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.

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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.

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 ≥ 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)

Table 1
Phenotypic characteristics of the strains included in the study.

Strain	OXA susceptibility	OXA MIC (mg/L)	OXA MET (mg/L)	MIC (mg/L)			MET (mg/L)		PAP/AUC	
				VAN	TEC	DAP	VAN	TEC	VAN	DAP
1A	R	32	256	1	≤0.25	≤0.25	4	4	VSSA	DAP-S
1B	R	64	128	2	≤0.25	≤0.25	16	6	VSSA	DAP-S
1C	R	64	256	2	2	4	16	4	hGISA	DAP-R
2A	R	256	256	1	2	0.5	16	6	hTISA	DAP-S
2B	R	256	192	1	1	≤0.25	12	4	hTISA	hDAP
2C	R	256	128	2	2	2	12	6	hTISA	DAP-R
2D	R	512	>256	1	0.5	0.5	12	4	hGISA	hDAP
3A	R	2	4	1	16	0.5	6	>32	hGISA	hDAP
3B	R	16	32	2	2	4	8	16	qVISA	DAP-R
3C	R	4	8	2	1	2	12	12	qVISA	DAP-R

OXA, oxacillin; MIC, minimum inhibitory concentration; MET, macro Etest; VAN, vancomycin; TEC, teicoplanin; DAP, daptomycin; PAP–AUC, population analysis profile–area under the curve; R, resistant; VSSA, vancomycin-susceptible *S. aureus*; hGISA, heterogeneous glycopeptide-intermediate *S. aureus* (i.e. heteroresistant to both VAN and TEC); hTISA, heterogeneous TEC-intermediate *S. aureus* (i.e. heteroresistant only to TEC); qVISA, quasi vancomycin-intermediate *S. aureus*; DAP-S, daptomycin-susceptible *S. aureus*; DAP-R, daptomycin-resistant *S. aureus*; hDAP, heteroresistant daptomycin *S. aureus*.

of each strain versus the daptomycin-susceptible *S. aureus* isolate within each set of isolates. For each analysis, three to five distinct biological replicates were carried out. Statistical expression analyses were performed using the Relative Expression Software Tool (REST) 2009 [16,25].

2.5. Sequencing and sequence analysis

Sequencing of *graR*, *walK*, *mprF* and *cls2* genes in all strains was performed using primers designed on GenBank-deposited sequences. All amplification products were purified using a QIAquick PCR Gel Extraction Kit (QIAGEN, Valencia, CA) and were sequenced with a DNA 4000L sequencer (LI-COR, Lincoln, NE). The DNA sequence was analysed by the gapped BLAST software.

3. Results

3.1. Minimum inhibitory concentrations, macro Etest and population analysis profile–area under the curve analysis

The three sets of isogenic strains were MRSA as defined by oxacillin MICs. Set 1 was composed of two DAP-S/VSSA strains (1A and 1B) and one DAP-R/hGISA (1C), whereas set 2 included one DAP-S/hTISA (2A), one hDAP/hTISA (2B), one DAP-R/hTISA (2C) and one hDAP/hGISA (2D). Set 3 was composed of one hDAP/hGISA (3A) and two DAP-R/qVISA (3B and 3C) (Table 1).

3.2. Molecular characterisation

All set 1 strains were ST398, SCC*mec* IVa and had an identical *Apa*I/PFGE profile (α1). Set 2 was ST5, SCC*mec* II and showed a *Sma*I/PFGE profile of USA100. Set 3 was ST8, SCC*mec* IV and belonged to the three subtypes of the same *Sma*I/PFGE profile, G (G1, G2 and G3) (Table 2).

3.3. agr group and δ-haemolysin

The results showed that the set 1 (A, B and C) and set 3 (A, B and C) strains were *agr* type I, whereas the set 2 (A, B, C and D) strains were *agr* type II. All strains were negative for δ-haemolysin production, indicating their proclivity to develop reduced susceptibility to DAP and glycopeptides, and confirming their glycopeptide heteroresistant phenotype, as confirmed by PAP analysis (Table 2).

