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Effects of Alterations in *Staphylococcus aureus* Cell Membrane and Cell Wall in Antimicrobial Resistance

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Abstract

Staphylococcus aureus is one of the most successful opportunistic pathogen able to cause serious infections due to its ability to produce virulence factors and acquire antimicrobial resistance. Recent reports indicate that the phenotypic changes in the cell wall and cell membrane are essential mechanisms related to the resistance to several antibacterial drugs (such as daptomycin and vancomycin). These alterations involve changes in cell wall composition and chemical modifications of some components (point mutation leading to modification in phosphatidylglycerol molecule, in the production of the aberrations in peptidoglycan structure and decrease in autolytic activity of the components of the cell envelope), leading to changes in electric charge of the cell surface, cell membrane fluidity and cell morphology. In fact, *S. aureus* develops several multifactorial and strain-specific adaptive mechanisms to survival in host. The study of such mechanisms is very important. The aim of this chapter is to review the phenotypic mechanisms related to drug resistance in *S. aureus*.

Keywords: *Staphylococcus aureus*, daptomycin, vancomycin, heteroresistance, stress stimulon response

1. Introduction

The lipopeptide and glycopeptide antibiotics are very effective against infections caused by Gram-positive cocci, showing good efficacy against *Staphylococcus aureus*. The mechanism of

action of these antibiotics is linked with their ability to block cell wall bacterial synthesis. As examples of the most used drugs to treat *S. aureus* infection, we have daptomycin (DAP) and vancomycin. However, currently, the indiscriminate use of these antimicrobials has decreased their effectiveness against *S. aureus* strains [1].

Daptomycin (DAP) is a cyclic lipopeptide antibiotic obtained from *Streptomyces roseosporus* with activity against the most Gram-positive bacteria, including vancomycin-resistant enterococci (VRE) and methicillin-resistant *S. aureus* (MRSA) [2–4]. DAP appears to have multiple effects on the Gram-positive bacterial cell membrane (BCM) [5]. The mechanism of action proposed is the aggregation between DAP and BMC, which alters the architecture of cell membrane and forms pores, leading to ion loss. This new arrangement leads to a rapid depolarization, resulting in a loss of membrane potential that culminates in bacterial cell death. Regarding the interaction mechanisms with the formation of cell wall and surface, a special mention should be given to the interference in peptidoglycan and lipoteichoic biosynthesis [6–8].

The glycopeptide antibiotics were introduced in therapy against infections caused by *S. aureus* in 1955 [9]. The vancomycin is glycopeptide produced by *Amycolatopsis orientalis* (actinomycete) and is the representative of this class of antibiotic widely used in medical practice today, especially after the appearance of resistant strains of *S. aureus* to methicillin [10]. However, the emergence of molecular mechanisms of resistance to vancomycin and the appearance of phenotypic resistance profiles in heterogeneous subpopulations of *S. aureus* as described susceptible have hindered the use of glycopeptide as first choice agent in antibiotic therapy [11]. Some reduced susceptibility phenotypes are classified in some profiles as vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) [11]. Subpopulations of heterogeneous *S. aureus* with heterogeneous resistance to vancomycin and notoriously sensitive to vancomycin are classified as heteroresistant vancomycin-intermediate *S. aureus* (hVISA) [1].

Heteroresistance is defined as resistance to antibiotics expressed by a subset of a microbial population that initially is considered susceptible to these antibiotics. These resistant subpopulations are able to adapt to increasing drug concentrations in a stepwise manner [12]. This phenomenon has been described in a wide range of bacteria and fungi, but attention has been directed toward *S. aureus* [13]. For the *S. aureus* hVISA strains containing subpopulations of vancomycin-intermediate daughter cells, the minimal inhibitory concentrations (MICs) for the parent strains of these daughter cells fall within the susceptible range of 1–4 µg/mL. However, if the lineage is exposed to increasing concentrations of vancomycin, a new generation of cells with reduced susceptibility profiles will be favored. This exhibition creates a selective pressure that favors the outgrowth of rare vancomycin-resistant clones leading to hVISA clones. Eventually, the continued exposure to vancomycin culminates in a uniform population of VISA phenotypes [14]. Evidence in clinical case studies which monitored *S. aureus* infections in 250 patients showed that the hVISA phenotype is closely related with significantly prolonged bacteremia events and associated with increased rates of endocarditis and osteomyelitis, compared with methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia [15].

