine bacterial resistance genes [31]. Besides, real-time PCR (Q-PCR) assays have also been used to detect and quantify genes correlated with resistance, as demonstrated in *S. aureus* to achieve more accurate and rapid detection of macrolide–lincosamide–streptogramin B resistance genes (i.e., the *erm* genes). These genes are commonly observed in Gram-positive bacteria, such as the genera *Enterococcus*, *Bacillus*, *Streptococcus* and *Staphylococcus* correlated to bacteria 23S rRNA

methylation, resulting in antibiotic resistance, and the Q-PCR test has been considered not only the most accurate and sensitive, but also the most rapid method used in these conditions [14,31]. In the case of methicillin susceptibility testing for *S. aureus*, conventional phenotypic methods use surrogate drugs and take about 48 h to become available from the time that positive cultures are detected, but in contrast, the detection by Q-PCR of the *mecA* gene, related to methicillin resistance, takes less than 2 h and yields a secure answer [26]. Therefore, together with the rapid turnaround time, the other main advantage of nucleic acid amplification is that it allows for the detection of low copy numbers of a specific organism's gene target in clinical samples, improving the therapy and patient outcome, but sometimes the polymicrobial infections may be missed [32].

The transcriptomic approach has been used to analyze a set of transcripts in a cell culture or a single cell that includes mRNAs encoding proteins and small non-coding RNAs (i.e. ribosome, tRNA, miRNA) in different pathogenic organisms, such as *M. tuberculosis* [33], *E. coli* [34], *Aggregatibacter actinomycetemcomitans* [35], *Campylobacter jejuni* [36] and *S. aureus* [37]. Kamensek and Zgur-Bertok evaluated the response of *E. coli* to colicin M, a bacericin that inhibits peptidoglycan synthesis, and demonstrated by transcriptomic analyses that subinhibitory concentrations of colicin M alter the gene expression involved in bacterial cell envelope, osmotic stress, cell motility and also in genes related to the production of exopolysaccharides and the CreBC system, already known to promote an increase in colicin M and E2 resistance [38].

DNA-based and proteomic analysis have been widely carried out in pathogenic bacteria through several experimental and analytic tools in order to build up a complete picture of the biochemical events that happen inside bacterial cells [39–41] and to characterize genes and proteins involved in the antibiotic resistance mechanisms [13]. These analyses when carried out together, may allow a better understanding of physiology and overall cellular metabolism, enabling the identification of connections between different metabolic and regulatory pathways that remain unclear [40,42].

In general, through proteomic analysis it has been possible to characterize a particular organism, tissue, or cell organelle structure by identifying the largest possible number of proteins, or even just comparing differentially expressed proteins, thus providing information complementary to that obtained through genomic techniques [43]. The use of this technology has been boosted with the emergence of ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) and with the application of liquid chromatography (LC), with high (HPLC) or ultra-efficiency (UPLC), which are used to allow measurement and identification of peptides with better sensitivity in complex biological samples [44,45]. The routine use of these techniques in clinical laboratories to identify resistance is still rare, probably due to the high cost of expensive capital equipment and the necessity of skilled labor. But advantages of these methods include the identification of a wide range of microorganisms resistant at one time and with minimal hands-on time, the high throughput capacity, the relative low per-test cost and the very rapid turnaround time [32,46].

MALDI-ToF MS has been applied to identify the different levels of bacterial resistance, for example in *S. aureus* strains resistant to methicillin [23,47], *Streptococcus pneumonia* strains resistant to fluoroquinolones [48] and *E. coli* resistant to multiple antibiotics [23,49]. Moreover, in MALDI-ToF MS AMPs resistance analysis, the technique was also seen to be efficient since it allowed the distinction between susceptible and magainin I-resistant *E. coli* strains [50]. Using this tool, the mass spectra acquired by MALDI-ToF MS for each isolate were used to construct a main spectrum profile (MSP), allowing a comparison with each other in a cluster tree. The distance between branches of these isolates showed that the subtle differences in molecular masses between the susceptible and resistant isolates permit the differentiation of the resistant strains [50]. The use of the MALDI-ToF MS tool in the clinical laboratory could rapidly and precisely discriminate the antibiotic-resistant pathogen

strains in infectious diseases, including the differentiating bacterial strains with varying degrees of antibiotic resistance, having a high throughput capacity, reducing therapeutic failure and consequently the dissemination of resistance [23,51,52].

Moreover, by several proteomic approaches, various peptides and proteins have been found with differential expression directly or indirectly correlated to antimicrobial bacterial resistance, such as the porins OprD, OprF, OprG, OprL, OmpH and the MexA protein of an efflux pump in *Pseudomonas aeruginosa* resistant to ampicillin, kanamycin and tetracycline [53,54]; the transmembrane channel TolC, F1-ATPA and DLD in *Vibrio parahaemolyticus*, related to efflux and phospholipids biosynthesis, increased, conferring resistance to AMPs [55]; there was also an increase in intracellular phosphoglycerate cynase and peptideoglycan hydrolase LytM in *S. aureus* resistant to methicillin and vancomycin [46,56] and in the 41 differential proteins related to metabolism and stress response in AMP magainin-I-resistant *E. coli* strains [10]. The identification of these proteins and their tabulation in a database may offer an additional support to discriminate the antimicrobial-resistant bacterial strains.

Therefore, the high-throughput methods available to identify the different bacterial resistance mechanisms outlined in this review may aid in the interpretation of relative gene expression profile, in the identification of potential genes and/or protein targets differentially expressed in drug-resistant strains and in the validation of essential gene expression for microorganism survival, including those activated or repressed in a hostile environment such as in the presence of antibiotics and AMPs.

