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Bacterial resistance to antimicrobial peptides: an evolving phenomenon

Osmel Fleitas[1,2], Caleb M. Agbale[2,3], Octavio L. Franco[2,4]

[1]Pos-Graduacao da Faculdade de Medicina, Campus Universitario Darcy Ribeiro, Universidade de Brasilia, Asa Norte, Brasília, DF, Brazil, [2]S-Inova, Programa de Pos-Graduacao em Biotecnologia, Universidade Catolica Dom Bosco, 79117-900 Campo Grande, MS, Brazil, [3]Department of Biochemistry, School of Biological Sciences, College of Agriculture and Natural Sciences, University of Cape Coast, Cape Coast, Ghana, [4]Centro de Analises Proteomicas e Bioquimicas, Programa de Pos-Graduacao em Ciencias Genomicas e Biotecnologia, Universidade Catolica de Brasilia, 70719-100 Brasília, DF, Brazil

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1. ABSTRACT

Bacterial resistance to conventional antibiotics is currently a real problem all over the world, making novel antimicrobial compounds a real research priority. Some of the most promising compounds found to date are antimicrobial peptides (AMPs). The benefits of these drugs include their broad spectrum of activity that affects several microbial processes, making the emergence of resistance less likely. However, bacterial resistance to AMPs is an evolving phenomenon that compromises the therapeutic potential of these compounds. Therefore, it is mandatory to understand bacterial mechanisms of resistance to AMPs in depth, in order to develop more powerful AMPs that overcome the bacterial resistance response.

2. INTRODUCTION

Since the first reports of bacterial resistance to conventional antibiotics the spread of antimicrobial resistance has been such that at present constitute one of the major health challenge. The threat to human health has reached such magnitude that the World Health Organization Antimicrobial Resistance Global Report on Surveillance (2014) by Dr Keiji Fukuda, Assistant Director-General, Health Security, stated that: “A post-antibiotic era - in which common infections and minor injuries can kill - far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century” (1).

Antimicrobial resistance is a complex and multifactorial phenomenon. A factor that facilitated the emergence of resistance to conventional antibiotics is that most of them target proteins involved in specific vital functions for bacteria, therefore exerting a great selective pressure that expedites the generation of mutations to counteract the antibiotic action (2).

It is currently necessary to search for and design new antimicrobial compounds that can be directed towards different targets and exercise a broad spectrum of activities. Among the compounds that carry these characteristics are the antimicrobial peptides (AMPs). They have become attractive as therapeutic drugs since they are less likely to develop resistance. However, resistance to AMPs has been reported since AMPs were first discovered as natural antibiotics. For example, in 1949 Joseph L. Stone reported resistance to bacitracin in cultures of *Staphylococcus aureus*, only four years after bacitracin had been launched as a new antibiotic by Johnson *et al.*, 1945 (3,4). Over the years, a growing number of AMPs resistance mechanisms have been discovered in bacteria, which include mechanisms at the

strain level as well as at the collective level. Moreover, resistance to AMPs as therapeutic drugs has become more dangerous because cross-resistance to effector AMPs of the innate immune system can occur. Bacteria can thus evade elimination by chemotherapy and the immune response (5).

It is therefore important to get a deep understanding of the structural and functional basis of the bacterial resistance to AMPs in order to design more powerful AMPs and efficient therapeutic strategies. In this review article we will shed some light on different bacterial resistance mechanisms to AMPs.

3. RESISTANCE MECHANISMS TO ANTIMICROBIAL PEPTIDE

3.1. Antimicrobial peptide degradation

One of the strategies that bacteria use to circumvent the action of AMPs is their degradation. For this purpose, bacteria make use of a diversity of proteases that include metallo-, serine-, cysteine- and aspartic-proteases (Table 1).These proteinases are secreted and/or anchored to the microbial cell surface, where they degrade the AMPs and prevent them from reaching the target site. At the bacterial surface, the proteases may be anchored directly or indirectly by the formation of complexes with cellular protein. Here the bacterial protease is fixed on the bacterial surface by the interaction of the host protein with its bacterial receptor. This has been described in the Gram-positive pathogen *Streptococcus pyogenes* (Group A *Streptococcus* (GAS)) (6). The pre-incubation of host protein alpha 2-macroglobulin with *S. pyogenes* strain KTL3 (expressing the surface-associated protein GRAB, which binds alpha 2-macroglobulin), followed by the addition of radiolabeled SpeB protease, showed that protease is bound to the surface of the strain KTL3. Only background levels of SpeB were associated with KTL3 when the bacteria was pre-incubated with phosphate-buffered saline or when alpha 2-macroglobulin was pre-incubated with strain MR4, a mutant of KTL3 lacking GRAB. In another experiment, KTL3 and MR4 strains were pre-incubated with alpha 2-macroglobulin followed by the addition of SpeB. The subsequent challenge with different concentrations of LL-37 showed that strain KTL3 survived to significantly higher concentrations of the antimicrobial peptide than MR4. Another interesting result was the fact that the trapping of SpeB by alpha 2-macroglobulin quenched the protease activity toward big protein substrates, but retained protease activity against small substrates like LL-37; even more, the SpeB- alpha 2-macroglobulin complexes cleaved more efficiently to LL-37 than SpeB alone. So, all these results suggest that expression of GRAB by *S.pyogenes* enable it to bind with high affinity and specificity to the host alpha 2-macroglobulin, which in turn traps the bacterial protease SpeB on its cellular surface and focuses its proteolityc activity towards host antimicrobial peptides. Johansson *et al.*, 2008 showed evidence that supports the possible occurrence of this mechanism *in vivo*. The analysis of tissue biopsy specimen from patients with various severe soft tissue infections caused by *S.pyogenes* showed that SpeB and GRAB are co-localized on coccus-like structures whose size corresponds to that of streptococci. This co-localization has been shown in LL-37, SpeB and bacteria (7).

Moreover, a new mechanism of evasion mediated by proteolytic activity in *S. pyogenes* has been described. The bacteria secrete the plasminogen activator streptokinase (Ska), which forms the complex Ska-plasminogen or Ska-fibrinogen-plasminogen. These complexes result in active protease plasmin that can be attached to bacterial surface by plasminogen and fibrinogen receptors, allowing the accumulation of protease activity on the cell surface (8). Hollands *et al.*, 2012 showed that this accumulated activity on the cell surface degraded and increased the resistance to the antimicrobial peptide LL-37. Additionally, it was shown that infection with GAS strain supplemented with aprotinin (plasmin inhibitor) or with Ska-knock-out strain in a murine model of necrotizing fasciitis in which LL-37 is abundantly expressed resulted in significantly smaller lesions. The interesting aspect of this mechanism is that bacteria use host protease instead of bacterial protease to evade the action of peptide-based innate host defenses (9).It has been shown that the abrogation of the SpeB activity preserves Ska protein, the host plasminogen and fibrinogen and their bacterial receptors, therefore accumulating plasmin activity on the bacterial surface (10). Maybe one of the implications of the SpeB- alpha 2-macroglobulin complexes attached on the cell surface is to allow the assembly of the plasmin-based proteolytic system. The fact that in the SpeB- alpha-macroglobulin complexes the proteolytic activity of SpeB can be directed toward small substrates like antimicrobial peptides could allow the preservation of the necessary components for the assembly of the plasmin-based proteolytic system and thus equip the bacteria with an additional system for the degradation of antimicrobial peptides. The degradation process could be performed by the action of a specific protease or by different proteases simultaneously. For example, Maisseta *et al.*, 2011 have demonstrated the involvement of metallo- and cysteine-proteases in the degrading of the antimicrobial peptide human beta-defensin 3(hBD3) by the *Porphyromonas gingivalis* supernatant culture. Pretreatment of the supernatant culture with a mix of protease inhibitors or each inhibitor individually suggested that metallo- and cysteine-proteases are involved in the degrading process. The subsequent treatment of the supernatant culture with the protease inhibitors leupeptin and cathepsin B inhibitor suggested the participation of Arg- and Lys-gingipains in the degrading process (11).