VISA and hVISA have an increased D-Ala-D-Ala moieties in their thickness cell wall that form false targets that sequester vancomycin, with reduced autolytic activity and slow growth in

vitro ([14]. This phenomenon is believed to “trap” vancomycin and this, together with the thickened cell wall, may act as a barrier to the diffusion of large molecules, like daptomycin (as cited below) [16, 17]. In this way, the emergence of these bacterial phenotypes cited above has put into question the effectiveness of these antibiotics against *S. aureus* [1, 18, 19]. These changes in susceptibility seem to be related to the difficulty of resolving cases of *S. aureus* infection, leading to increased mortality of patients [20]. Altered host-pathogen interaction due to hVISA strains may change the course of the infection to worst prognostic for the patient. In a macrophage model of infection, hVISA/VISA strains showed increased capsule and reduced protein A (SpA, a protein surface cell), associated with reduced NF- κ B activation and reduced TNF- α and IL-1 β expression [21]. The persistent infections associated with hVISA/VISA strains can be a result of changes in host-pathogen interactions that culminate in attenuated host immune response [14].

2. Mechanisms for daptomycin resistance associated to modifications in cell membrane of the *Staphylococcus aureus*

A few years ago, antibiotic therapy using DAP to treat MRSA infections appeared to be a good choice; however, there are reports in the literature about bacterial strains with resistance to daptomycin (DAP-R) and with intermediary resistance (DAP-I) [19, 22]. *S. aureus* DAP-R infection has been associated with endocarditis and abscesses; it is characterized by high concentration of microorganisms in infected area with oral administration of low doses of DAP (i.e., ≤ 6 mg/kg/day) [23, 24]. In addition, it was observed the emergence of DAP non-susceptible *S. aureus* phenotypes in a patient with persistent VISA bacteremia [1]. The resistant phenotypes of VISA strains have developed an increased DAP resistance during therapy [25, 26]. Besides, it could be associated with the previous use of vancomycin [27]. DAP-I mechanisms in *S. aureus* have not yet been elucidated completely, but a possible predominant phenomenon for the appearance of this phenotype would be an increase repulsion of DAP molecules of the surface cell. This modification is generally associated with an overall net charge change on the cell surface (for a more positive charge) [23].

The lysyl-phosphatidylglycerol (Lys-PG) is an important membrane lipid in bacteria, more common in Gram-positive cells [28]. The Lys-PG is incorporated into the microbial cell membrane by the activity of the multiple peptide resistance factor (MprF) gene product (encoded by the *mprF/fmtC* gene). The MprF is a bifunctional protein composed by C-terminal part responsible by synthesis of the Lys-PG and the N-terminal hydrophobic protein domain is essential for efficient translocation of Lys-PG from the inner to the outer leaflet of the cytoplasmic membrane bacteria [28, 29].

The MprF protein catalyzes the modification of the negatively charged in phosphatidylglycerol (PG) with l-lysine and translocation of Lys-PG from the inside to the outside of the cell membrane leaflet [30]. MprF proteins in *S. aureus* encompass a lysyl-phosphatidylglycerol synthase and a Lys-PG flippase domain, responsible for aiding the movement of phospholipid molecules between the two leaflets in cell membrane (“flip-flop” transition). Moreover, in *S. aureus*

the Lys-PG flippase and synthase in the MprF can understand two different proteins fused with distinct functional subunits [29].

The neutralization of the cell membrane surface leads to MprF triggered by bacterial resistance to cationic antimicrobial peptides (CAMPs) [31]. MprF was first described as a virulence factor of *S. aureus* by Peschel et al. [32], analyzing a mutant strain observed for MprF gene, which was unable to modify phosphatidylglycerol with L-lysine, was considerably faster killed by human neutrophils and exhibited an attenuated virulence in mice. This study indicated a key role of MprF for the resistance to defensins and cathelicidins (secreted peptides in host mucosal) and implied a higher pathogenicity of *S. aureus*. Interestingly, MprF is involved in the development of DAP-R in *S. aureus* [33]. These observations related to cells with DAP-R proliferate take into account several factors. Among these are mechanisms that can interfere with the balance in incorporation of the PG into membrane. Also, it was verified that the co-expression of the alanyl-PG (Ala-PG) synthase with flippase domains of Lys-PG synthesizing MprF proteins led to a wild-type level of daptomycin susceptibility, indicating that Ala-PG can also protect bacterial membrane against DAP [33]. Moreover, the incorporation of the point mutations leading to amino acid exchanges in the MprF proteins of *S. aureus* strains leads to a decreased susceptibility to daptomycin [34].

