Review

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Mechanisms of resistance to daptomycin in *Staphylococcus aureus*

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ABSTRACT

Daptomycin is a cyclic lipopeptide active against multidrug-resistant Gram-positives, including methicillin-resistant *Staphylococcus aureus* (MRSA) and *S. aureus* with reduced susceptibility to vancomycin. It is 4-8 fold as active as vancomycin against methicillin-susceptible *S. aureus* (MSSA) and MRSA, and retains most of this activity against *S. aureus* with reduced susceptibility to vancomycin. The mechanism of action of daptomycin is not fully understood. Daptomycin binds to the bacterial cytoplasmic membrane, leading to depolarization due to the loss of potassium ions from the cytoplasm. Daptomycin non-susceptibility is unusual in the clinical setting. Different mechanisms have been proposed to explain daptomycin-resistance, most of them associated to changes in composition, charge and fluidity of the cell wall. The *mprF* mutations, which lead to an increase in the lysyl-phosphatidyl glycerol production, and *rpoB* and *rpoC* mutations (*rpo* genes encode for bacterial RNA polymerase subunits) have been proposed as associated to daptomycin-resistance, but a number of mutations in other genes (*walK, cls, ggrA*...) have been proposed.

Keywords: Daptomycin, resistance mechanisms.

Mecanismos de resistencia a daptomicina en *Staphylococcus aureus*

RESUMEN

Daptomicina es un lipopéptido cíclico, activo frente a microorganismos grampositivos multirresistentes, incluyendo *Staphylococcus aureus* resistente a meticilina (SARM) y *S. aureus* con sensibilidad reducida a vancomicina. Es 4-8 veces más activa que vancomicina frente a *S. aureus* sensible a meticilina (SASM) y SARM, y mantiene prácticamente la misma actividad frente a *S. aureus* con sensibilidad reducida a vancomicina. El mecanismo de acción de daptomicina no está completamente explicado. Daptomicina se une a la membrana citoplasmática bacteriana y da lugar a su despolarización, como consecuencia de la pérdida de iones potasio. La resistencia a daptomicina es todavía infrecuente en el ámbito clínico. Se han propuesto diversos mecanismos de resistencia, en su mayor parte asociados a cambios en la composición, carga y fluidez de la membrana celular. Se ha propuesto la asociación de la resistencia a daptomicina con mutaciones en los genes *mprF*, que dan lugar a un aumento en la producción de lisil-fosfatidil-glicerol, a mutaciones en *rpoB* y *rpoC* (los genes *rpo* codifican diferentes subunidades de la ARN polimerasa bacteriana) pero también a mutaciones en otro numeroso grupo de genes (*walK, cls, ggrA, etc.)*.

Palabras clave: Daptomicina, mecanismos de resistencia.

INTRODUCTION

Staphylococcus aureus is one of the main human bacterial pathogens. It shows a high pathogenic capacity, associated to a wide enzymes and toxins production capacity. Moreover, *S. aureus* has shown a high capacity for acquiring and accumulating mechanisms of resistance to antibiotics.

Methicillin-resistant *S. aureus* (MRSA) are resistant to all β-lactam antibiotics, excepting some recently released cephalosporins, and have become a main epidemiological problem worldwide. They are associated to longer hospital stays, longer antibiotic regimens, higher costs and greater mortality, compared to methicillin-susceptible *S. aureus* (MSSA). For years, the main alternative against MRSA were glycopeptide antibiotics (vancomycin, teicoplanin), especially in severe infections[1].

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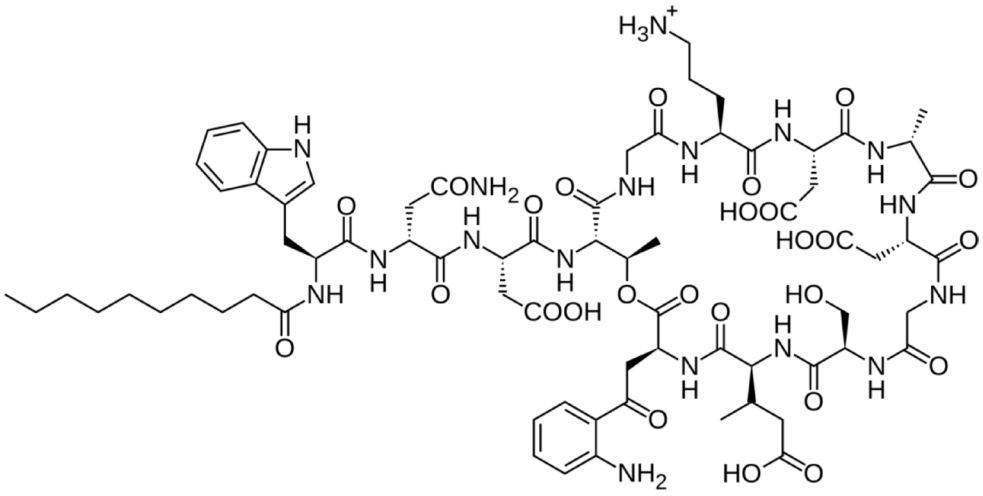