3. Membrane alterations that cause antimicrobial peptide resistance

Most AMPs are capable of permeabilizing microbial membranes causing an osmotic cellular imbalance [57]. In general terms, the electrostatic forces start the interaction between the negatively charged cell surface and the positively charged peptide. This initial interaction leads to a second step in which the peptide with hydrophobic patches binds to the lipidic membrane, resulting in membrane disruption. Barrel-stave, toroidal, and carpet are the main models of this concept [58]. In barrel-stave, peptides seem to oligomerize and form transmembrane pores. Otherwise, toroidal pores could be formed by monomer peptides, which induce a local membrane curvature that also results in membrane disruption. Finally, a carpet mechanism occurs when the AMPs cover the membrane surface, causing a detergent-like effect that is able to disintegrate the membranes. Although these three classical mechanisms of action have been extensively discussed in the last two decades, some authors have proposed several other possibilities [8,57,58].

The mechanisms of bacterial resistance to AMPs are still not fully established, but the modifications in the physical–chemical interaction between the bacterial cell membrane and the AMP molecule seem to be the first step commonly involved in the resistance process [59,60]. In general, bacterial resistance can be achieved by bacteria changing the AMP target to make it less susceptible to AMP action or even by mechanisms related to the removal of AMPs from their site of action in the bacterial membrane (Fig. 1) [61,62]. Often, fluidity and permeability of the bacterial cell membranes decrease due to alterations in the architecture of the outer and inner membranes [62,63]. Reduced levels of specific membrane proteins and ions (such as Mg^{2+} and Ca^{2+}) [63–65], and changes in membrane lipid composition afford protection to the site of action of various AMPs. This was observed in bacterial resistance to polymyxins, defensins and cathelicidins, where these compounds main target is the cytoplasmic bacterial membrane [64,66].

Included in the main constituents of bacterial membranes are various types of phospholipids, such as phosphatidylethanolamine (PE), phosphatidyglycerol (PG) and cardiolipin (CL) [67,68]. The bacterial membrane structure fulfils several vital tasks, while the phospholipid membrane composition prevents cell damage in unfavorable growth
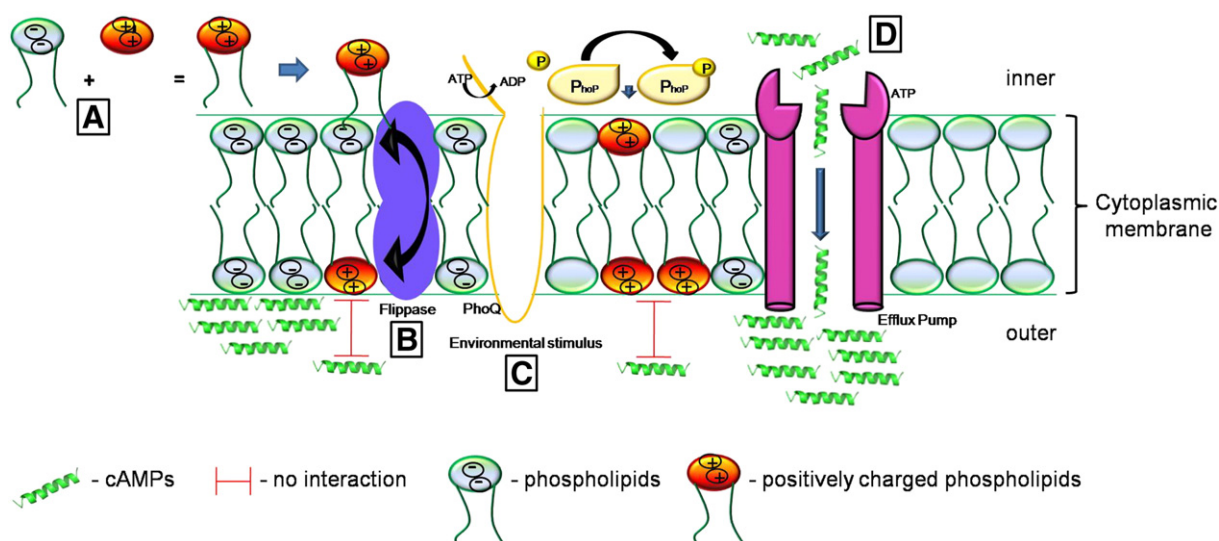
Fig. 1. Membrane alterations that influence cAMPs bacterial resistance. (A) The bacterium modulates its relative positive membrane charge (such as in the cardiolipin inclusion or in the increase of aminoacylated phospholipids content), changing the membrane lipid composition and reducing the negative membrane charge, avoiding the insertion of the positively charged AMPs, as a protective tactic to prevent cell damage and consequently to resist cAMP action; (B) The expression of flippases proteins seems to be important in the translocation or flipping of positively charged lipids from the inner to the outer leaflet of cytoplasmic membranes, resulting in changes in the membrane charge and composition, giving it a stable profile against antimicrobial action and reducing the affinity between cell membrane and the AMP and consequently the effectiveness of AMPs binding and action; (C) Under the control of the two-component signal regulatory systems (TCS) (i.e. PhoP–PhoQ), the bacteria perceive an environmental stimulus, activate signal transduction and respond to the presence of AMPs, controlling the bacterial membrane modifications and resulting in increased AMP resistance; (D) The efflux pumps transduce the electrochemical energy to displace the antimicrobial drugs or cAMPs out of the periplasm and their overexpression in bacteria closely correlated to their antibiotic resistance.

conditions, and this modulation seems to be crucial for bacterial survival [69]. The roles of the various phospholipids, their biosynthesis, turnover and regulation are very important in bacterial membrane structure and function, showing a close relation to antibiotic resistance. One example is about the expression of postulated flippase proteins that are required for translocation of lipids from the inner to the outer leaflet of cytoplasmic membranes, resulting in changes in the membrane composition and giving it a stable profile against antimicrobial action [69].