Table 1. Bacterial proteases that confer resistance through degradation of AMPs

Protease	Type	Peptide	Bacteria	Localization	Reference
Elastase	Metallo	LL-37 alpha-defensin	*Pseudomonas aeruginosa*	Extracellular	(15)
Gelatinase	Metallo	LL-37	*Enterococcus faecalis*	Extracellular	(15)
ZapA	Metallo	Protegrin LL-37 hBD-1 Protamine SLPI Elafin	*Burkholderia cenocepacia* *Proteus mirabilis*	Extracellular	(16,17)
ZapB	Metallo	Protamine SLPI Elafin hBD-1	*Burkholderia cenocepacia*	Extracellular	(16)
Aureolysin	Metallo	LL-37	*Staphylococcus aureus*	Extracellular	(18)
Metalloproteases	Metallo	LL-37	*Bacillus anthracis*	Extracellular	(19)
SepB	Cysteine	LL-37 BRAK/CXCL14 hBD-2 hBD-3 Midkine	*Streptococcus pyogenes*	Extracellular Surface associated	(15)
Gingipains	Cysteine	hBD-3	*Porphyromonas gingivalis*	Extracellular	(11)
SufA	Serine	Midkine BRAK/CXCL14 LL-37 MIG/CXCL9	*Finegoldia magna*	Surface associated Extracellular	(20,21)
DegP	Serine	Lactoferricin B	*Escherichia coli*	Periplasmic side of inner membrane	(22)
OmpT	Aspartic	Protamine LL-37 C18G	*Escherichia coli*	Surface associated	(23,24)
CroP	Aspartic	C18G CRAMP	*Citrobacter rodentium*	Surface associated	(25)
Pla	Aspartic	LL-37	*Yersinia pestis*	Surface associated	(26)
PgtE	Aspartic	C18G	*Salmonella enterica* Typhimurium	Surface associated	(27)
OmpP	Aspartic	Protamine	*Escherichia coli*	Surface associated	(28)
Arl C	Aspartic	HD-5[1] hBD-2[1] MIG[1] LL-37[1] CP10A[1] CP28[1]	*Escherichia coli*	Surface associated	(29)
[1]arlC mutant is more sensitive to these peptides					

Intracellular proteolysis of AMPs also appear be involved in the resistance. Shelton *et al.,* 2011 showed evidence that suggested that in non-typeable *Haemophilus influenzae* the AMPs could be internalized via Sap ABC transporter and, once in the cytoplasm, the AMPs are degraded by intracellular proteases. The coupling of intracellular proteolytic activity to internalizing transport could protect the bacteria from AMPs action. Additionally, the authors suggest that the intracellular proteolysis of AMPs could be beneficial nutritionally to bacteria (12).

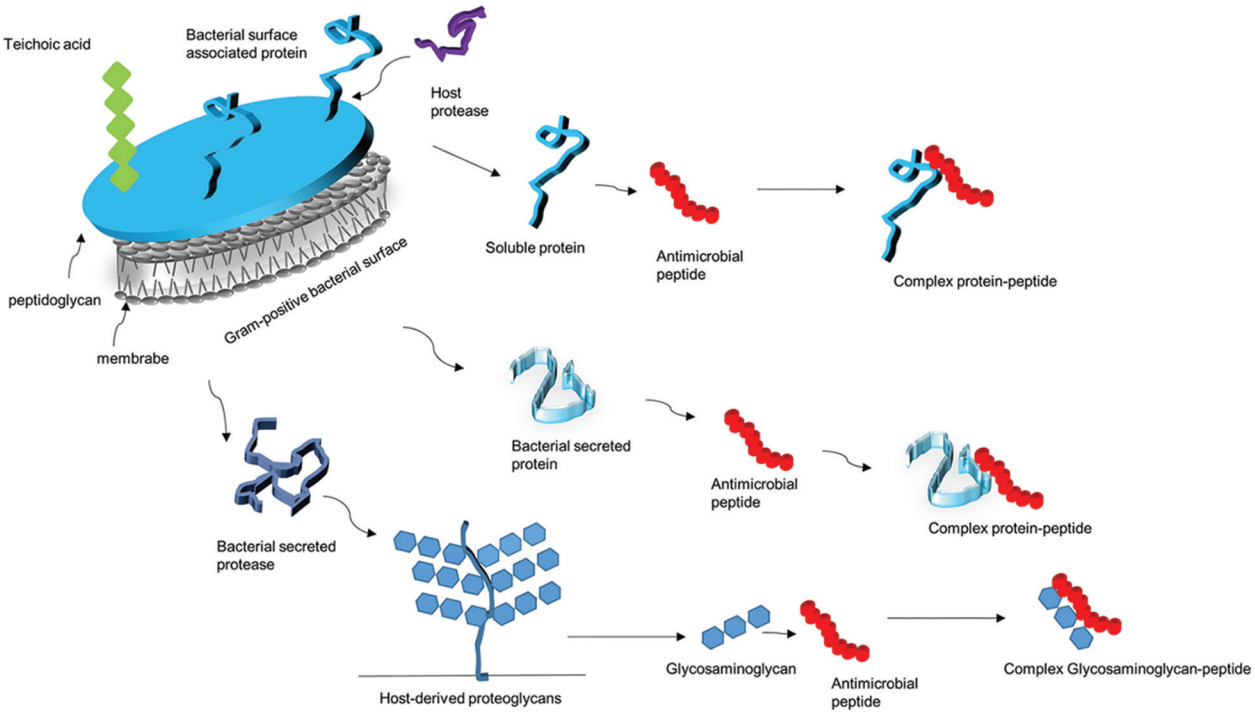

Figure 1. AMP trapping at the extracellular level in Gram-positive bacteria. Host-derived proteases release bacterial surface-associated proteins that bind and neutralize the AMP. Bacteria secrete proteins that bind to AMP and proteases that degrade host-derived proteoglycans, yielding glycosaminoglycans that bind and neutralize the AMP (this is a strategy shared with Gram-negative bacteria). Figure designed by the authors.

Moreover, various mechanisms of action have been described for AMPs where the targets are intracellular processes, such as cell wall synthesis, DNA replication and transcription, protein synthesis, chaperone assisted protein folding and enzymatic activity (13, 14). So one of the strategies for evading the intracellular action of the AMPs could be via intracellular degradation of the AMPs after their internalization. Another element that could be in favour of the intracellular proteolytic degradation of AMPs is that the bacterial cytoplasm tends to be a reducing environment, and this could facilitate the degradation of antimicrobial peptides that contain multiple disulphide bridges which are more resistant to proteolytic degradation.

3.2. Antimicrobial peptide trapping and neutralization

Another strategy in which the bacteria are able to limit the action of the AMPs is by trapping and neutralization, thus preventing them from reaching the bacterial target. This could be achieved by bacterial molecules and/or bacterial cellular structures. The resistance mediated by molecules implies the direct and indirect binding of the AMPs at extracellular level or bacterial surface level.The resistance mediated by molecules at the extracellular level consists of the secretion/liberation of bacterial molecules that can bind directly to the AMPs or an indirect way in which bacterial factors promote the liberation of host molecules that bind to the AMPs (Figure 1). Among the molecules secreted by bacteria that bind directly to the AMPs are the proteins SIC and DRS produced by GAS (30, 31). *Streptococcus dysgalactiae* subsp. *equisimilis*a related bacterium to GAS, producing DrsG, a homologue protein to SIC and DRS. DrsG is secreted to the extracellular medium and binds to LL-37, inhibiting its antimicrobial action (32). Furthermore, the protein staphylokinase, secreted by *Staphylococcus aureus,* not only functions as a plasminogen activator but also binds to alpha-defensins and mCRAMP (33, 34).

Moreover, bacterial surface-anchored proteins could be released to the extracellular medium by the action of proteases and bind to their AMPs targets. Nilsson *et al.*, 2008 showed that polymorpho-nuclear neutrophil-derived proteases could liberate from the surface of *S.pyogenes* the proteins H and M1, which in their soluble form bind to the beta-2-glycoprotein I-derived antimicrobial peptides (35). Another strategy employed by bacteria consists of the utilization of host-derived molecules to mediate the neutralization of AMPs. Secreted bacterial proteases from *S. pyogenes*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* degraded host-derived proteoglycans which yield glycosaminoglycans like dermatan sulfate, and these bind and inactivate neutrophil-derived peptides (36). At the surface level, the resistance could be mediated by anchored bacterial molecules that directly bind to AMPs or indirectly through host molecules (Figures 2, 3).

