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dltA overexpression: A strain-independent keystone of daptomycin resistance in methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

The mechanisms leading to reduced susceptibility to daptomycin (DAP) are multifactorial and have not been fully elucidated. We analysed, by sequencing and expression studies, the role of the major molecular targets (cell-envelope charge genes, *dltA*, *mprF*, *cls2*; cell-wall turnover and autolysis genes, *sceD*, *atl*) involved in the emergence of DAP resistance in three series of isogenic clinical methicillin-resistant *Staphylococcus aureus* (MRSA) in which DAP resistance emerged after a heterogeneous glycopeptide-intermediate *S. aureus* (hGISA) step under teicoplanin and DAP therapy. All of the isolates had different genotypes and were δ-haemolysin negative, reflecting a strain proclivity to acquire DAP/glycopeptide non-susceptibility under antibiotic pressure. DAP exposure led to the emergence of DAP resistance after an hGISA step probably in parallel with the timing of the two antimicrobial administrations and, in two of three cases, in conditions of DAP underdosage. Real-time qPCR data revealed that all DAP-resistant (DAP-R) isolates had *dltA* overexpression, whereas *mprF* upregulation was found only in DAP-R strains with the S295L and T345I amino acid substitutions. Strains that were heteroresistant to DAP did not possess DAP-R-like characteristics. DAP-R strains presented high *cls2* expression and no known *cls2* mutations, and moreover exhibited *sceD* and *atl* upregulation. In conclusion, these findings highlight that *dltA* overexpression is the common pathway of resistance among genotypically different series of isolates and may represent the keystone of DAP resistance in MRSA, leading to electrostatic repulsion and, indirectly, to a reduction of autolysin activity. *mprF* mutations related to increased transcription may play a role in this complex phenomenon.

1. Introduction

Daptomycin (DAP) has been increasingly used in the treatment of various types of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) [1]. However, since 2005 cases of DAP-resistant (DAP-R) isolates have been described in the literature [2,3]. Although the incidence remains low, non-susceptibility to DAP appears to be the result of single or combined strain-related and/or infection-related events. Strain-related causes of DAP resistance[1] are thought to be due to accumulation of multiple mutations in different targets, affecting different cell wall and membrane pathways [4], whilst infection-related causes can be due to: (i) potential exposure of the micro-organisms to subinhibitory concentrations of the drug owing to large variations in serum peak and trough levels at the currently recommended doses [5]; (ii) prior exposure to other antimicrobial agents, particularly vancomycin (VAN) [6,7]; (iii) a high bacterial inoculum and prolonged antibiotic exposure and/or biofilm-related infections, such as infectious endocarditis or bone infections [8]; and (iv) exposure to host-derived cationic peptides [9].

The emergence of *S. aureus* with diminished DAP susceptibility during glycopeptide [VAN or teicoplanin (TEC)] therapy represents a challenge for the medical community, as VAN treatment may account for loss of DAP susceptibility [2,7].

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) [10] and the Clinical and Laboratory Standards Institute (CLSI) [11] define an *S. aureus* strain as DAP-R or DAP non-susceptible at a minimum inhibitory concentration (MIC) cut-off value of >1 mg/L. Strains with a heterogeneous phenotype, i.e. strains with subpopulations growing at antibiotic concentrations above the MIC, have also been described [12].

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[1] In this study, although the official terminology is 'daptomycin nonsusceptibility', the term 'daptomycin resistance' was used for ease of understanding.

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The phenotypic and molecular features of DAP-R *S. aureus*, commonly related to glycopeptide reduced susceptibility, can be grouped into: (i) changes in cell-wall arrangement and turnover [13]; (ii) changes in membrane composition, structure and membrane potential [14]; and (iii) modifications in sensitivity to autolysis and permeabilisation [14]. Several genetic loci have been implicated in *S. aureus* non-susceptibility to DAP and glycopeptides, such as *dltABCD* (alanylation of wall teichoic acids), *mprF* (lysinylation of phosphatidylglycerol), *sceD*, *atl* and *lytM* (autolysins), two-component regulatory systems such as WalRK and GraRS, and the *agr* (accessory gene regulator) locus [15,16]. In particular, *mprF* encodes a bifunctional membrane protein mediating both the lysinylation of phosphatidylglycerol and its translocation to the outer leaflet of the membrane. The *dltABCD* operon controls the alanylation of wall teichoic acids in response to antimicrobial challenge; its pathway is linked to cationic antimicrobial peptide resistance in *S. aureus*, and the positive charge of D-alanine residues repels positively charged molecules such as defensins [17,18]. A greater net positive surface charge, as also mediated by *mprF*, would theoretically reduce the overall access of calcium-decorated DAP to its membrane target [17]. *dltABCD* also indirectly regulates the activity of the autolytic system, since a decrease of the cell-wall positive charge accelerates autolysin activity [18].

The cardiolipin synthase 2 gene *cls2*—responsible for the synthesis of cardiolipin, a negatively charged phospholipid that makes up 30% of the *S. aureus* cell membrane [19]—also plays a role in osmotic stability as a proton reservoir.

The *S. aureus* major autolysis gene *atl* and the lytic transglycosylase *sceD* (SAV 2095 *sceD*-like gene) play a key role in controlling cell-wall expansion, remodelling and daughter cell separation, but primarily participate in peptidoglycan turnover [17].