In a model membrane it was shown that inclusion of only 10% of CL was already able to effectively suppress membrane translocation and pore formation in liposomes by antibiotic action, resulting in resistance [68]. The methicillin-resistant *S. aureus* strains seem to utilize adaptations in phospholipid cell membrane, mediated by *mprF* (multiple peptide resistance factor), *cls* (cardiolipin synthase) and *pgsA* (phosphatidylglycerol synthase), together with other mechanisms, to modulate its relative positive surface charge as a protective tactic, probably against the insertion of positive charges of cationic AMPs (cAMPs) and also calibrate its cell membrane order (fluidity versus rigidity) to resist the action of AMPs [66,70,71]. In another membrane model, the presence of aminoacylated phospholipids (Lys-PE, Gln-PE), a common secondary modification of bacterial lipid bilayers of pathogenic bacteria, both Gram-negative and Gram-positive, is required for its stabilization, protecting it against AMP activity [72].

The reduction in negative charge resulting from alteration in the phospholipid bilayer composition in association with the function of the two-component signal regulatory systems (TCS), for example the PhoP–PhoQ described in *Salmonella enterica* and *P. aeruginosa* [64]; the ParR-ParS and PmrA–PmrB in *P. aeruginosa* [64,73]; the ApsR–ApsS in *Staphylococcus epidermidis* and *S. aureus* [62], and the TCS/ABC of *B. subtillis* (BceSR two-component system (TCS)/BceAB ABC transporter) [74] and of *S. aureus* (BraSR/BraDE transporter module) [75], which control the bacterial membrane modification according to the environmental stimuli response, could reduce the affinity between cell membrane and the AMPs and boost the effectiveness of AMP binding, increasing resistance [10,62,64,76]. In *S. aureus* most AMP-inducible AMP-resistance mechanisms, including *dlt*, *mprF* and *vraFG* genes, were found to be under *aps* (ApsR–ApsS) control, and the regulatory response and target mechanisms are mainly for cationic AMPs [62,77].

Under the control of the TCS systems, the bacteria perceive environmental stimulus, activate signal transduction and respond to the presence of AMPs, resulting in increased AMP resistance [75,78]. Along with this, the resistance is often also increased by the activity of transmembrane transporters, the efflux pumps that transduce electrochemical energy to displace the drugs out of the periplasm [79]. Active efflux plays a major role in this resistance, and multidrug efflux pumps decrease the accumulation of drugs within cells [80].

The tripartite-transport systems composed of inner and outer membrane proteins, connected by a periplasmic membrane fusion protein (i.e. AcrABTolC in *E. coli*), are mainly an important efflux system to pump cytotoxic compounds and antimicrobial drugs. Their overexpression in bacteria is really correlated with their antibiotic resistance [80,81]. Thus, the bacteria can adapt to a wide range of environmental conditions, including the presence of antimicrobial compounds, antibiotics and AMPs, which constitute environmental chemical stresses for bacterial cells, through appropriate developed mechanisms that confer protection against this external attack, such as the mechanism involving the cell membrane that seems to be pivotal in bacterial survival and resistance.

4. Modifications in cell wall of AMP-resistant bacterial strains

The bacterial cell envelope primarily consists of the cytoplasmic membrane and cell wall [82]. The cytoplasmic membrane makes the first physical barrier to protect the intracellular content. The cell wall gives strength and structure to the bacterial cell [83] and ensures the survival of most bacteria. Mycoplasma lacks a cell wall, but has a limited lifestyle, needing a completely predictable environment. In that case mycoplasma can survive without oxygen, but not in very high or very low salt concentrations due to the absence of cell walls [84]. Other bacteria turn into L-forms (without a cell wall) under conditions of extreme nutritional limitation or by mutation [85,86].

Structural differences in the bacterial cell wall led to the classification of bacteria into Gram-positive and -negative. The Gram-positive bacterial cell wall includes a thick peptidoglycan layer and another polysaccharide coat, mainly formed by teichoics and teichuronic acids. Furthermore, the Gram-negative bacterial cell wall proves to be more

complex, including thin layers of peptidoglycans, lipoproteins, lipopoly-saccharides and an outer membrane [82].

All antimicrobial peptides establish some kind of interaction with the cell wall to unleash their bactericidal activity, including ionic and hydrophobic interactions [87,88], since bacteria possess an overall negative surface charge. Therefore, bacterial defense toward this kind of action often relies on cell wall modifications, which usually alter the ionic cell wall potential, essentially on interaction spots, reducing the attachment of antibiotic peptides [89–91].

4.1. Modifications in the polysaccharide layer

Gram-positive bacteria usually resist antimicrobial peptides by partially neutralizing their cell wall [89]. Gram-positive cell walls have a large amount of anionic polysaccharides (up to 60%) attached to cytoplasmic membrane or the peptidoglycan layer. Lipoteichoic acid (LTA) and lipoglycans bind to the cytoplasmic membrane, while wall teichoic acid (WTA), teichuronic acid and other anionic polysaccharides connect to peptidoglycans [92,93]. LTA and WTA are composed of repeating monomeric alditol units (i.e. ribitol and glycerol) joined by anionic phosphodiester linkages, which confer an anionic character on these teichoic acids [93].