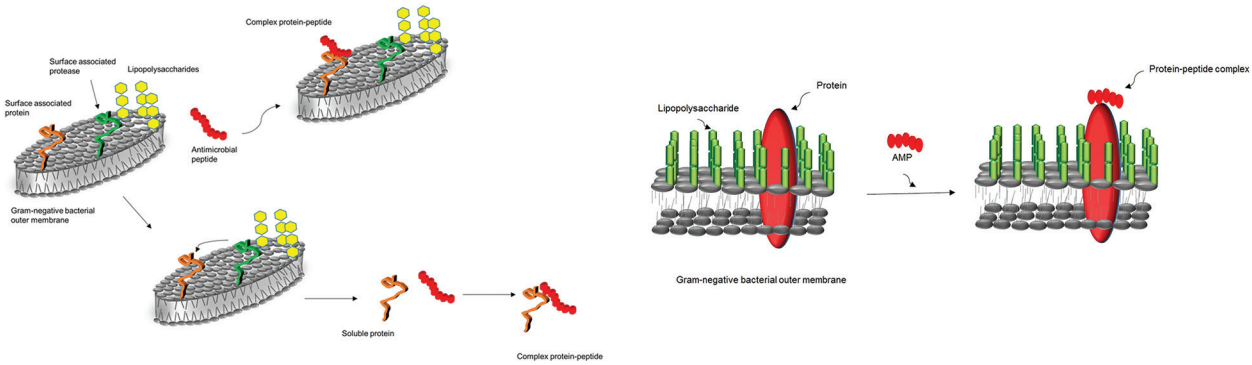

Figure 2. AMP trapping in Gram-negative bacteria. Surface-associated proteins directly bind and neutralize the AMP. Figure designed by the authors.

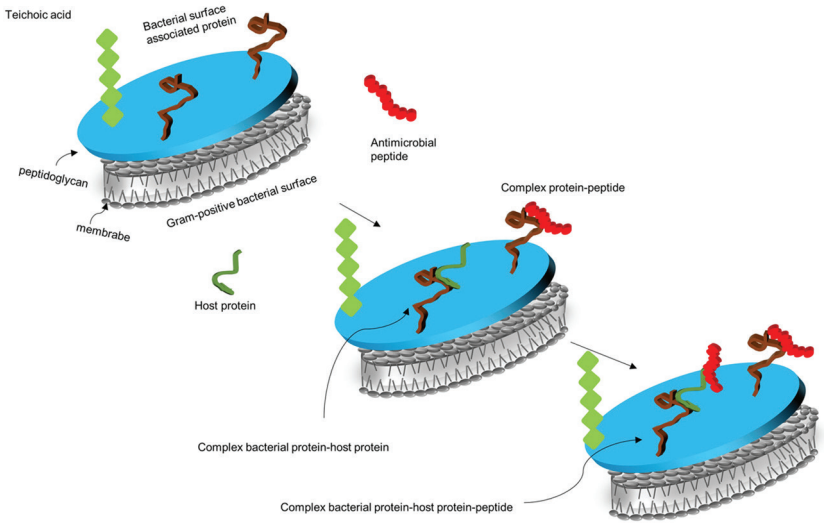

Figure 3. AMP trapping at the surface level in Gram-positive bacteria. Surface-associated proteins directly bind the AMP or alternatively bind host-derived proteins forming complexes that trap the AMP. Figure designed by the authors.

The expression of the protein M1 on the surface of GAS confers resistance to the antimicrobial activity of LL-37. The resistance appears to be due to sequestration of the peptide by interactions with the N-terminal hyper variable domain of the protein (37). Additionally, the protein M1 could bind to beta-2-glycoprotein I on the bacterial surface and prevent its degradation by polymorpho-nuclear neutrophil-derived proteases with the subsequent inhibition of the production of beta-2-glycoprotein I-derived antimicrobial peptides. The surface protein H also binds to beta-2-glycoprotein I with highest affinity (35).Another surface molecule that has been involved in the binding to AMPs is the group B *Streptococcus (GBS) pilus* protein subunit PilB. However, there are contradictory data about its role. Maisey *et al.*, 2008 showed that knockout GBS strain lacking PilB was more susceptible than wild-type GBS NCTC 10/84 strain to killing by macrophages and neutrophils. This susceptibility appear be linked to the lack of AMP-protecting function of PilB, since a significant difference was observed in bacterial survival after exposure to mCRAMP, polymyxin B and LL-37. Subsequent heterologous expression assays in *Lactococcus lactis* suggested that PilB functions by binding LL-37 and thus impeding AMP access to the bacterial membrane (38). However, a study performed by Papasergi *et al.*, 2011 found that there are no significant differences in the survival and susceptibility to antimicrobial peptides AMP-1D, LL-37, colistin, polymyxin B and mCRAMP between the wild-type GBS NEM316 strain and its isogenic *pil*B mutant (39).

In reference to the indirect binding of AMPs on the bacterial cell surface, it has been shown that binding of human serum albumin (HSA) to the surface of Group G streptococci mediated by HSA-binding protein G protects the bacteria against the antimicrobial action of MIG/CXCL9, which is a major bactericidal peptide released at the epithelial surface. The HSA add to the bacterial surface binds directly to the MIG/CXCL9

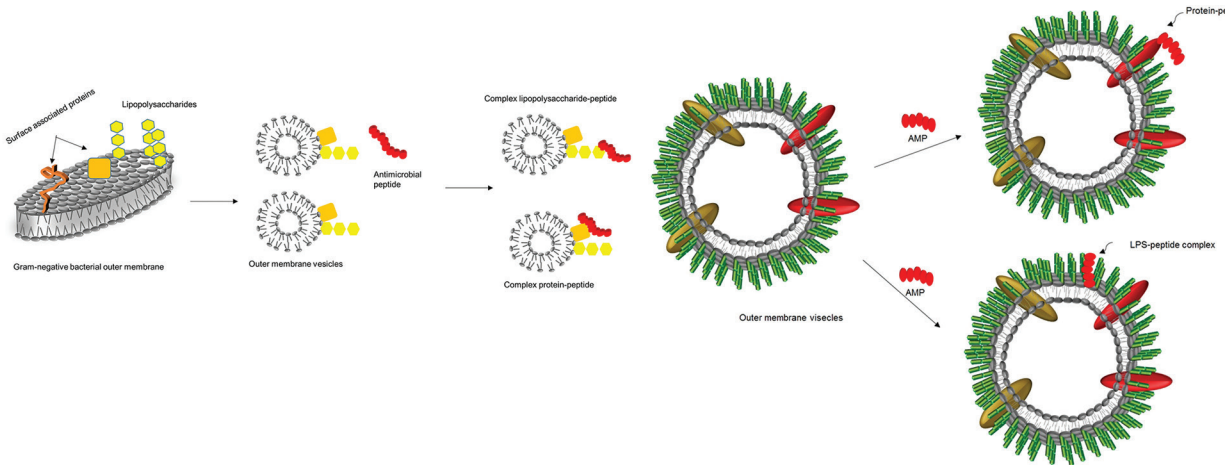

Figure 4. AMP trapping through cellular structure. Gram-negative bacteria secrete outer membrane vesicles that bind the AMP via lipopolysaccharides or proteins. Figure designed by the authors.

peptide and interferes in its antibacterial activity (40). The resistance mediated by cellular structures consists of the secretion of outer membrane vesicles (OMVs) that can act as "pickers" (Figure 4). OMVs are spheroid particles, approximately 20-200nm in diameter, that are secreted constitutively by Gram-negative bacteria but secreted in high amounts in stress situations (41, 42). The presence of AMPs constitutes a stress situation for bacteria, which increase their production of OMVs, especially because AMPs act as bacterial membrane stressors (43). It has been observed that a hyper-vesiculating mutant of *Escherichia coli* has a higher survival rate than wild-type when subject to the action of polymyxin B and colistin. Additionally, the culture of wild-type strain supplemented with OMVs showed a higher survival than non-supplemented culture. It has also been revealed that in an enterotoxigenic *E. coli* (ETEC) strain the presence of AMPs induces the production of OMVs, and these vesicles have a protective function (43). The probable mechanism by which OMVs mediated the protection was by adsorption of polymyxin B via interaction with the lipopolysaccharide (LPS) constituents of OMVs, since the OMVs from a polymyxin B-resistant strain of ETEC (which have a modified lipid A) did not mediate the protection of the susceptible strain. Other lines of evidence suggest that these OMVs could constituted an ``innate defense´´ mechanism that acts early in the presence of the stressor and permits the subsequent build-up of adaptive mechanisms of resistance (43).