Several hypotheses have been raised about the interference mechanisms of a mutant gene for the MprF protein in the dynamics of PG synthesis in *S. aureus*. It is considered that the biophysicist repulsion process of DAP molecules from the bacterial surface would be assigned to an increased PG incorporation by $\Delta mprF$ strain with singly point-mutated *mprF* gene (*mprF*_{S295L} or *mprF*_{T345A}) [35]. More recently, it was noted that the decrease in susceptibility to DAP in some mutant strains for the MprF gene was the result of the critical effects caused by PG poor incorporation, resulting in failures in the oligomerization in the cell membrane, which compromise its antibacterial activity [23]. In addition, the possibility of antibiotic repulsion (such as DAP) triggered by the increase in positive charge can be seen in some *S. aureus* strains with a significant increase in cell wall thickness. These strains exhibit increased MICs for drug antibiotic and VISA-DAP-R phenotype [1].

3. Mechanisms for vancomycin resistance associated to modifications in cell envelope of the *Staphylococcus aureus*

The resistance mechanisms for both VISA and hVISA appear to have common features. However, it is always observed in patterns that distinguish them from vancomycin-susceptible *Staphylococcus aureus* (VSSA). Although the gene expression of hVISA or VISA during exposure to vancomycin denotes some patterns, subtle differences in the contribution of transcripts of these two phenotypes are found. One basic evidence regarding decrease in susceptibility is related to the cell wall thickening in bacterial cells. This process seems to initiate with the acquisition of a reduced autolysis caused by downregulation of autolysin genes, such as *atl/lytM* [1]. A thickened cell wall has been highlighted as a characteristic phenotype commonly found in clinical VISA. This feature is intimately associated with peptidoglycan-clogging theory that explains vancomycin resistance by passage delay of antibiotic molecules across the thickened

peptidoglycan layers [18]. Punctual mutations by genetic manipulation in *S. aureus* strains that could lead to cell wall thickening were investigated thoroughly. However, these studies did not conclude that active point mutations involving genes of regulatory systems, autolysins and major catalytic enzymes could be involved in the appearance of VISA phenotypes [36, 37].

Studies conducted on gene transcription analysis showed that after exposure of DAP, the cells exhibit a regulation of the cell wall “stimulon,” similar to that observed in response to vancomycin [1, 38]. Comparison of DAP-I and DAP-S. *aureus* strain pairs, which are located in the genes that have been associated with VISA phenotype, indicated that the genes involved in the synthesis and/or homeostasis of the cell wall play an important role in the resistance to vancomycin observed to DAP strains [39].

Signal transduction mediated by two-component systems (TCSs) *YycFG* or sensor histidine kinase *WalK* (also called *WalRK*) is a regulatory system of two essential components of cell wall synthesis and homeostasis, which has also been implicated in DAP-R and VISA/VSSA cell phenotypes [1, 40]. Members of the two-component regulatory system *WalK/WalR* that regulate genes are involved in autolysis, biofilm formation and cell wall metabolism [1]. *WalK* functions as a sensor protein kinase, which is autophosphorylated at a histidine residue and transfers its phosphate group to *walk* [40]. The impacts of the single substitutions in either *WalR* or *WalK*, dramatically change the bacterial cell physiology, with significant reductions in autolytic activity and increases in cell wall thickness linked to the insertion of *WalR* or *WalK* alleles from the VISA strain into vancomycin-susceptible *S. aureus* (VSSA).

The impacts of the single substitutions in either *WalR* or *WalK* dramatic changes the cell bacterial physiology, with significant reductions in autolytic activity and increases in cell wall thickness linked to the introduction of the *walR* or *walk* allele from the VISA strain into the room fully vancomycin-susceptible *S. aureus* (VSSA). Vancomycin-intermediate *S. aureus* (VISA) is generated from vancomycin *S. aureus* sensible by multiple spontaneous mutations in two-component regulatory systems as standing for vancomycin resistance-associated sensor/regulator (*VraSR*) and inducible by cationic antimicrobial peptides (CAMPs), which could be important for bacterial cells in resisting the harmful effects of CAMPs and possibly other antimicrobial agents [41].