Finally, peptidoglycans comprise the main constituents of the Gram-positive cell wall: a disaccharide formed by two sugars (N-acetylglucosamine and N-acetylmuramic acid) linked to a short peptide chain (three to five amino acid residues). The disaccharide portion varies slightly in different bacteria, while the peptide chain, linked to the carboxyl group of N-acetylmuramic acid, shows wide variation in its composition [94,95]. The WTA is covalently linked to the peptidoglycan by a special coupling to O-6 or N-acetylmuramic acid [92]. Together with peptidoglycan, teichoic acids (WTA and LTA) determine the negative surface of most Gram-positive bacteria [93], which is essential for many cell functions, such as maintenance of homeostasis, cell shape and the activity of autolytic enzymes [96].

Gram-positive bacteria do not possess outer membrane protection [82]. The layer of peptidoglycans and teichoic acids receives all the extracellular pressure and makes the first contact with harmful molecules,

likely host defense peptides [97]. Some Gram-positive bacteria evade the attack of antimicrobials. Extensive studies on this ability revealed that bacteria with the *dlt* operon promote D-alanylation of teichoic acids, reducing their anionic charges [98] (Fig. 2). Because many Gram-positive bacteria have the *dlt* operon, Devine and Hancock [99] suggested the D-alanylation of teichoic acids as a natural defense of this Gram-type. Also, Peschel et al. [100] observed that D-alanine-esterified teichoic acids may represent a virulence factor due their occurrence in several pathogenic bacteria, such as streptococci, enterococci, clostridia, listeria, and bacilli.

Peschel and co-workers [98] reported that *S. aureus* and *Staphylococcus xylosus* with many copies of *dlt* operon were resistant to cationic peptides (defensin, protegrins, tachyplesins, magainin II, gallidermin and nisin) and a neutral peptide (gramicidin D). But mutant bacteria, lacking *dlt* operon, were sensitive up to 50-fold more to cationic peptides only. The mutant teichoic acid lacked D-alanine esters and remained highly charged by deprotonized phosphate groups in its glycerolphosphate repeating units, allowing the action of cationic peptides, but not of neutral gramicidin.

Peschel et al. [101] reported similar results in glycopeptide antibiotics, such as vancomycin and teicoplanin: *S. aureus* lacking D-alanine esters in teichoic acid proved three-fold more sensitive to glycopeptide antibiotics. Chan et al. [102] investigated the role of D-alanylation of lipoteichoic acid (LTA) in *Streptococcus gordonii*. The authors concluded that D-alanylation of LTA in this strain influences the host immune system by modulating cytokine production and promoting relative resistance to antimicrobial peptides. When they knocked out the *dltA* gene of *S. gordonii*, its susceptibility to polymyxin B, nisin, magainin II, and human β defensins 1 and 2 markedly increased [102]. Kristian et al. [96] obtained similar results in Group A *Streptococcus* mutant with allelic replacement of the *dltA* gene with the chloramphenicol acetyltransferase gene.

However, Saar-Dove et al. [103] investigated the interactions of the human pathogen Group B *Streptococcus* with cationic antimicrobial peptides with different properties. According to them, D-alanylation probably alters LTA conformation, which increases the cell wall density, flexibility and permeability, acting as a physical barrier to linear peptides.
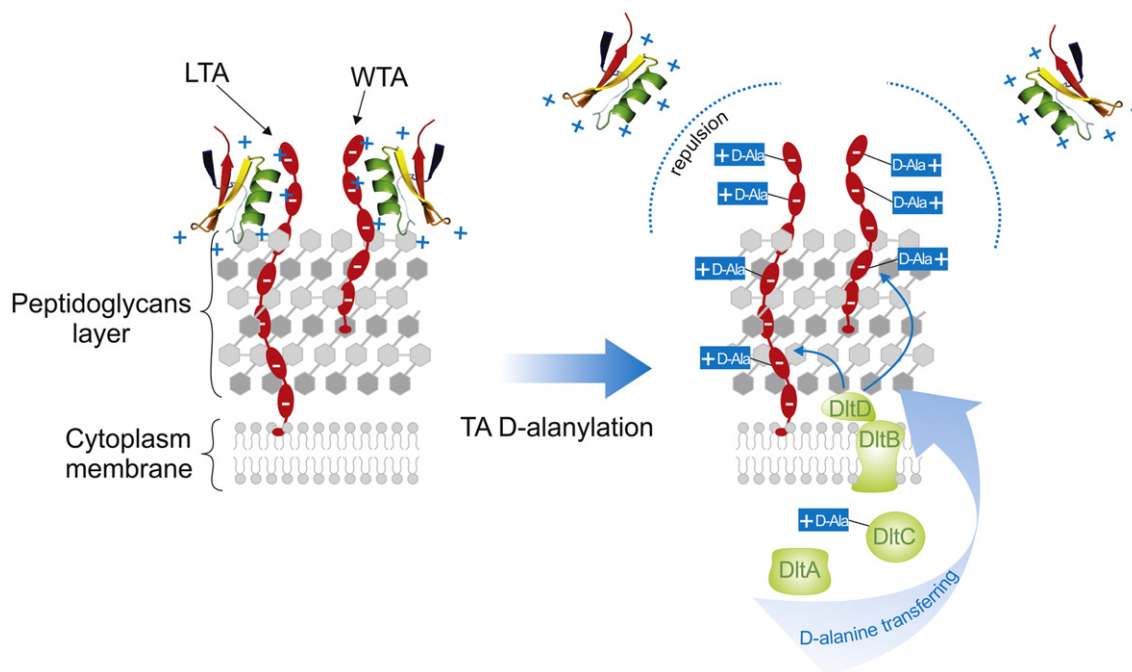
Fig. 2. AMP resistance from D-alanylation of teichoic acids. Schematic representation of the cell envelope of a Gram-positive bacterium before (left) and after D-alanylation of teichoic acids (right). D-Alanine transferring to teichoic acids (TA) through DltABCD proteins provides the counter ion that partially neutralizes the net anionic charge of the TA. Then, the repulsion force precludes the ionic interaction between cAMP and LTA and WTA and the cAMP action on Gram-positive cell wall.