One of the most interesting findings in the study of Manning and Kuehn, 2011 (43) was the fact that OMVs protect bacteria against bacteriophage action, since there is a quick and irreversible binding of phage to OMVs with the subsequent virus inactivation. More recently, it has been shown that OMVs of *Komagataeibacter intermedius* adsorb phage particles (44). The fact that OMVs interact and inactivate phages could have implications for the use of engineered phages as alternative antibiotic therapy. Another characteristic of the antimicrobial resistance mediated by OMVs is the fact that they may participate in the cross-resistance to different antimicrobial peptides. OMVs delivery by *Vibrio cholerae* when its culture is supplemented with sub-minimal inhibitory concentrations of polymyxin B protected it from the action of LL-37. This cross-resistance was mediated by the trapping of LL-37 by protein Bap1, which is presented only in the OMVs induced by polymyxin B (45).

3.3. Efflux pumps

There are different classes of multidrug efflux pumps, constituted by five efflux protein superfamilies: the adenosine triphosphate (ATP)-binding cassette (ABC), the major facilitator superfamily (MFS), the small multidrug resistance (SMR) superfamily, the resistance-nodulation-cell division (RND) superfamily and the multidrug and toxic compound extrusion (MATE) superfamily (46). Unlike the ABC transporter, which uses ATP as an energy source, the MFS, SMR, RND and MATE transporters use the electrochemical potential of the membrane for the extrusion of drugs (46). The efflux pumps that have been linked to development of resistance to AMPs are listed below (Table 2). Although the major function of the efflux pumps in resistance response to AMPs is to expel them, other functions have been described. In Gram-positive bacteria there are specialized structures called peptide sensing and detoxification modules (PSD) that are composed of a two-component system (TCS) linked functionally to an ABC transporter. In PDS the TCS generally senses the presence of the antimicrobial peptide and induces the expression of the ABC transporter that expels the peptide. However, there are evidences that in these modules the ABC transporter could be involved in sensing the peptides. In *Bacillus subtilis* bacitracin binds directly to the BceB permease component of BceAB transporter. BceB forms a sensory

Table 2. Efflux pumps that mediate resistance to AMPs

Efflux pump	Superfamily	Peptides	Bacteria	Reference
MtrC-MtrD-MtrE	RND	Polymyxin B LL-37 PG-1	*Neisseria meningitidis*	(54)
MtrC-MtrD-MtrE	RND	TP-1 PC-8 PG-1 LL-37 CRAMP-38	*Neisseria gonorrhoeae*	(55,56)
MtrC-MtrD-MtrE	RND	LL-37 hBD-3	*Haemophilus ducreyi*	(57)
QacA	MFS	tPMP-1	*Staphylococcus aureus*	(58)
RosA	MFS	Polymyxin B Cecropin P1 Melittin	*Yersinia enterocolitica*	(59)
AcrAB	RND	HNP-1 hBD-1 hBD-2 Polymyxin B	*Klebsiella pneumoniae*	(60)
KpnGH	MFS	Polymyxin B	*Klebsiella pneumoniae*	(61)
KpnEF	SMR	Colistin	*Klebsiella pneumoniae*	(62)
MexAB-OprM	RND	Colistin	*Pseudomonas aeruginosa*	(63)
MeFE/Mel[1]	RND ABC	LL-37	*Streptococcus pneumoniae*	(64)
AcrAB/TolC EmrAB/TolC	RND	Protamine	*Escherichia coli*	(65)
CmeABC CmeDEF	RND	Polymyxin B	*Campylobacter jejuni*	(66)

[1]MefE belonging to the MFS superfamily, Mel incomplete ABC-transporter, For updated information about ABC-transporter refer to (67)

complex via direct interaction with the BceS histidine kinase component of the BceRS TCS. This signaling complex is thought to trigger the production of more BceAB transporters that could mediate resistance via extrusion of the peptide (47, 48). On the other hand, in *S. aureus* the BraDE transporter senses the presence of nisin or bacitracin, and through BraSR TCS induces the expression of the VraDE transporter, which acts by expelling the AMPs (49). Similarly, the VraFG transporter acts as a sensor in the GraSR/VraFG system, in which after sensing peptide, signaling through GraSR TCS induces the expression of the *dlt* and *mprF* genes, which mediate resistance via peptide repulsion. Interestingly, in this system, the signaling via GraSR TCS regulates the expression vraFG genes in a positive feedback fashion, thereby ensuring the production of more transporters for the sensing (50). Recently, a network in which the transporter EF2752-2751 sensed bacitracin and via sensor kinase EF0927 activated the expression of the EF2050-2049 transporter that removed the AMP has been described in *E. faecalis* (51).

Despite the existence of literature supporting the role of efflux pumps in resistance to AMPs (Table 2), there are data that contradict such information. Rieg *et al.*, 2009 showed that *E. coli* AcrAB, *P. aeruginosa* MexAB RND-type efflux pump and *S. aureus* NorA MFS efflux pump were unable to protect these microorganisms against LL-37, HNP-1-3, HD-5, hBD-2 and hBD-3 (52). Moreover, Bayer *et al.*, 2006 highlight that in *S. aureus* the carriage of plasmids bearing genes encoding the QacB MFS-type efflux pump and QacC SMR-type efflux pump do not confer resistance to thrombin-induced platelet microbicidal protein 1 (tPMP-1) peptide, and the low resistance conferred by QacA MFS-type efflux pump is unrelated to proton motive force-dependent peptide efflux (53).

3.4. Cellular surface remodelling

The cytoplasmatic membrane constitutes the major target for the action of the AMPs. To reach its target the AMPs have to interact and traverse the surface components of the bacteria. Therefore, one of the

resistance mechanisms used by bacteria to circumvent the action of the AMPs is remodeling their surface so that AMPs cannot interact and/or pass through this surface. To achieve these aims the bacteria change the net charge or the properties of their surface. Given the fact that there are structural and chemical differences between the surface of Gram-negative and Gram-positive bacteria it is expected that mechanisms involved in the remodeling will be different. Cellular surface remodeling changes the net charge on the bacterial surface to make it more positive, therefore facilitating the electrostatic repulsion of cationic AMPs.

The surface of Gram-positive bacteria is constituted by a cell wall that surrounds the cytoplasmatic membrane. The cell wall consists of peptidoglycan sacculus made up of linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) units via β 1,4 bonds. Peptides are bound to the MurNAc units and in turn could be cross-linked directly or indirectly to form a three-dimensional structure. Additionally there are glycopolymers that can be attached either to the peptidoglycan or to lipid membranes (68, 69). To achieve a reduction in the net negative charge at the cell wall level, the bacteria target major anionic glycopolymers: the teichoic and lipoteichoic acids. The strategy consists of the esterification of the carboxyl group of the amino acid D-alanine to the monomeric subunits (or its glycosidic substituents) of the glycopolymers, resulting in exposure of the amino group of the D-alanine which contains a positive charge. This process is performed by several proteins encoded by the *dltABCD* operon (70). The presence of the homologue of the *dtl*B gene that confers immunity to bactofencin A, a new type of cationic bacteriocin, has been shown recently in *Lactobacillus salivarius* DPC6502. Inherently, bactofencin A shares a similarity with eukaryotic cationic antimicrobial peptides, and the D-alanylation of teichoic acids appears to be the mechanism that confers specific protection against bacteriocin (71). Moreover, in GBS it has been proposed that D-alanylation of teichoic acids could mediate the resistance to AMPs by modifying the rigidity and permeability of the cell wall more than by electrostatic repulsion (72). The protection mediated by D-alanylation has been described in a broad range of Gram-positive genera that include: Staphylococcus, Listeria, Enterococcus, Bacillus, Clostridium, Streptococcus and Lactobacillus (67), and it is effective against a diversity of cationic AMPs, like colistin, nisin, polymyxin B, Mangainin II, HNP-1-3, beta-defensin 2, gallidermin, mCRAMP, animal-derived protegrins, tachyplesins, indolicidin, cecropin B, bactofencin A, vancomycin, daptomycin and LL-37 (71,73-81).