Figure 1 | **Chemical structure of daptomycin.**

The emergence of glycopeptide-intermediate *S. aureus* (GISA) in 1997[2], and then glycopeptide-resistant *S. aureus*[3], and the increasing antibiotic resistance in other Gram positive pathogens such as coagulase-negative staphylococci and enterococci, boosted the development of newer antibiotics active against multidrug-resistant (MDR) Gram positive pathogens, such as oxazolidinones, newer glycopeptides and lipopeptides.

DAPTOMYCIN: CHARACTERISTICS AND MECHANISM OF ACTION

Daptomycin is a cyclic lipopeptide produced by *Streptomyces roseosporus*. It consists of a 13-amino-acid depsipeptide, harboring a cyclic decapeptide core with three extra-cyclic amino acids attached to an aminoterminal fatty acid tail (figure 1), and is active against MDR Gram positives, including MRSA and *S. aureus* with reduced susceptibility to vancomycin[4]. Daptomycin has been shown 4 to 8-fold as active as vancomycin, and 30-fold as active as linezolid against MSSA and MRSA[5]. MICs of daptomycin against GISA are similar or slightly higher than MICs observed against MSSA and MRSA[5]. Some studies have shown that, in bacteremia caused by *S. aureus* with MIC of vancomycin >1mg/L, the early administration of daptomycin leads to a significant better clinical outcome[6,7], though other authors have not found significant differences[8].

Daptomycin was the first lipopeptide antibiotic approved by the FDA, being available since 2003 for soft-tissue infections and from 2006 for *S. aureus* bacteremia and right-sided endocarditis.

The mechanism of action of daptomycin can be considered as unique, is currently not fully understood, and is probably much more complex and multifactorial than other antimicrobials. Daptomycin binds to the bacterial cytoplasmic membrane in the presence of physiological concentrations of calcium ions (50 µg/ml), both in the growing and stationary phase, leading to depolarization due to the loss of potassium ions from the cytoplasm. This process leads to the interruption of multiple factors of the bacterial cell membrane without penetrating the cytoplasm. The alteration of cellular homeostasis leads to inhibition of bacterial vital processes, and thus to cell death[9-12].

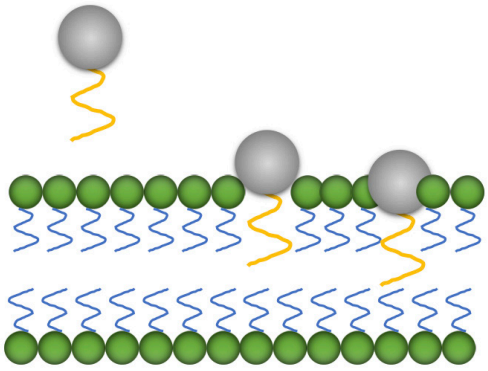
Therapeutic failures have been described with daptomycin when administered at low concentrations[6,13].

EMERGENCE OF DAPTOMYCIN RESISTANCE IN *S. AUREUS*

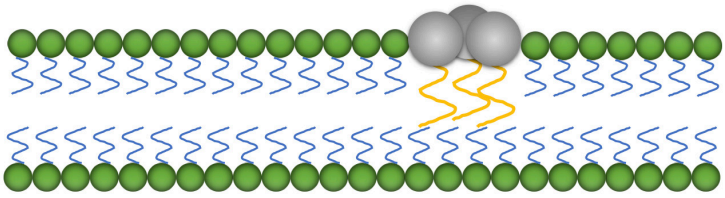
The term non-susceptibility is preferred to resistance concerning daptomycin, since a MIC that determines resistance has not yet been established. The CLSI guidelines (2016)[14] consider susceptible those microorganisms with daptomycin MIC <1 mg/L, and non-susceptible those microorganisms with daptomycin MIC $\geq$ 1 mg/L.