3.4. Mutations and expression studies

Sequencing of *graR*, *walK*, *mprF* and *cls2* in all strains was performed. Comparing the hDAP or DAP-R strains with their parental

strains, it was found that *graR*, a regulator of genes involved in cell-wall charge, did not present the point mutation Asn197→Ser in all strains. *walK* (*yycG*) sequencing also revealed the absence of the truncating mutation of three nucleotides (CAA) from position 1111 to 1113 in all tested strains. *mprF* sequencing showed the mutation giving the S295L amino acid substitution only in strain 1C and the mutation leading to the T345I amino acid substitution in strain 2C, whereas a new *mprF* point mutation causing the amino acid substitution L291I in hDAP isolate 2D was found. No *cls2* mutations were found in any hDAP or DAP-R isolates.

Fig. 1 shows the relative quantitative expression of genes related to the cell-envelope charge (*dltA*, *mprF* and *cls*2) and genes involved in cell-wall turnover, growth and cell separation (*sceD* and *atl*), expressed as fold changes compared with the DAP-S strain in sets 1 and 2 or compared with hDAP in set 3.

With regard to the cell-envelope charge genes, the DAP-R strain of series 1 (1C) having S295L in MprF and no mutation in *cls2*, showed that *dltA*, *mprF*, *cls2*, *atl* and *sceD* were upregulated compared with the DAP-S counterpart (1A). The DAP-S/VSSA strain (1B) showed no significant differences in *dltA*, *mprF* and *atl* expression levels versus strain 1A, whereas a significant downregulation was found in *sceD* and *cls2* transcription, but not related to DAP or VAN reduced susceptibility.

In series 2, the DAP-R strain (2C) had the T345I amino acid substitution in MprF and no mutation in *cls2*. This strain showed an upregulation of all genes in the study compared with the DAP-S strain (2A).

In the hDAP strains of set 2 (2B and 2D), no *dltA*, *mprF* and *cls2* profiles of expression associated with a DAP-R phenotype were found. The only exception was strain 2D, having L291I in MprF, in which a high transcription level of *mprF* was found.

Table 2
Molecular characteristics of the strains included in the study.

Strain	ST	SCC*mec*	PFGE	*agr* type	δ-Haemolysin production
1A	398	IVa	*Apa*I/α1	I	–
1B			*Apa*I/α1		–
1C			*Apa*I/α1		–
2A	5	II	USA100	II	–
2B			USA100		–
2C			USA100		–
2D			USA100		–
3A	8	IV	G1	I	–
3B			G2		–
3C			G3		–

ST, sequence type; SCC*mec*, staphylococcal cassette chromosome *mec*; PFGE, pulsed-field gel electrophoresis; *agr*, accessory gene regulator.
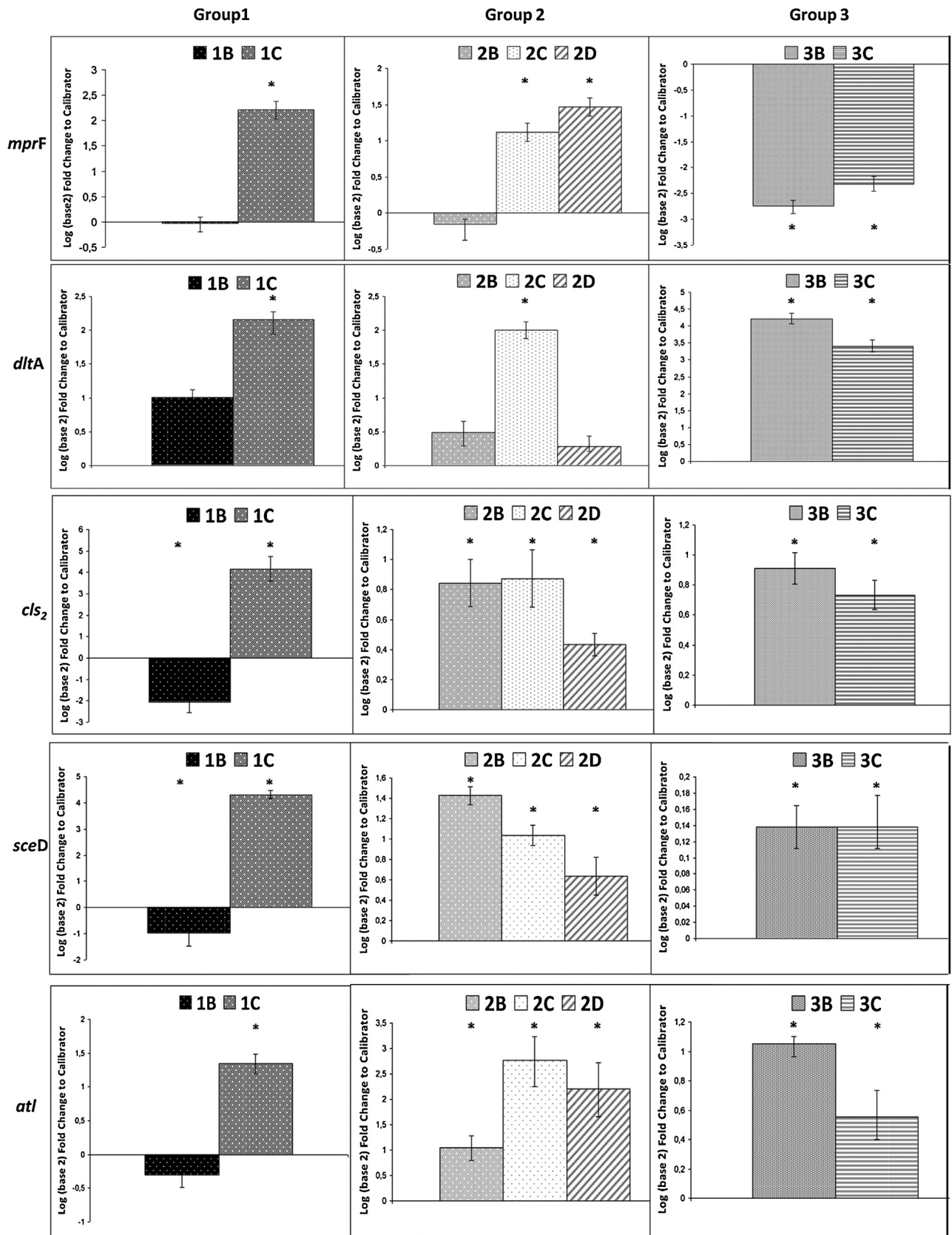
Fig. 1. Relative quantitative expression of all the studied genes in each group of strains. *Statistically significant differences ($P < 0.05$) between samples and controls.