On the surface of Gram-negative bacteria, the major modifications take place in the LPS and lipooligosaccharides (LOS), specifically in the lipid A moiety. These modifications implicate the removal of the phosphate groups, the addition of amine substituents like phosphoethanolamine (pETN), 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the disaccharide backbone or glycine and diglycine residues in the acyl chains. The removal of the phosphate groups from the C´1 and C´4 position at the disaccharide backbone is mediated by lipid A phosphatases. *Helicobacter pylori* mutants in the genes *lpxE* (1´-phosphatase), *lpxF* (4´-phosphatase) and the double mutant *lpxE/*F showed an increased susceptibility to polymyxin B, LL-37, beta-defensin 2 and P-113 (82). Similarly, *lpxE, lpxF* and *lpxE/*F mutants of *Rhizobium etli* and *lpxF* of *Francisella novicida* are more susceptible to polymyxin B than wild type strains (83, 84). Engineered *E. coli* strains that express the enzymes LpxE and/or LpxF of *F. novicida* are more resistant to polymyxin B, with the greatest resistance observed in the species with lipid A dephosphorylated at C`4 (85).

Moreover, pETN could be attached directly to the position C´1 of the glucosamine backbone of lipid A, which implies the removal of the phosphate group at this position. *Helicobacter pylori* $LpxE_{HP}$ mutants were unable to remove the C´1 phosphate with the subsequent addition of pETN making the strain more vulnerable to polymyxin B than the wild type strain, which produces LPS, decorates with pETN in the C´1 and lacks the phosphate group in the C´4 position (86). Additionally pETN could be linked to phosphate groups in the C´1 and/or C´-4 position. Blockage in successful transfer of pETN to the phosphates groups renders strains more susceptible to AMPs. This has been shown in *Campylobacter jejuni*, *Neisseria gonorrhoeae*, *Neisseria meningitidis* and *Salmonella enterica* (54, 87, 88).

Another AMP resistance strategy employed by bacteria to reduce the anionic charge is the addition of L-Ara4N to lipid A. However, some data suggest that the structure and/or localization of the L-Ara4N modification to lipid A could be involved in the inhibition of the binding of polymyxin B to the lipid A (89). The resistance via Ara4N modification has been described in *Salmonella enterica, Proteus mirabilis, Pseudomonas aeruginosa and Klebsiella pneumoniae* (90). More recently, a new lipid A modification which confers resistance to polymyxin B has been described in *Vibrio cholerae* El Tor strains. This consists of the linking of glycine or diglycine amino acids to the 3-OH group of the secondary acyl chain attached at the 3´position. Implicated in this modification process are a series of proteins, such as AlmE, which that acts as a glycine carrier ligase that ligates the amino acid residue to AlmF, which in turn transfers it to AlmG, which finally transfers it to LPS. The machinery is encoded by the *almEFG* operon and is under the control of the VprAB two-component system, which is up-regulated by the presence of polymyxin B (91, 92). Additional modifications of lipid A consist of the addition of glucosamine, galactosamine and acylation. In *Bordetella pertussis* the addition of glucosamine

mediated the resistance to polymyxin B, colistin, LL-37, indolicidin, HHC-10 and CP28 (93). Similarly, in *Bordetella bronchiseptica* the resistance to polymyxin B and porcine beta-defensin 1 was associated with the glucosamine modification of the LPS lipid A (94). Moreover, in *Acinetobacter baumannii* colistin-resistant laboratory-adapted strain and colistin-resistant clinical-relevant strains, the addition of galactosamine to lipid A together with other modifications appears to be involved in the resistance to the AMP (95). Due to the acylation process, it has been shown that a *K. pneumoniae* mutant in the *lpx*M gene which encodes the enzyme that mediates secondary acylation of lipid A was more susceptible to polymyxin B, colistin, CP-28 and C18G peptides. This susceptibility appears to be the cause of the *in vivo* attenuation of the mutant in BALB/c mice (96). A recent study suggests that in this mutant the underacylation could provoke a more fluid outer membrane as a consequence of weak lateral interactions between LPS fatty-acyl chains, thus facilitating the insertion of the N-terminal domain of polymyxins (97). Moreover, the involvement of the acyltransferase MsbB (LpxM) in the resistance to the AMPs LL-37, mCRAMP, magainin 2 and polymyxin B has been shown in El Tor *Vibrio cholerae* strain while in the Classical strain it mediated the resistance to all AMPs except to polymyxin B (98). A further study demonstrated that resistance to polymyxin B by *V. cholerae* requires the activity of hydroxy-acyltransferase enzyme LpxN, which transfers 3-hydroxylaurate to the lipid A (99). Again, the addition of palmitate to lipid A mediated by the enzyme palmitoyl transferase (PagP) is also involved in the resistance to AMPs. Hittle *et al.*, 2015 showed that *Bordetella parapertussis* PagP mutant was more susceptible to C18G than the wild-type strain (100). The *P. aeruginosa* PagP mediated the resistance to C18G but appears not to be involved in the resistance to polymyxin B (101). Other microorganisms in which palmitoylation plays a role in the resistance to AMPs are Salmonella and Legionella (102, 103).

To reduce the net negative charge on cytoplasmic membrane the bacterium modifies its major anionic phospholipids, phosphatidylglycerol (PG) and cardiolipin. One of the modifications consists of the addition of amino acids via ester bond between the α-carboxyl group of amino acids and the hydroxyl group of the terminal glycerol moiety of PG or the hydroxyl group of the middle glycerol moiety in cardiolipin. This arrangement permits the presence of a NH2-terminal free, which carries a positive charge and, in the case of basic amino acids, there are additional free positive charges in the side chains. Although lysine is the major amino acid added, addition of other amino acids, such as alanine and arginine, has been described (104,105). The synthesis and translocation of the aminoacylated-PG is performed by the multipeptide resistance factor protein (MprF) family, which are present in a variety of Gram-positive and in Gram-negative bacteria (106). Some members of the MprF family have a restricted substrate specificity, while others have a broad substrate specificity which could confer a more elaborate cell membrane remodeling (104). The MprF protein is also required for the lysinylation of cardiolipin (107). It has been suggested that in addition to the electrostatic repulsion of the AMPs, the incorporation of aminoacylated phospholipids in lipid membrane bilayers stabilizes them against peptide-induced disturbances (108).

Another modification of PG that promotes resistance to AMPs is the addition of palmitate. A recent study showed that in *Salmonella typhimurium* the sensor system PhoPQ and the palmitoyltransferase enzyme PagP are involved in the palmitoylation of PG, increasing levels of palmitoyl-PG in the outer membrane and mediating the resistance to AMPs. The resistance likely occurs by increasing the hydrophobicity and saturation level of the membrane bilayer (109). While some changes in the membrane composition facilitate the repulsion of the AMPs others cause variations in the membrane properties like fluidity that in turn affect the microbicidal activity of the peptides. Recently it has been shown that a reduction in the content of branched fatty acid could increase the rigidity of membrane-mediated resistance of *Enterococcus faecalis* to cationic AMPs together with an increase in the positive charge content (110). Similarly, a previous study showed that a less fluid membrane could mediate the resistance to daptomycin in *E. faecalis* and *E. faecium* (111). It has been documented that in *Listeria monocytogenes* the resistance to nisin is accompanied by changes in the constituent fatty acids of the membrane, which increase its rigidity (112). Moreover, an increase in the *S. aureus* membrane rigidity was a common characteristic in a strain with induced resistance to magainin 2 and another to gramicidin (113). While a decrease in membrane fluidity mediates the resistance in some microorganisms, for others an increase in the fluidity is the main mechanism of resistance. Lather *et al.*, 2015 showed that *S. aureus* strain resistant to pediocin has increased levels of unsaturated, branched chain, and hydroxy fatty acids with respect to the susceptible strain. This peculiar composition results in a higher fluidity of the membrane, which prevents the oligomerization of the peptide (114). An increase in membrane fluidity also appears be involved in the resistance of *S. aureus* to tPMP, HNP-1 and polymyxin B and nonsusceptibility to daptomycin (115, 116). The decreasing sensitivity of laboratory-derived *Staphylococcus aureus* mutant to telavacin is attributable to the increase in the fluidity of the membrane (117). Moreover, in daptomycin resistant methicillin-resistant *S. aureus* strains there is a reduction in the content of cell membrane carotenoids, which increase the membrane fluidity (118). However, in another study a mutant strain that overproduces the Staphyloxanthin carotenoid showed reduced susceptibility to daptomycin and was resistant to other

AMPs, like tPMP, HNP-1, RP-1 and polymyxin B (119). A new mechanism of resistance to daptomycin has been described in *Enterococcus faecalis*. This consists of the redistribution of microdomains enriched in cardiolipin and a reduction in the content of PG, causing a diversion of the AMP away from the septum division, its target site of action (120).