The *dlt* operon contains four genes, *dltA*, *dltB*, *dltC*, and *dltD*, all of them being involved in the teichoic acids' D-alanylation [93,104]. The exact process of the D-alanine transferring to external polymers of the Gram-positive cell wall remains uncertain [89], but it is known that the *dltA* encodes a D-alanine-D-alanyl carrier protein ligase (Dcl), which activates and transfers D-alanine to the D-alanine carrier protein (encoded by *dltC*); DltB participates in D-alanine incorporation into teichoic acids and transfer of activated D-alanine across the cytoplasmic membrane; and DltD assists the transfer of D-alanine from the membrane carrier to teichoic acids [93].

4.2. Lipid A alterations

Gram-negative bacteria possess an extra barrier, the outer membrane (OM), in comparison to Gram-positive strains. The OM maintains cell integrity in the face of environmental changes and controls the entrance of hostile molecules into the cell. The outer membrane is an asymmetrically organized bilayer membrane that has an inner layer composed of phospholipids and proteins; its externally exposed layer is composed of specific proteins and lipopolysaccharides (LPS), the latter being its major surface component [105]. Lipopolysaccharide molecular structures include three different subunits: an endotoxin (lipid A), a core oligosaccharide and an O-antigen polysaccharide [106]. The lipid A anchors the LPS unit to the outer membrane. This endotoxin corresponds to a β-1′,6-linked glucosamine disaccharide, hexa-acylated and bisphosphorylated. The lipid A negative charge, and consequent anionic cell surface of Gram-negative bacteria, results from its core phosphorylation [91,106].

Some Gram-negative bacteria incorporate positive charges into lipid A, which reduces the negative net charge from the cell envelope and the electrostatic repulsion between neighboring LPS molecules, thus decreasing affinity for antimicrobial peptides [107,108] (Fig. 3). Lipid A modifications often result from changes in at least one the following substituents that occurs at 1- or 4′-phosphate groups: palmitate, phosphoethanolamine and 4-amino-4-deoxy-L-arabinose (4-aminoarabinose) [106]. High levels of lipid A modifications with these substituents appear to be related to cationic antimicrobial peptide resistance [90] and bacterial virulence, as seen in polymyxin B [109–111]. The 4-aminoarabinose attachment to lipid A occurs by means of a phosphodiester bond in its anomeric carbon; phosphoethanolamine attachment often occurs as a pyrophosphate [90]; and palmitate residue transfer occurs from a phospholipid to the N-linked hydroxymyristate on the proximal unit of lipid A [109]. The two-component regulatory system, PhoP–PhoQ (PhoPQ), usually controls all three modifications of lipid A.

The PhoPQ system induces the expression of the *pmrD* gene, which activates the PmrA–PmrB two-component system (PmrAB) by post-translational modifications. These two regulatory systems control a group of genes required for antimicrobial resistance and pathogenesis [111]. The polymyxin resistance operon (*pmr*) is required for amino-arabinose LPS modification. In *Salmonella enterica* serovar Typhimurium, Bijlsma and Groisman [112] reported that the binding of PmrD protein to phosphorylated PmrA prevents dephosphorylation, activating PmrA-regulated genes. Johnson et al. [111] reported that, in *Salmonella typhimurium*, cation chelating properties of extracellular DNA activate PhoPQ/PmrAB regulatory systems and, consequently, the antimicrobial peptide resistance (for an extensive study of *Salmonella* PmrAB regulation, see Gunn [113]). In Murray's work [114], a *msbB Salmonella* expressing PmrA(Con) protein confers polymyxin resistance by lipid A modification with phosphoethanolamine. Navarre et al. [115] reported an unusual linkage between PhoPQ and SlyA regulatory systems of *S. typhimurium*. The expression of *pagC* and *mig*-14, genes required for virulence and antimicrobial peptide resistance, known to be controlled by PhoP/PhoQ, appeared also regulated by SlyA.

However, *P. aeruginosa* promotes LPS modification independently of the PmrAB or PhoPQ system [116]. Since its PhoQ protein lacks the AMP-binding domain and depends on divalent cation-limitation,

P. aeruginosa mediated regulation of the *arn*BCADTEF (*pmr*HFIJKLM) LPS modification operon to become resistant to antimicrobial peptides [117]. Kawasaki et al. [118] observed that a strain of *S. enterica* lacked the conventional lipid A modifications, and the deacylation of lipid A by PagL promoted bacterial resistance to polymyxin B. Although PagL expression is also under the control of the PhoP–PhoQ two-component regulatory system, the deacylation of lipid A is not usual in vivo due to PagL latency. PagL needs to be released from latency to compensate for the loss of resistance to polymyxin B. Additionally, Reinés et al. [119] reported the temperature-dependence of *Yersinia enterocolitica* resistance to antimicrobial peptides, governed by PhoPQ and PmrAB two-component systems. They demonstrated the down-regulation of lipid A modifications with aminoarabinose and palmitate at 37 °C when compared to 22 °C (room temperature).