In contrast, the real-time qPCR data of *sceD* and *atl* evidenced an expression pattern related to their reduced susceptibility to glycopeptides phenotype, i.e. upregulation of *sceD* and no *atl* downregulation, characteristic of the hVISA phenotype (see [16]).

Series 2 isolates also included strains with an hTISA phenotype (2B and 2C). As traits in common with the DAP-R phenotype, strain 2C showed *dltA* and *mprF* upregulation, whereas strain 2D showed only *mprF* upregulation. As the trait in common with the hVISA phenotype, both strains exhibited *sceD* upregulation (see [16]).

The two DAP-R strains of series 3 (3B and 3C), having no mutations in any of the analysed genes, showed *dltA*, *cls2*, *sceD* and *atl* upregulation compared with hDAP, whereas only *mprF* was downregulated.

4. Discussion

To date, different single cases of DAP resistance development among clinical *S. aureus* strains under DAP therapy have been described [2,4,26]. Here we report our experience on three sets of DAP-S, hDAP and DAP-R MRSA isogenic clinical isolates in which we examined: (i) the development of DAP resistance and correlation with therapies; (ii) mutations and expression of the main targets involved in DAP resistance; and (iii) the co-occurrence of phenotypes of DAP resistance with glycopeptide reduced susceptibility.

Cell-wall structure modifications, depolarisation of the cell membrane and autolysis are not present at the same time in all DAP-R strains, confirming that a DAP non-susceptibility mechanism is multifactorial and is the result of multiple steps not easily detectable until full expression occurs. Identification of which molecular mechanism is the most important and/or appeared first has not yet been fully clarified.

A number of interesting findings emerged from this study. (i) The evaluation of DAP resistance development under therapy. The first consideration was regarding the development of resistance to DAP under TEC and/or DAP exposure as therapeutic regimens. In all sets, the phenotype of reduced susceptibility to both families of drugs emerged after consecutive administration of TEC and then DAP; in particular, TEC exposure induced only the hTISA/hGISA phenotype, whereas DAP treatment led to hGISA/qVISA and hDAP/DAP-R strains. This timing of antimicrobial administration favours a possible co-evolution of DAP resistance with the GISA phenotype, in agreement with other findings [14]. It is interesting to note that in only one patient (case 1) was DAP used at the suggested dosage (6–8 mg/kg/day). In the other two cases, the TEC trough level was adequate for clinical efficacy [27], but DAP was underdosed in both cases.