Other modifications that take place on the bacterial surface are the production of capsular polysaccharides (CPS) and exopolysaccharides. The CPS play a direct role in the resistance to AMPs by preventing their interaction with the bacterial membrane. *Klebsiella pneumoniae* mutants in the production of CPS were more sensitive than the wild-type strain to a range of AMPs, such as HNP-1, HBD-1, lactoferrin, protamine sulphate and polymyxin B. It was observed that clinical strains with increased production of CPS were more resistant to polymyxin B and that a threshold amount of CPS was necessary for protection (121). Moreover, it has been proposed that CPS can act as a decoy for AMPs, interfering in their microbicidal activity by direct trapping of the peptides. This has been confirmed in anionic CPS, while cationic or uncharged CPS do not exert this trapping effect. Supporting the decoy role of CPS was the fact that polymyxin B and HNP-1 provoked the release of CPS from capsulate *K. pneumoniae* K2, *Streptococcus pneumoniae* serotype 3 and *P. aeruginosa* overexpressing CPS. The protection conferred by CPS was evidenced by the fact that the transfer of a purified CPS from the above-mentioned strains protects against polymyxin B and increases the minimum inhibitory concentration (MIC) to polymyxin B and HNP-1 of a *K. pneumoniae* uncapsulated strain (122). Additionally, other studies demonstrated that serogroup B and C polysaccharide meningococcal capsules are involved in the resistance to defensins, cathelicidins, protegrins and polymyxin B (123,124). In enteropathogenic *E. coli* the protection to HD-5 appears to be mediated in part by the production of group 4 capsule (125). A recent study show that *Acinetobacter baummannii* mutans in the K locus which encode for the machinery involved in the processing of CPS exhibited an increased sensivity to colistin (126).

With regard to the production of exopolysaccharides, it has been observed that *Staphylococcus epidermidis* mutant in the *cap* genes which encode for the enzymes involved in the production of the polyanionic polymer poly-gamma-DL-glutamic acid (PGA) was more sensitive to LL-37 and beta-defensin 3 than the wild-type strain (127). *S. epidermidis* also produces an exopolysaccharide called exopolysaccharide intercellular adhesin (PiA) which is subject to deacetylation to make it cationic. This allows PIA to participate in the defence against AMPs like LL-37 and beta-defensin 3 (128).A study in opportunistic lung pathogens *P. aeruginosa, Inquilinus limosus* and *Burkholdelia cepacia* complex showed that the exopolysaccharide produced by these bacteria could bind to human, *Pongo pygmaeus* and *Presbytis obscurus* orthologue cathelicidins LL-37. The strongest inhibition of AMP activity was observed in more negatively charged exopolysaccharides (129). Meanwhile, Benincasa *et al.*, 2009 have demonstrated that the exopolysaccharides produced by *P. aeruginosa, K. pneumoniae* and *Burkholdelia cepacia* complex inhibit the microbicidal activity of LL-37, hBD-3, SMAP-29, PG-1 and Bac7 (1-35) and could be involved in the persistence of these microorganisms in the lung of cystic fibrosis patients (130).

3.5. Induction of AMP expression downregulation

Manipulation of genetic expression of the immune cells to impair the production of antimicrobial peptides is among the diverse strategies employed by microorganisms to circumvent antibacterial response. This strategy appears to be widespread in several enteric pathogens. Early observations by Islam *et al.*, 2001 showed downregulation of LL-37 and hBD-1 in biopsies of patients with bacillary dysenteries and LL-37 in Shigella infected cell lines. It was also observed that downregulation of LL-37 was mediated by bacterial plasmidic DNA, suggesting that downregulation occurs via interference with signalling pathways (131). Sperandio *et al.*, 2008 showed that *Shigella flexneri* repressed the expression *in vitro* of hBD-3 and LL-37 via the injection into the host cells of virulence plasmid-encoded effectors controlled by MxiE, which could impair the signalling pathways that trigger the expression of AMPs. The role of gene expression regulation as mechanism to escape the immune response was also confirmed *in vivo* (132). Moreover, enteric pathogens like *Vibrio cholerae* and enterotoxigenic *Escherichia coli* through their virulence factors, cholera toxin (CT) and labile toxin (LT), respectively, induced the downregulation of the cathelicidin (hCAP-18/LL-37) and hBD-1 genes expression in the intestinal epithelial cells. This suppression was found to occur at the transcriptional level and was mediated by the activation of several signalling pathways downstream of cAMP accumulation and the transcriptional repressor ICER (133). But not only enteric pathogens display this strategy. *Neisseria gonorrhoeae* downregulated the levels of LL-37 in a cervical epithelial cells line ME180, while *Neisseria meningitidis* downregulated the levels of peptidase inhibitor 3(PI3) in the 16HBE14 bronchial epithelial cell line (134,135).

Moreover, one of the strategies developed by *Klebsiella pneumoniae* for survival in the lung is through its CPS, which evades recognition by the pattern recognition receptors. Non-recognition implies that the signalling pathways that trigger the expression of human beta-defensins are not activated, and they therefore downregulate AMP expression (136).

3.6. Osmotic stabilization

In a recent study, address by Wenzel *et al.,* 2014 was described a new mechanism of resistance to AMPs. In this, the bacteria overcome to the stress caused by membrane-targeting bacteriolytic peptides through the production and secretion of osmoprotective amino acids. The treatment of *Bacillus subtilis* with the cationic hexapeptide MP196 induced the up-regulation of enzymes involved in amino acid anabolism. Also was detected a reduction in the intracellular levels of glutamine/glutamate, asparagine/aspartate, lysine and proline. While the extracellular levels of glutamine/glutamate and asparagine/aspartate were substantially increased and the arginine, lysine, and proline levels were moderate increased. These changes in the amino acid pools suggested that in response to the stress caused by MP196 *B. subtilis* increase the production and release of amino acids. Additionally was found that the levels of glutamine/glutamate released by *B. subtilis* when is under treatment with MP196 exceed the sum of intracellular and extracellular levels in the untreated bacteria and the supply of exogenous glutamate increase the MIC of MP196. Moreover, the supply of sodium chloride and potassium chloride had the same effect on the MIC of MP196. All these evidences suggested that the release of glutamate is involved in the protection against MP196 but this protection is not specific of glutamate and is mediated by osmotic effects. Also was observed release of glutamate in response to others peptides like gramicidin S, aurein 2.2., nisin and gramicidin A suggesting that glutamate release constitutes a general response to membrane-targeting bacteriolytic peptides. Besides, the release of glutamate appeared be linked to mechano sensitive channels because a *B. subtilis* mutant in the four MscL and MscS-type mechanosensitive channels showed high intracellular levels of glutamate and greater sensitivity to MP196. However, these channels are not the only route for release glutamate because when the quadruple *msc* mutant was challenged with MP196 there was an increase in the extracellular levels of glutamate (137).