Recently, Hankins et al. [120] characterized for the first time another lipid A modification, lipid A glycylation, and associated it with polymyxin resistance of El Tor *Vibrio cholerae*. The proteins encoded by the *almEFG* operon, responsible for glycine addition, remain from the Gram-positive system required for D-alanylation of teichoic acids. In their next work, the same research group reported the detailed mechanism of how aminoacyl esterification occurs in the glycine or diglycine for lipid A in *V. cholerae* [89]. Their model infers that AlmE starts the process, activating glycine by adenylation. Then AmlE transfers it to the 4′-phosphopantetheine group of the aminoacyl carrier protein AlmF, which becomes active. AlmG transfers the glycine substrate from the AlmF to the hydroxylauryl chain of lipid A.

However, a single bacterial species may resort to many resistance mechanisms at the same time. This is what happens with *Neisseria meningitides* [121] and *P. aeruginosa* [64]. The data obtained by Tzeng and co-workers [121] indicate that meningococci use multiple mechanisms to modulate levels of cationic antimicrobial peptide resistance, including the MtrC–MtrD–MtrE efflux pump, lipid A modification and the type IV pilin secretion system. Skiada et al. [64] reviewed the adaptive resistance to cationic compounds in *P. aeruginosa*. In the presence of aminoglycosides, the MexXY-OprM efflux pump mediated *P. aeruginosa* resistance; while the ParR–ParS two-component system regulated the resistance induced by cationic peptides through incorporation of 4-aminoarabinose in lipid A.

4.3. Other cell wall modifications

Cell wall thickening is a common feature of *S. aureus* resistance to several classic antibiotics such as erythromycin [122], vancomycin [123,124] and acriflavine [125], but just recently it was observed in antibiotic resistance to peptides [126]. Indeed, Cui et al. [127] correlated the reduced daptomycin susceptibility with vancomycin resistance in vancomycin-intermediate *S. aureus* (VISA), relating both to cell wall thickening. However, Yang et al. [128] reported the evidence of multiple resistance mechanisms to daptomycin in *S. aureus*, including cell wall thickening. Kramer et al. [129] concluded that nisin tolerance of *Micrococcus flavus* and *Listeria monocytogenes* previously exposed to antibiotic pressure results from changes in cell wall thickness and cell wall charge. In a comparative proteomic analysis of magainin II resistant and susceptible *E. coli* strains, Maria-Neto and co-workers [10] suggested that cell wall thickness is one of the multiple defense mechanisms used by *E. coli* against antimicrobial peptides. The authors found the glutamine synthetase up-regulated, exerting a direct influence on the synthesis of the cell wall peptidoglycan layer.

Recently, Taneja et al. [130] reported the association of D-alanine incorporation and resistance to AMPs in *Bordetella pertussis*, a Gram-negative bacterium. The *B. pertussis dra* locus shows high homology with the *dlt* operons that decrease the negative charge of teichoic acids in Gram-positive bacteria (previously described). The *dra* operon inactivation resulted in enhanced sensitivity of *B. pertussis* to structurally different antimicrobial peptides, such as the human neutrophil peptides, HNP-1 and HNP-2 (β-sheet structure and disulfide bridges);
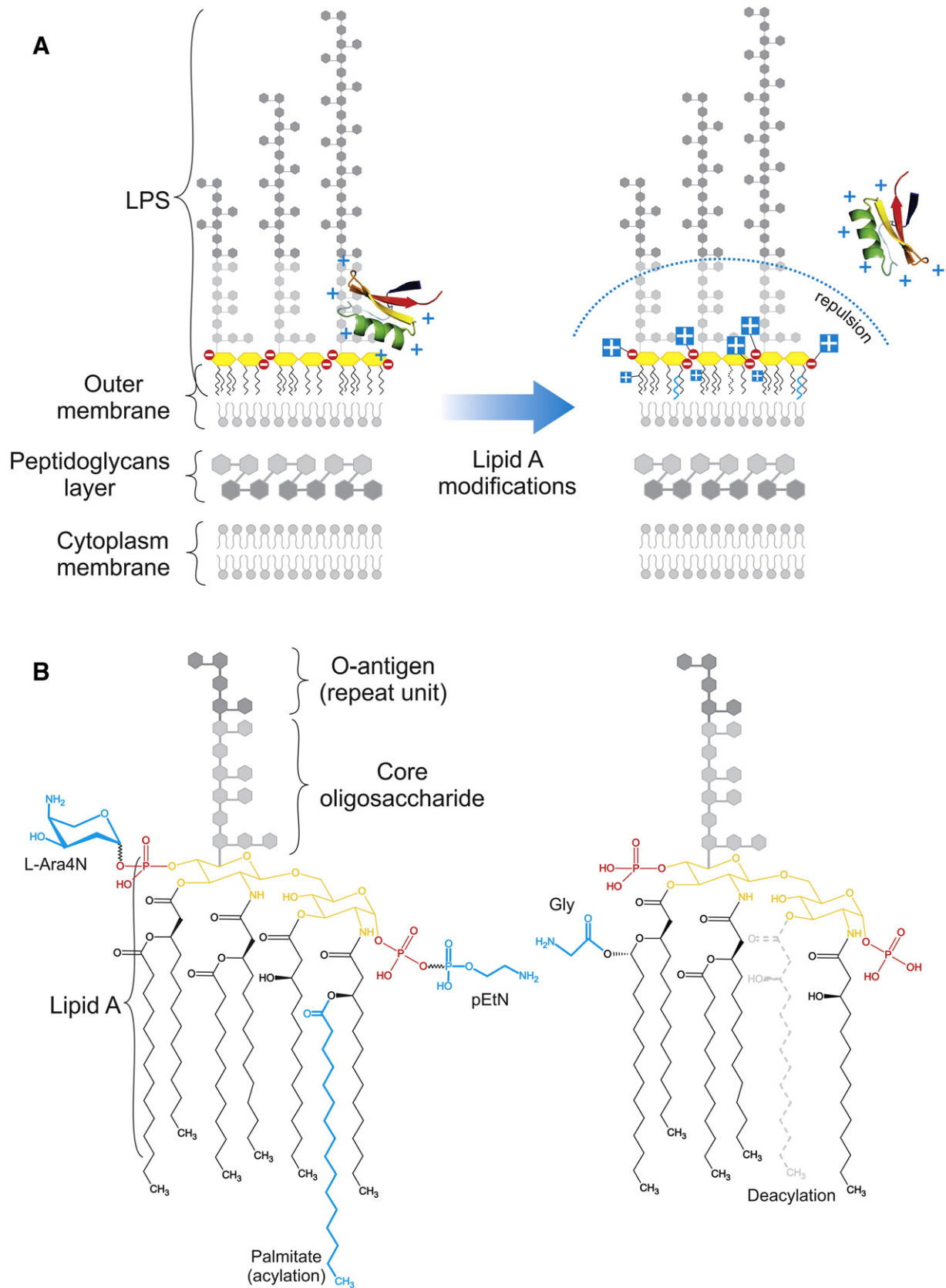
Fig. 3. AMP resistance from lipid A modification. (A) Schematic representation of the cell envelope of a Gram-negative bacterium before (left) and after reduction of cell surface negative net charge (right). Substituents on lipid A incorporate positive charges into LPS and promote electrostatic repulsion of antimicrobial peptides. (B) Major lipid A modifications and substituents: L-Ara4N (4-aminoarabinose), pEtN (phosphoethanolamine), glycylation (incorporation of glycine), acylation (incorporation of palmitate), deacylation (removal of long-chain fatty-acyl).