Daptomycin non-susceptible *S. aureus* clinical isolates have been obtained both from patients treated with daptomycin, from patients who received other antibiotics and even from untreated patients[13,15,16]. Nevertheless, daptomycin non-susceptibility is unusual in the clinical setting. Prior exposure to other drugs such as vancomycin does not seem to affect the clinical efficacy of daptomycin[17]. Different mechanisms have been proposed to explain the non-susceptibility to daptomycin[18]:

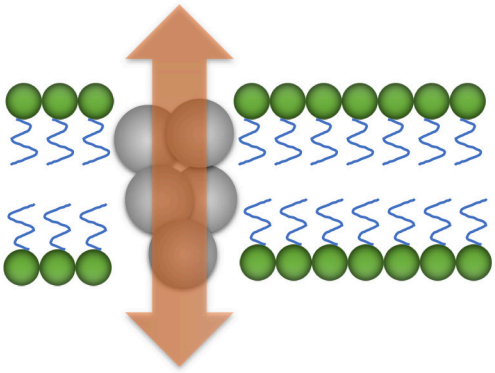
- Increase in the bacterial membrane positive surface charge, due to the increase of phospholipids in its outer layer.
- Alteration in the bacterial membrane fluidity due to changes in the fatty acids composition.
- Increased carotenoid pigment content.



2-1. Daptomycin access the cell wall



2-2. Daptomycin disrupts the cell wall



3.3. Ion channel formation

Figure 2 Mechanism of action of daptomycin

- Increased teicoic acid synthesis in the cell wall.

Obviously, combinations of several of these factors are also possible.

Song et al.[19] developed a genomic, transcriptomic, ultrastructural and wall autolysis study on two daptomycin non-susceptible *S. aureus* isolates obtained *in vitro* (table 1). The emergence of daptomycin non-susceptible mutants was very uncommon ($<1x10^{-10}$). Nevertheless, the emergence of daptomycin non-susceptible mutants seems to be much more frequent when they are selected by using passages in increasing concentrations of daptomycin[20]. Daptomycin non-susceptibilty seems to be linked to multiple mutations in a number of genes[20,21]. Moreover, the kinetics of emergence of mutations associated to daptomycin non-susceptibility seems to be different in clinical isolates and in *in vitro* selected isolates (table 1). Friedman et al.[22] compared non-susceptible clinical isolates obtained after treatments with daptomycin (MIC 4 mg/L), their parent susceptible isolates (MIC 0,2-0,5 mg/L), and daptomycin non-susceptible mutants obtained *in vitro* from *S. aureus* MW2, showing that mutations profiles can be very heterogeneous among mutants obtained *in vitro*, among mutants obtained *in vivo* and between both groups (table 1).

Nevertheless, despite this heterogeneity, in most cases, the genes affected are genes involved in cell membrane homeostasis[19,23]. A study published by Jones et al.[13] shows that daptomycin non-susceptible isolates have a more fluid cytoplasmic membrane, with increased lysyl-phosphatidyl glycerol translocation towards the outer face of the cytoplasmic membrane, and a greater transmembrane potential, as compared to daptomycin-susceptible isolates.

GENES INVOLVED IN DAPTOMYCIN NON-SUSCEPTIBILITY

The *mprF* gene encodes for lysyl-phosphatidyl glycerol synthetase, an enzyme involved in the phospholipid metabolism. This is a protein with two functional domains[21], which transfers positively charged lysine molecules and adds them to phosphatidyl glycerol in the cell membrane[21,24,25]. Mutations in this gene lead to an increase in the lysyl-phosphatidyl glycerol production. The increase of this compound in the outer layer of the membrane results in a lower susceptibility to daptomycin and cathionic antimicrobial peptides[22,26]. Mutations in this region have been associated to daptomycin non-susceptibility both in mutants obtained *in vitro* and

Table 1 **Mutations associated to daptomycin-resistance in *S. aureus*.**

Genes	Origin of daptomycin-resistant isolates				
	*In vitro**	Clinical isolates	*In vitro**	*In vitro**	Clinical isolates
mprF	P314L	S295L		L826F	G61V
	T345I	L826F			S295L
	T345A				S337L
					I420N
					T345I
					L826F
yycG (walK)	S221P	Adenine 26121	L9F		I471T
	R263C	insertion			
rpoB	I953S				L468Q
	A1086V				A477D
rpoC	F632S				
	Q961K				
cls2				T33N	A23V
					L52F
					F60S
pgsA				V59N	
				A64V	
				K65R S177F	
agrA			Y100Stop		Adenine 712
					deletion
prs			A234V		
pnpA			L346P		
Reference	22	22	19	27	27