3.7. Activation of bacterial regulatory systems

For assembly of resistance response, initially bacteria have to sense the presence of AMPs with the subsequent transmission of the information to inside of the cell. This produce changes in the transcriptional status of the bacteria. Between the several sensing systems which are equipped the bacteria for detect the changes in their environment, the TCS appear to be the most important in orchestrate the resistance response to AMPs. The TCS is a ubiquitous system in bacteria and generally involve a membrane-integrated histidine kinase receptor and a cytoplasmic response regulator protein that mediate the transcriptional activity. The flux of information between the histidine kinase receptor and the response regulator occur via phosphorylation and dephosphorylation reactions (138).

The PhoP/PhoQ two-component system was originally described in the pathogen *S. typhimurium* but is found in other Gram-negative bacteria. In this system, PhoQ constitute the sensor kinase protein that sense stimulus like low levels of Mg^{2+}, acidic pH and AMPs like polymyxin B, C18G, LL-37 and protegrin. Each one of these stimulus provoke a differential expression of Pho-P regulated genes (139).

When *S. typhimurium* detect via PhoQ an environment with low levels of Mg^{2+} occur the activation of the response regulator protein Pho-P by its phosphorylation. The activate Pho-P promote the transcriptions of a series of genes like *mgtA*, *slyA*, *ugtL*, *pagP*, *mig-14*, *virK* and *prmD* that are involved directly or indirectly in the resistance to AMPs. The gene *mgtA* encode the Mg^{2+} transporter MgtA that could remove Mg^{2+} from the periplasm of the bacteria triggering the transduction of the signal via PhoP/PhoQ TCS which the subsequent transcription of the Pho-P regulated genes *prmD* and *ugtL* which are involved in the modifications of LPS that support the resistance to polymyxin B (140). The gene *slyA* encode for the protein SlyA that acts as transcriptional activator of *ugtL* gene which encode for protein UgtL, an oligomeric inner membrane protein that mediate the formation of a monophosphorylated lipid A. Mutant strains of *slyA* and *ugtL* were sensitive to magainin 2 and polymyxin B (141,142). The gene *pagP* encode for a palmitoyltransferase enzyme PagP that are involved in the palmitoylation of PG and lipid A (109,142). Moreover, *mig-14* and *virK* encode for Mig-14 and VirK proteins, which showed be involved in the resistance to CRAMP within murine activated macrophages (143). Meanwhile *prmD* encode for the protein PrmD that mediated cross-talk between the PhoPQ TCS and the PrmAB TCS, here the indirect activation of PrmAB TCS via PrmD imply LPS modifications that mediated the resistance to AMPs (144). Recently was observed that in *E. coli* also occurred this cross-talk between PhoPQ TCS and the PrmAB TCS mediated by PrmD but interestingly appear be involved another mechanisms of activation PrmD independent of PhoP/PhoQ. This show that the crosstalk between both TCS is a complex process (145). Analysis of the *S. thyphimurium* proteome after infection of non-phagocyte epithelial cells showed an increased in the PhoPQ TCS as well as PgtE, a protease that is involved in the resistance to AMPs and whose expression is controlled by PhoPQ TCS (146).

Other of the TCSs associated to resistance to AMPs is the PmrAB TCS, which has been described in a variety of bacteria such as *S. thyphimurium*, *E. coli*, *K. pneumoniae*, *Y. pestis*, *C. rodentium* and *P. aeruginosa*. Most of the studies in PmrAB TCS have been carried out in *Salmonella* but many of the findings can be applied to others Gram-negative pathogens (147). How was discussed above the PmrAB TCS can be activated indirectly by cross-talk with PhoPQ TCS mediated by

PmrD. Here PmrD bind to the phosphorylated form of the response regulator protein PmrA and inhibit its dephosphorylation by the histidine kinase PmrB allowing the transcription of the PmrA-regulated genes (148-150). Additionally PmrB can sense directly high concentration of Fe 3+, Al3+, mildly acidic pH and cationic AMPs (147). Within the PmrAB regulon, there are genes that encode for proteins that are involve in the modifications of the different structural elements of the LPS, thus mediating the resistance to AMPs. The major modifications in the lipid A are the addition of pEtN and L-Ara4N mediated by the *pmrC* gene product the first and the *pmrHFIJKLM* operon and *ugd* gene products the second (151-153). Beside through the expression of PmrR, a PmrA-activated inner membrane peptide, the PmrAB TCS control the activity of the enzyme LpxT that is responsible of generate a lipid A specie diphosphorylated at the position 1. PmrR interact and inhibit LpxT, therefore reduce the formation of lipid A diphosphorylated with the consequent reduction of the net negative charge on the LPS (154). Otherwise, the PmrA-regulated wzz_{ST} and wzz_{fepE} genes encoded for proteins involved in the polymerization of the O-antigen of the LPS and have been linked to the resistance to polymyxin B (155,156).

In the resistance response of *P. aeruginosa* to polymyxins in addition to PhoPQ and PmrAB TCS participate others TCS. The ColRS and CprRS TCs interact with the PhoPQ TCS influencing on the L-Ara4N modification of the LPS but also appear regulate additional cellular factors necessary for the resistance response. Moreover, in the peptide-induced adaptive resistance response appear be involved the ParRS TCS which activate among others the *arnBCADTEF* operon, *pmrB* and *pagL* genes (157,158). Additionally have been described a gene encoding MIG-14 like protein that is involved in the recognition of colistin and Novispirin G10 and is required for the development of colistin-tolerant subpopulations in *P. aeruginosa* hydrodynamic flow chamber biofilms (159).

In GAS the CsrRS TCS have been linked to the induction of an invasive phenotype. The histidine kinase CsrS specifically sense LL-37 and activate the transcription of a series of genes as *hasABC* operon, *sda 1*, *slo*, *ska* and *spyCEP/scpC* which increase the resistance to opsonophagocytic killing by human leukocytes, reduce the influx of neutrophil, degrade neutrophil extracellular traps and damage of phagocytes (160-162). Beside in *S. aureus* the GraRS TCS mediate the resistance to several AMPs like hNP-1, tPMPs, daptomycin, polymyxin B and RP-1. GraS acts as a sensor kinase that sense certain AMPs like LL-37, RP-1 and polymyxin B via 9-amino acid -external loop at the N-terminus and then induce the transcription via GraR of downstream target genes such *mprF* and *dltABCD* provoking change in the surface charge (163-165). Other of the TCS that operate in *S. aureus* is the LytSR TCS. Here LytS sense decrease in the electrical transmembrane potential and trough LytR induce the transcription of the *lrgAB* operon, which can influence in biofilms formation, programmed cell death and autolysis. Additionally have been shown that a *lytS* mutant was more susceptible *in vitro* to hNP-1, tPMPs, RP-1 and daptomycin, interestingly the increase in the susceptibility was *lrgAB*-independent suggesting that other downstream pathways could be involved in the resistance response (166-168). Recently have been proposed in a study developed by Weatherspoon-Griffin *et al.*, 2014 a new mechanism that involved the CpxRA TCS through which *E. coli* modulate the resistance to protamine and probably to others AMPs. In this mechanism, the CpxRA TCS activate the transcription of the *marRAB* operon that via MarA activator enhance the expression of TolC-dependent tripartite multidrug transporters. Beside CpxRA TCS activate the transcription of the *aroK* gene permitting the subsequent production of aromatic metabolites among whom salicylate and 2, 3 dihydroxybenzoate can derepress *mar* operon transcription by the liberation of the repressor MarR from the operator site *marO*. Additionally the produced indol could acts as stimuli for the activation of CpxR trough phosphorylation by the CpxA sensor kinase, hence activate the *mar* operon transcription (65). Furthermore, a previous study showed that CpxRA TCS is involved in the resistance to several AMPs in *Salmonella* and *E. coli* by activate the transcription of *amiA* and *amiC* genes that encoded for two peptidoglycan amidases (169). Other study showed that CpxRA TCS participate in the tolerance of *E. coli* to ApoEdpL-W, polymyxin B and melittin (170). Other of the TCS that has been implicate in the resistance to AMPs is the VprAB TCS, how was discus above in *V. cholerae* this system controls the expression of the genes necessary for the addition of glycine to the LPS (92). Recently it have been described that CarRS TCS also is involved in the control of LPS modification by glycine addition in *V. cholerae*. CarR perform a positive regulation of the *almEFG* operon by directly bind to the regulatory region of the operon (171).