GraRS, a two-component regulatory system, controls the expression of several genes, including some involved in cell-wall synthesis or global regulation [17].

Several studies have investigated changes in the presence of mutations and expression of the above genetic loci in clinical and/or laboratory-derived DAP-R strains, but which molecular mechanism is a priority in the reduced susceptibility is still not well understood [14,16,20].

To address all of the above mentioned issues on the possible mechanism of resistance and their interconnections, a sample of three series of MRSA clinical isogenic isolates obtained after antimicrobial exposure to TEC and DAP were included in this study. Different approaches, i.e. sequencing to detect single nucleotide polymorphisms (SNPs) in the target genes and real-time quantitative PCR (qPCR) to analyse their expression levels, were used.

This work aims to demonstrate that *dltA* overexpression represents a strain-independent keystone of DAP resistance (i.e. common to all DAP-R strains). This *dltA* overexpression was found in more than one set of DAP-R MRSA clinical isolates (in all our three sets) from different patients, in diverse geographical areas and with different genomic backgrounds. This trait, as previously reported [14,15], confers an increase in D-alanylation of teichoic acids responsible for a drug electrostatic repulsion mechanism and a reduction in autolysis. Furthermore, this study showed a connection between *mprF* overexpression and the presence of specific point mutations in the same gene. All these hallmarks may co-operate to affect both DAP and/or glycopeptide reduced susceptibility.

2. Materials and methods

2.1. Strains and molecular characterisation

The three series of DAP-susceptible (DAP-S) and DAP heteroresistant (hDAP)/DAP-R MRSA, isolated from different patients, were epidemiologically unrelated clinical isolates from skin and soft tissue (sets 1 and 3) and bloodstream infections (set 2), collected from three different Italian hospitals (set 1 from 'Ospedale Civico di Cristina Benfratelli Palermo', set 2 from 'Santa Maria della Misericordia di Udine' and set 3 from 'Ospedali Riuniti di Bergamo'). Each series included an initial pre-DAP therapy strain (1A, 2A and 3A) and its isogenic isolates after development of DAP resistance during DAP administration (1B, 1C; 2B, 2C, 2D; and 3B, 3C). To verify the isogenicity of strains within each series, isolates were evaluated by the following genotypic assays: pulsed-field gel electrophoresis (PFGE); *agr* typing; multilocus sequence typing (MLST); and staphylococcal cassette chromosome *mec* (SCC*mec*) typing [21].

2.2. Minimum inhibitory concentrations, macro Etest (MET) and population analysis profile–area under the curve (PAP–AUC) analysis

MIC and MET determination for glycopeptides and DAP were conducted according to CLSI guidelines [11]. The MET procedure was performed following a protocol previously published and then evaluated for growth following the manufacturer's instructions (EAS003; AB BIODISK, Solna, Sweden) [22]. Mu3 [heterogeneous VAN-intermediate *S. aureus* (hVISA)], Mu50 [VAN-intermediate *S. aureus* (VISA)] and ATCC 29213 [VAN-susceptible *S. aureus* (VSSA)] were used as control strains. DAP categorisation was defined according to the EUCAST guidelines [10].

The PAP/AUC procedure was performed as previously described [23]. Colonies were counted and the log CFU/mL was plotted against the VAN concentration using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). The ratio of AUC of the tested isolates to the AUC of *S. aureus* Mu3 was calculated and was then interpreted as previously described [23]. Mu3 (hVISA), Mu50 (VISA) and ATCC 29213 (VSSA) were used as control strains. In this study, strains were categorised as heterogeneous glycopeptide-intermediate *S. aureus* (hGISA) (i.e. heteroresistant to both VAN and TEC), heterogeneous TEC-intermediate *S. aureus* (hTISA) if the strain exhibited a PAP of heteroresistance versus TEC alone, hDAP (heteroresistant to DAP), quasi VISA (qVISA) if the strain showed a PAP of VAN and TEC as Mu50 but with a VAN MIC of <8 mg/L, and DAP-R if the strains had a DAP of MIC $\geq$1 mg/L.

2.3. Screening of δ-haemolysin activity on 5% sheep blood agar plates

agr operon functionality was measured by δ-haemolysin production testing the strain by cross-streaking perpendicularly to *S. aureus* RN4220 as previously described [24].

2.4. RNA extraction, retrotranscription and real-time quantitative PCR

An aliquot of an overnight culture was diluted 1:50 and bacterial cells were grown in brain–heart infusion to exponential phase (optical density at 600 nm = 0.4 at 3 h). RNA was then extracted, treated and quantified [16]. mRNA of the studied target genes, i.e. *dltA*, *mprF*, *cls2*, *atl* and *sceD*, was retrotranscribed as previously described [16].

Real-time qPCR was conducted as previously published [16]. Primers for quantification were selected to amplify a fragment of <300 bp. *gyrB* was used as a normaliser (internal control) as previously published. *dltA*, *mprF*, *atl* and *sceD* real-time primers were as previously published [16], whereas the *cls2* primers, amplifying a fragment of 225 bp, were CV_{158} 5′ATTAGAGTTAATCGTTGATGAGCAAT3′ and CV_{159} 5′TTACGGATGTCTTGTATTAGGTCAT3′. Expression of the studied genes is represented as the increment/decrement (fold changes)