LL-37 (α-helical); polymyxin B (cyclic and amphipathic); and hSPLUNC1, similar to a BPI (bactericidal/permeability increasing) protein (helixes and a β sheet mixer structure [131]). The authors suggested *dra* involvement in the incorporation of D-alanine into an outer membrane component and subsequent surface modification. Further studies are needed to more comprehensively understand the role of *dra* in antimicrobial peptide resistance.

5. Subverting cellular metabolism

The exposure to antimicrobial peptides results in the cellular stress often noted in cell metabolism. Bacteria avoid the imminent threat of death by regulating the expression of several pathways, such as proteases, modification of surface structures, biofilm formation and suppression of vulnerable features [132].

Recent research reported by Rodrígues-Rojas and co-workers [12] showed that AMPs targeting the cell wall do not stimulate the bacterial mutation rates or promote the stress-pathway induction, as occurs with classic antibiotics. The authors treated *E. coli* for four continuous hours with the cAMPs cecropin A, melittin, magainin II, pexiganan, and LL-37, at 50% of minimal inhibitory concentration. None of these AMPs showed changes in mutation rate when compared to the control, while the antibiotics ampicillin, ciprofloxacin and kanamycin increased it three or four times. Similarly, AMP treatments did not result in differential expression of genes related to stress-induced mutagenesis. These data suggest that bacterial adaptation and resistance development to AMPs is not a result of by accelerated mutation rate.

On the other hand, proteolytic cleavage of antimicrobial peptides has been referred to as an inherent mechanism of bacterial resistance and virulence in several common pathogenic bacteria [133]. Sieprawska-Lupa et al. [134] and Jusko et al. [135] directly correlated the level of secreted endogenous extracellular proteases to the level of bacterial susceptibility to antimicrobial peptides. In Sieprawska-Lupa's work [134], *S. aureus* with larger amounts of the metalloprotease aureolysin degraded cathelicidin LL-37, contributing to the natural resistance of this pathogen. Aureolysin inactivates LL-37 bactericidal activity by cleaving C-terminal peptide bonds. Similarly, a metalloprotease of *Tannerella forsythia*, karilysin, also cleaved the antimicrobial peptide LL-37, significantly reducing its bactericidal activity [136]. Karilysin high-expressing *T. forsythia* strains were more resistant than low-expressing strains [135]. Similarly, the extracellular metalloprotease ZapA of *Proteus mirabilis* cleaves human β-defensin 1 (hBD1) into six short peptides and LL-37 into at least nine, reducing their antimicrobial activity [137].

Proteolytic cleavage of nisin represents a novel mechanism for antimicrobial resistance in strains of non-nisin-producing *Lactococcus lactis* [138] and of nisin-controlled gene expression systems (NICE) [139]. The protein encoded by the nisin resistance gene (*nsr*) inactivated nisin in vitro by removing six amino acids from the carboxyl terminal of nisin [138]. A recent study indicates that cytosolic peptidases also might cause resistance to antimicrobial peptides active in the bacterial cytosol [140]. The authors observed that the overexpression of the serine peptidase oligopeptidase B in *E. coli* reduces the susceptibility of bacterial cells to proline-rich antimicrobial peptides up to 30 residues in length, such as bovine peptides Bac5 and Bac7, and porcine peptide PR-39.