Aside from TCS, the Rcs phosphorelay system has been implicated in the resistance response to AMPs in *S.typhimurium*. The Rcs phosphorelay system consist in a complex arrangement proteins that create a signalling platform that control the expression of genes involve in motility, biofilm formation, virulence, periplasmic quality control and AMPs resistance. The system are conformed by a histidine kinase protein (RcsC) and RcsB as response regulator protein, additionally are involved an intermediate inner membrane phosphorelay protein (RcsD), an auxiliary nonphosphorylatable transcription factor (RcsA) and two proteins that participate in the signal sensing IgaA and RcsF (172). The mechanisms of activation implicate autophosphorylation of RcsC with the subsequent phosphotransfer to RcsD follow by phosphotransfer to RcsB that through formation of homodimers or heterodimers with RcsA control the gene

expression (173). In *S. typhimurium* RcsF is required for the activation of the Rcs system by AMPs like polymyxin B, polymyxin B nonapeptide, LL-37 and C18G (173). Erickson and Detweiler, 2006 showed that in the resistance to polymyxin B by *S. typhimurium* appear be involved RcsB-regulated genes like STM1284-1285 operon that encode for two putative cytoplasmic proteins, STM 1951-1953 operon that encode for a putative ABC transporter system, STM1589 that encode for a putative oxidoreductase and STM1515 that encode for the putative periplasmic protein YdeI (174).

3.8. Collective resistance

There are growing evidences that the arrangement of bacteria in populations has a direct influence on antibiotic resistance. Bacterial populations deploy different mechanisms to develop coordinated and protective responses to antimicrobial substances at the collective level. Once of the mechanisms is growing in biofilms, which constitute special structures in which different mechanisms converge, making this an effective form of persistence. The arrangement in biofilm provides the bacterial population with a way to circumvent the effect of AMPs. Studies performed by Chan *et al.*, 2004, 2005 show that in biofilms of *P. aeuroginosa* the arrangement of the exopolysaccharide alginate matrix could form structures like hydrophobic ``pockets´´ that create a microenvironment in which the peptides may be sequestered to promote their self-association before they reach the target bacterial membrane. This makes the alginate act as a kind of scavenger (175,176). Another element that constitutes the matrix of biofilms of *P. aeuroginosa* is extracellular DNA. It could act as a chelating agent, creating a cation-limited environment that is sensed by the bacteria, which respond by expressing the LPS modification operon PA3552-PA3559 (177). More recently it has been shown that in *Salmonella enterica* serovar Typhimurium biofilms the extracellular DNA induces a similar resistance response to that in *P. aeuroginosa* biofilms (178).

Curli fimbriae are a major component of the extracellular matrix of urophatogenic *E. coli* biofilms and have been associated with resistance to cathelicidins. It was specifically demonstrated that curli fimbriae bind to LL-37 and prevent it from reaching the bacterial membrane (179). Moreover, in Gram-negative bacteria biofilms, reversible palmitoylation of lipid A domain of LPS takes place. This modification triggers a weak inflammation response and promotes resistance to the peptide protegrine-1, which could be associated with the observed biofilm persistence *in vivo* (180).

It has been shown that structural organization of the biofilms could influence the resistance to AMPs. Different structural organization could create diversity of microenvironments from which different subpopulations could arise. In *E. coli* biofilms with complex structure organization subpopulations tolerant to colistin were observed, but not in less structured biofilms. The tolerant phenotype appears be linked to genetically encoded mechanisms regulated via the *basR/basS* two-component system (181). In *P. aureginosa* biofilms there is the presence of heterogeneous subpopulations. The subpopulations associated with the cap of the mushroom-shaped multicellular structures were found be tolerant to colistin, and this tolerance is linked to the *pmr* operon which is involved in the modification of LPS (182). Additionally, metabolically active subpopulations of cells in *P. aeruginosa* biofilms that express the *mexAB-oprM* genes developed tolerance to colistin, while the low metabolically active cells subpopulations were susceptible (63).

4. SUMMARY AND PERSPECTIVE

The high levels of resistance to conventional antibiotics have plunged the scientific community into a search for new antimicrobial compounds. In this regard, one of the most promising classes are the AMPs. However, their microbicidal activities are also subject to a resistance response by bacteria. Over the years the bacteria have appropriated diverse mechanisms that allow them to counteract the potent action of AMPs. Most of the mechanisms prevent AMPs from reaching the bacterial membranes, which seem to be the main targets. To achieve this, bacteria secrete and/or anchor proteases or molecules at the cell surface that degrade or neutralize the AMPs respectively. Additionally, bacteria could secrete outer membrane vesicles that trap the peptides. Other mechanisms involve the expulsion of AMPs mediated by efflux pumps and the remodelling of the bacterial surface components that affect the binding of peptides to membranes. Bacteria can also manipulate host factors, such as proteases, human serum albumin, proteoglycans and alpha 2-macroglobulin to reverse AMPs actions and also induce AMP downregulation expression by immune cells. At the population level a mechanism for resistance has also been created, essentially consisting of the formation of biofilms. Knowledge of the molecular basis underlying the bacterial resistance response allows the design of more powerful AMPs and/or therapeutic strategies that circumvent the resistance response. To this end, antimicrobial peptides could be engineered via manipulation of their structural elements to confer on them resistance to the degradative action of proteases. Moreover, the nanoencapsulation of AMPs could protect them from proteases and trapping molecules. Additionally, the inclusion in chemotherapeutic schemes of drugs that impair the AMP-sensing systems in bacteria could prevent resistance mechanisms to AMPs from being assembled.

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Abbreviations: WHO: World Health Organization; AMPs: antimicrobial peptides; GAS: Group A *Streptococcus*; GRAB: protein G-related alpha 2M-binding protein; SpeB: streptococcal pyrogenic exotoxin B; Ska: plasminogen activator streptokinase; hBD: human beta-defensin; SLPI: secretory leukocyte protease inhibitor; BRAK/CXCL14:breast-and-kidney-expressed chemokine/CXC chemokine ligand 14; MIG/CXCL9: monokine induced by interferon-gamma; C18G: human platelet factor IV 18; CRAMP: cathelin-related antimicrobial peptide; mCRAMP: mouse cathelin-related antimicrobial peptide; CP10A: Indolicidin derivative peptide; HD-5: human defensin 5; SIC: streptococcal inhibitor of complement; DRS: distantly related to SIC; GBS: Group B *Streptococcus*; PilB: group B *Streptococcus (GBS) pilus* protein subunit; HAS: human albumin serum; OMVs: outer membrane vesicles; ETEC: enterotoxigenic *Escherichia coli*; LPS: lipopolysaccharides; Bap 1: biofilm-associated extracellular matrix protein; ABC: adenosine triphosphate (ATP)-binding cassette; MFS: the major facilitator superfamily; SMR: the small multidrug resistance superfamily; RND: the resistance-nodulation-cell division superfamily; MATE: the multidrug and toxic compound extrusion superfamily; PSD: peptide sensing and detoxification modules; TCS: two-component system; BceB: bacitracin export permease protein; HNP: human neutrophil peptide; tPMP-1: thrombin-induced platelet microbicidal protein 1; GlcNAc: N-acetylglucosamine; MurNAc: N-acetylmuramic acid; LOS: lipooligosaccharides; pETN: phosphoethanolamine; L-Ara4N: 4-amino-4-deoxy-L-arabinose; *lpxE*: 1´-phosphatase; *lpxF*: 4´-phosphatase; PagP: enzyme palmitoyl transferase; PG: phosphatidylglycerol; MprF: multipeptide resistance factor protein; CPS: capsular polysaccharides; MIC: minimum inhibitory concentration; PGA: poly-gamma-DL-glutamic acid; PIA: exopolysaccharide intercellular adhesin; CT: cholera toxin; LT: labile toxin; cAMP: cyclic adenosine monophosphate; ICER: inducible cAMP early repressor; PI3: peptidase inhibitor 3

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Send correspondence to: Octavio L Franco, Full address Universidade Catolica Dom Bosco, S-Inova Biotech, Campo Grande, MS, Brazil, Tel: 556799854942, E-mail: ocfranco@gmail.com