The assessment of the effects of mutations directed at specific targets, which are responsible for the metabolism of the cell wall in *S. aureus*, indicated that the synergistic mutations (double mutation) might have a more significant effect on the appearance of the VISA phenotype. Recently, it was observed that a deletion mutation in genes of the two-component regulatory system *walRK* (synonyms: *vicRK* and *yycFG*) might result in an increased resistance to vancomycin and appearance of VISA phenotype from the *S. aureus* LR5P1-V3 strain. On the other hand, LR5P1 strain with double mutation (*walk*clpP**) exhibited a thickened cell wall, slow growth and decreased autolytic activity [37]. Similarly, Hu et al. [42], also studying the participation of point mutation in *WalK* (G223D) gene in *S. aureus* MW2 (community-acquired methicillin-resistant), reported a decreased expression of genes associated with the cell wall metabolism, decreased autolytic activity and a reduced vancomycin susceptibility. In addition, the electrophoretic mobility shift assay (EMSA) indicated that *WalK* (G223D)-phosphorylated *WalR* had a reduced capacity to bind the *atIA* promoter. The *atIA* promoter is one of the component potential members of the *WalK/WalR* regulon and is involved in cell wall metabolism in *S. aureus*.

The ATP-dependent Clp protease (ClpP) consists of two heptameric rings and belongs to the family of serine peptidases [43]. ClpP plays an essential role in the degradation of pathogen cell wall but also in the regulation of their virulence [44]. The ClpP components in *S. aureus* are responsible for initiating physiological adaptive responses against different external pressures, including extreme changes in temperature, osmolarity and oxidative stress [45]. In addition, the ClpP protease presented chaperone-like functions, recognizing and refolding misfolded proteins accumulated or aggregated proteins in the cell, consequently participates in the cell envelope turnover in microbial cells [46, 47].

In various pathogenic microorganisms, such as *Listeria monocytogenes*, *Streptococcus pneumoniae* and *S. aureus*, ClpP proteases have been identified in in vivo expression studies as an important virulence factor [44, 48, 49]. Knockout studies of the *clpP* ATP-dependent gene in *S. aureus*, followed by global transcriptional analysis using DNA microarray technology, showed that the loss of *clpP* leads to complete derepression of transcription of the *CtsR* gene (or *yacG*, a polypeptide transcriptional regulator). The ClpP proteins are regulated by the *CtsR* heat-shock repressor controlled by HRCA (heat-inducible chaperone, a heat-shock regulon) and a partial derepression of genes involved in response to oxidative stress and DNA repair system SOS response pathway. Moreover, the expression of genes whose products are involved in autolysis of microbial cells was unregulated. This culminated in a dramatic increase in autolysis processes in *S. aureus* $\Delta clpP$ mutant [46]. In addition, other observations on the effect of the mutation identified in *clpP* system in *S. aureus* LR5P1-V3 strain indicated that deregulation is a new mechanism, which can lead to resistance to vancomycin. It was observed that the LR5P1-V3 mutant, derived from N315LR5P1, exhibited increased resistance to vancomycin, as MIC values rose from 1 to 8 $\mu\text{g/mL}$ [37].

Recently, a genetic evaluation of vancomycin-intermediate *S. aureus* (VISA) isolated from the same patient at different times tightly corroborates an association of this phenotype with evidence mutations in the novel class of genes, encoding LPXTG motif-containing cell wall-anchoring proteins. These proteins contain an LPXTG sequence motif at their C-terminus, which is a cleavage signal that leads to the covalent binding of the proteins to the cell wall [50]. The presence of covalently bound LPXTG proteins has been shown to contribute to the regular cross-linked structure of the cell wall and anchoring of surface proteins to the peptidoglycan layer [51]. It is hypothesized that the origin of the VISA phenotype may be also related to mutations in the genes encoding LPXTG-associated proteins, leading to an aberrant peptidoglycan structure [50]. This new architecture would provide false binding sites for vancomycin, thus reducing the permeation of this antibiotic in to the cell wall [50]. Mutations evidenced in the anchor domain LPXTG family proteins indicate that the phenotype VISA can have a reduced susceptibility to vancomycin in vivo resulting from spontaneous or induced mutations of little-known character [52].

4. Mechanisms resistant to β -lactam antibiotics associated to penicillin-binding protein in *Staphylococcus aureus*

With regard to β -lactam antibiotics, one mechanism-conditioned resistance to these antibiotics, mainly to methicillin and others related drugs, is the expression of penicillin-binding protein

modified (PBP2s). The PBP2 is a bifunctional protein produced by *S. aureus* and has binding specificity to β -lactam antibiotics. This protein presented a functional transpeptidase domain and carboxypeptidase domain; it is directly linked to the cell wall metabolism in bacteria [53]. In *S. aureus*, both PBPs and (PBP2) are membrane-associated proteins that catalyze the final step of peptidoglycan biosynthesis. The transpeptidase and carboxypeptidase activities of PBPs occur at the D-Ala-D-Ala terminus of a peptidoglycan precursor containing N-acetylglucosamine and N-acetyl-muramic acid L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (pentapeptide chain) [53].