In *Streptococcus pyogenes*, the proteolytic inactivation of LL-37 by the cysteine protease SpeB was reported both in vitro [141] and in vivo, in patients with several streptococcal infections [142]. The SpeB-mediated inactivation of LL-37 occurs through the α2-macroglobulin-protease complexes at the streptococcal surface. *S. pyogenes* expresses surface-attached GRAB (G-related α2-macroglobulin-binding) protein, which binds to the protease inhibitor α2-macroglobulin. Next, the GRAB-α2-macroglobulin complex traps Speb, which is retained at the bacterial surface. Speb remains proteolytically active and readily cleaves small peptides that have penetrated the complex, such as LL-37 [143], leading to the bacterial resistance mechanism.

Schmidtchen et al. [144] reported that *P. aeruginosa*, *Enterococcus faecalis* and *S. pyogenes* use a common mechanism to modulate and evade α-defensin action. Extracellular proteinases secreted by these strains degrade dermatan sulfate-containing proteoglycans, subsequently releasing free dermatan sulfate into the environment. Then, the released compound binds to neutrophil-derived α-defensin and completely neutralizes its bactericidal activity. Another extracellular trap contributes to Group A *Streptococcus* resistance to cathelicidin [145]. M1 protein on the *Streptococcus* surface might mediate resistance to LL-37 through its entrapment and inactivation into the fimbrial-like extension of M1 protein before the cathelicidin reaches its cell membrane target of action. Similarly, a pilus protein of Group B *Streptococcus* seems to capture cathelicidin antimicrobial peptides [146]. In this work, Maisey and co-workers studied the heterologous expression of PilB, a protein subunit forming the Gram-positive pilus backbone, in *L. lactis*. They suggested the association of cathelicidin with the cell wall-anchored PilB, which may capture or trap the peptide.

The development process of biofilm formation is an important mechanism of resistance to antimicrobial compounds and environmental stresses [147]. In biofilm formation, the autonomous bacterial cells are encased in an extracellular matrix, consolidating a multicellular surface-association. The biofilm matrix produces an extracellular polymeric substance (EPS) where the cells are embedded. EPS has a characteristic structure and is mainly composed of amyloid and adhesive fimbriae, non-fimbrial large surface proteins, exopolysaccharides, and extracellular DNA [148]. Biofilm-formation bacteria resist the majority of conventional antibiotics [149], but do not resist several antimicrobial peptides that act as antibiofilm agents [150,151]. However, Mulcahy and co-workers [152] reported that extracellular DNA of *P. aeruginosa* biofilm induces resistance to polymyxin B and colistin. Extracellular DNA chelates cations from the environment, and *P. aeruginosa* understands this as a signal to induce LPS modification genes and resistance to antimicrobials. Kai-Larsen et al. [151] reported another biofilm structure involved in antimicrobial peptide resistance, a curli fimbriae. They suggested the LL-37 resistance of a curliated *E. coli* resulted from a curli–LL-37 interaction. At the same time that LL-37 inhibits curli formation by preventing the polymerization of the major curli subunit, CsgA, curli traps LL-37, blocking its action on bacterial biofilm.

6. Outlook and conclusive remarks

As previously described, bacterial resistance is a complex problem that needs to be solved or at least reduced. In order to successfully control resistant bacterial infections, we must adopt multiple strategies that include the discovery of novel antibiotic compounds or the extension of the lifespan of our current repertoire of antibiotics. There are several strategies to do this that include a restriction on the quantity of antibiotics in agriculture; a reduction in the number of antibiotics that are prescribed for virus diseases such as influenza; better education in the community so that patients are aware that they must finish their whole antibiotic prescription; and the use of antibiotic adjuvants [153].

Nevertheless, for efficient drug development, it is essential to gain a complete understanding of bacterial resistance at the different developmental stages in an infection. One pitfall is that each antibiotic has a distinct expression profile, which clearly makes it difficult to identify proteins, carbohydrates and lipids involved in the resistance process. At this point it is tremendously difficult to select a single technique that could solve the bacterial resistance enigma. In fact, all techniques present their positive points and their drawbacks. For example, proteomics has been extremely important for protein panorama visualization, but does not show the coverage of transcriptomics and genomics techniques [154].

Indeed, the two latter techniques do not elucidate what is really occurring in the bacterial cell, since nothing guarantees that the transcripts produced will effectively be transformed into real proteins. Given this, the union of several techniques and a multi-task team are

necessary to make a real contribution in this field. No less important or urgent is the development of new methods to detect resistance in real time. Despite the speed of MALDI ToF and other techniques described here, we will need portable and inexpensive equipment that can be used in any part of the world [155]. Such technology, which also needs to be easy to handle, will open possibilities for rapid detection of resistant pathogens and pave the way to more accurate antibiotic prescriptions.

In summary, despite clear efforts by the scientific community, we are just starting to comprehend bacterial resistance, and many great strides are made daily, with more needed. The battle against resistant microbes is just beginning, and further studies will be vital to control lethal pathogens and decrease the harm produced.

Conflict of interest

The authors declare no conflict of interests and all agree to send the work *Understanding bacterial resistance to antimicrobial peptides: from the surface to deep inside* to BBA — Biomembranes. Furthermore, this manuscript has not been published or accepted for publication and it is not under consideration at another journal.

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