***Resistant mutants obtained in vitro from type strains.**

in clinical isolates[22]. It is the only gene whose association to decreased daptomycin susceptibility has been demonstrated conclusively by gene deletion and complementation molecular studies. It is accepted that the lower daptomycin susceptibility is associated, in this case, to the increased synthesis of lysyl phosphatidyl glycerol, and an enhanced passage of this molecule to the outer layer of the membrane[26]. This generates an increased electric repulsion force that hinders daptomycin to anchor to the phospholipid bilayer. *mprF* mutations are usually the first to appear, and they emerge relatively early in the selection process[22]. Peleg et al.[27] argued that mutations at the amino-terminal end would affect the transmembrane domains of the protein, while changes at the carboxy terminal end of *mprF* would result in an increase in protein lisinylation and in its translocation towards the outer layer of the membrane, so leading to an increase in the outer layer positive charge which would increase the electrical repulsion against the daptomycin molecule. Cameron et al.[23], observed that the suppression of the *mprF* gene in *S. aureus* leads to a decrease in lysyl-phosphatidyl glycerol translocation to the outer layer, and causes a reduction of the positive charge and a decrease in daptomycin MIC up to 4-fold. Nevertheless, though *mprF* mutations are the most frequent changes in daptomycin non-susceptible isolates, reduced susceptibility to daptomycin can also emerge in absence of *mprF* mutations[28].

The *yycG* gene, also known as *walK*, encodes for the synthesis of a histidine kinase sensor, and belongs to the *yycF/yycG* regulator. This regulator modulates the synthesis of a number of Gram positive proteins, including proteins involved in wall metabolism and permeability[18,22,29,30], as well as some virulence factors in *S. aureus, B. subtilis* and *S. pneumoniae*[19,32]. The emergence of mutations in this gene has been associated to resistance to other antibiotics, such as vancomycin[16,30], and both alone and combined with mutations in *mprF*, have been also associated to daptomycin non-susceptibility in *S. aureus*[16].

The *rpoB* and *rpoC* genes encode for bacterial RNA polymerase β and β' subunits[18,22]. Mutations described in these two genes associated to daptomycin non-susceptibility are different from those affecting to other antibiotics, such as rifampicin. Unlike *mprF* mutations, which usually emerge at relatively early times during the selection process, *rpoB* and *rpoC* mutations appear later[22]. Some authors[24,31] have shown that a single point mutation in the *rpoB* gene, such as A477D or A621E, can reduce the susceptibility both to daptomycin and vancomycin. Such mutations have been shown to cause cell wall thickening and reduction of the negative charge of the outer layer in *S. aureus*[31].

Point mutations in *cls2* have also been associated to daptomycin non-susceptibility. This gene encodes for a cardiolipin synthase[23]. Cardiolipin synthases in *S. aureus* are involved in the synthesis of cardiolipin from phosphatidyl-glycerol in some metabolic situations, such as the evolution from logarithmic to stationary growth phase[32]. Hypothetically, mutations in *cls2* might impair this metabolic path, leading to accumulation of phosphatidyl glycerol in the wall.

The *agrA* locus[19] encodes a quorum sensing system in *S. aureus* which controls the expression of several genes, including virulence genes, through a two-component system[33,34]. AgrA is a response regulator that upregulates its own promoter, P2, in the *agr* locus, and the RNAIII promoter. RNAIII is the effector of the *agr* system. Hundreds of genes have been identified to be upregulated by RNAIII[35]. Otherwise, *agr* mutants show decreased autolysis[36,37].

The *pgsA* gene encodes for a phosphatidyl transferase involved in the synthesis of phosphatidyl glycerol[23], the most important anionic phospholipid in the cytoplasmic membrane. Mutations in this gene have been associated to daptomycin insensitivity in *B. subtilis*[38], but still not in *S. aureus*. *pgsA* mutations associated to daptomycin non-susceptibility have only been obtained *in vitro*, thus their trascendence in clinical isolates remains to be elucidated.

The *pnpA* gene encodes for a polynucleotide phosphorylase. Among other functions, this enzyme is required for the expression of certain virulence genes[39].

The products of the *dltABCD* operon are involved in the cell wall teicoic acid D-alanination in many Gram-positive bacteria[21]. Mutations in this operon would lead to a cell membrane positive charge increase, as happens with *mprF* mutations.

The suppression of *clpX* gene has been shown[24] to produce a small reduction in susceptibility to daptomycin and its inactivation decreases the virulence of *S. aureus*. The ClpX chaperone associates with ClpP peptidase for protein degradation and facilitates protein folding and interaction.

In whole, daptomycin resistance is still infrequent in the clinical settings, but is feasible especially when the microorganisms undergo steadily increasing antibiotic concentrations, and can emerge both from methicillin-resistant and methicillin-susceptible staphylococci, though high-level resistant mutants seem to emerge more easily from MRSA (unpublished data). Daptomycin resistance is a complex process involving a wide number of genes and functions, though the most important mechanisms of resistance seem to be associated to the wall charge, metabolism and permeability.

Further studies are necessary to know the frequency and the real transcendence of each mutation *in vitro* and *in vivo*, and how these mutations, impact in the phenotype and in the biology of the microorganism.

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