The β -lactam antibiotics inhibit PBPs by competing with the precursor [undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)] for binding to the active site of the enzyme. PBP2s enzymes are associated with decreased bacterial susceptibility to oral cephalosporins and recently, they have been implicated in the emergence of the “MRSA superbugs” [54]. One of the most worrisome evidence on MRSA is related to high mortality rates among infected patients. It is estimated that for MRSA patients, the mortality rate is two to three times higher than for patients infected with *S. aureus* susceptible strains [55].

In methicillin-resistant *S. aureus* strains (MRSA), the enzymatic function can be replaced by PBP2 and PBP2s, proteins encoded by the *mecA* gene, which act as substitutes for the transpeptidase [56]. The *mecA* gene is present on the staphylococcal cassette chromosome (SCC *mec*), a genomic island in staphylococci and other Gram-positive bacteria that encode methicillin resistance. Its mobile nature allows it to become widespread among microorganisms [57]. Generally, in susceptible *S. aureus* cells, the vancomycin molecules bind on the C-terminal D-Ala-D-Ala, thereby inhibiting the catalytic reactions of the transglycosylation and transpeptidation, mediated by PBPs. Moreover, PBP2 also plays an important role in the resistance in MRSA; it is related to expression of high-level resistance to vancomycin [58]. This resistance profile is an associated expression of *VanA* operon, one complex genetic in *S. aureus* and *Enterococcus* species designated as vancomycin-resistant enterococci (VRE) [59]. The *vanA* operon was acquired by VRSA from serving of the transposon *Tn1546* residing on a conjugal plasmid; possibly, its origin is related to a transfer of the genetic element by association with *Enterococcus faecalis* in the same niche located in the host [60].

5. The cell-wall-stress stimulon response in *Staphylococcus aureus*

Some antibiotics related to cell wall biogenesis and its stability can influence gene expression and produce a cell-wall-stress stimulon (CWSS) in the *S. aureus* cells. The oxacillin antibiotics, widely used until recently against *S. aureus* infections, appear to interfere in transcription of genes required for the synthesis of peptidoglycan in the bacterial cells. Analysis of the dynamics of gene expression and proteomic studies using GeneChips™ approach hypothesized on an upregulation for some proteins and transcription factors involved in cell wall metabolism and response to stress. The same experimental approach that indicated the main influence of amoxicillin during cell exposure is related to increased expression of genes encoding involved in cell wall metabolism, including *pbpB*, *Vras* and *Murz* genes. This pattern of transcriptional response can represent the signature of a stimulon cell wall induced in response to antibiotics, which interfere with the synthesis and structure of the cell wall [61].

Recently, by using a reporter gene system based on a highly sensitive luciferase fused with *tcaA* and *sa0908* genes, it was demonstrated that gene expression in *S. aureus* occurs in different kinetic patterns [62]. The *tcaA* and *sa0908* genes relate to encoding a membrane protein related to signature resistance to glycopeptide antibiotics and encoding protein for the cell envelope (member of the LytR-CpsA-Psr family), respectively. These patterns were determined from observations of the fluorescence intensity during growth of *S. aureus* (susceptible strain BB255) in the presence of various antibiotics at subinhibitory and inhibitory concentrations. From their results, it was possible to establish that all drugs tested induced the CWSS and that the induction patterns varied according to the drug. One of the hypotheses raised in this study would be that the standard kinetics observed could perhaps be related to the specificity or antibiotic mechanism action. These findings are based on fluorescence results obtained for different drug groups such as tunicamycin, flavomycin, oxacillin and fosfomicin, which presented high levels of maximal induction (RLU > 40,000); and daptomycin and lysostaphin exhibiting fluorescence indices below 10,000. Moreover, some antibiotics such as fosfomicin and D-cycloserine showed a lag-phase induction for all tested concentrations of about 30 and 10 min, respectively. Possibly, these antibiotics act in the early stages of peptidoglycan synthesis, which could be linked to delays in the induction CWSS in the *S. aureus*.

6. Conclusion

Multiple mechanisms contribute to the increased antimicrobial resistance in *S. aureus*, culminating in a more robust cellular adaptation against antibiotics that act on the synthesis of components of the cell envelope. These multiple mechanisms of adaptation in *S. aureus* include heteroresistance or mutational events, make the bacteria to evolve from a susceptible strain in to a resistant clone; thus significantly interfering with the bacterial response to antibiotics. Finally, further knowledge of these mechanisms may considerably impact the development of new drugs designed to specific targets in the microbial cell.

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