All DAP-R isolates, but also DAP-S isolates, were δ-haemolysin negative, indicating that alterations in δ-haemolysin activity predispose the micro-organism to different factors such as reduced susceptibility to glycopeptides and DAP [16,24], reduced response to VAN treatment [28], reduced susceptibility to host defence cationic peptides [9] and increased biofilm formation [29].

(ii) Mutation and expression of main targets. Looking at the molecular basis of DAP resistance, this study focused on the analysis of genes related to the cell-envelope charge, cell-wall turnover, and cell growth and separation, as well as their regulation. On the basis of our recent findings and from other published studies [14], involvement of the *dlt* operon and *mprF* in DAP resistance owing to their role in the maintenance of positive staphylococcal surface charge has been highlighted. *dlt* operon activity has been related to increased alanylation of wall teichoic acids, resulting in a high net cell positive charge [18]. In *mprF*, gain-in-function point mutations were identified and were associated with either excess production or increased outer cell membrane translocation of the positively charged lysyl-phosphatidylglycerol. The net result of these effects is believed to be the enhancement of relative positive surface charge [18].

On the strength of the current data, *dltA* overexpression is the only mechanism in common among our genotypically different series of isolates and represents a strain-independent keystone of DAP resistance in these MRSA strains. This gene, increasing the D-alanylation of teichoic acids, should confer an increased net positive charge responsible for the mechanism of resistance, essentially due to an electrostatic repulsion against Ca^{2+}-DAP and, indirectly, due to a reduction of the autolytic system activity. These findings are in line with other prior observations performed on laboratory-induced DAP-R MRSA strains [14] or found in meticillin-susceptible *S. aureus* (MSSA) clinical isolates [15].

One of the consistent features of the DAP-R strains published in the literature has been the progressive accumulation of SNPs within the *mprF* open reading frames. The most frequently identified are those leading to S295L [30] and T345I [20] amino acid substitutions obtained both in clinical and in vitro selected isolates [14,20]. Together with the hypothetical involvement of SNPs in this resistance, other authors failed to find *mprF* mutations [30]. In the current study, only two of four DAP-R strains, despite showing this common phenotype, possessed mutations in *mprF* and were overexpressed. These results are in agreement with other findings on laboratory-induced DAP-R strains [14], MSSA clinical isolates [30] and MRSA [13]. In the DAP-R strains with no *mprF* mutations, the resistance was only due to *dltA* expression. Owing to the presence of the DAP-R phenotype in strains with or without *mprF* mutations [16], we can hypothesise that mutations represent an adjunctive secondary trait responsible for an enhancement of *mprF* expression, associated with an augmentation of LP-G synthesis and/or flipping, ultimately increasing cell-envelope repulsion [18].

A different situation was found in hDAP strains where *dltA* was not upregulated, similarly to the hTISA and hGISA strains, reinforcing the involvement of *dltA* overexpression only in DAP-R strains. Moreover, a high mutation-independent transcription level of *mprF* was found in only one of the three hDAP strains.

The hypothetical involvement of cardiolipin synthase (*cls2*) mutations in DAP resistance was recently described [20]. Cardiolipin, responsible for a net negative cell-envelope charge, was not downregulated in our strains, the only situation in which this gene could be involved in the repulsion mechanism. A recent report, in which no difference in cardiolipin content was found in nine DAP-S/DAP-R MRSA clinical strain pairs [31], supports our results on the non-involvement of this trait in DAP resistance.

With regard to the genes involved in cell-wall architecture, our data show that all DAP-R and hDAP strains exhibited *sceD* upregulation, a feature correlated with high cell-wall turnover.

(iii) DAP resistance co-occurrence with glycopeptide reduced susceptibility. All of our DAP-R strains had a glycopeptide reduced susceptibility phenotype. With the exception of *dltA* overexpression, characteristic of DAP-R strains, *mprF*, *atl* and *sceD* follow a pathway due to the co-occurrence of DAP and glycopeptide resistance, as recently published [16]. On the contrary, the hTISA phenotype showed only the previously observed hVISA profile [16] of cell-wall turnover, whereas the maintenance of cell-wall envelope charge was due to the expression profile of *dltA* and *mprF*, dependent on their hDAP or DAP-R phenotype.

This investigation underlines the multifactorial nature of DAP resistance owing to a concomitance of different molecular and phenotypic events that lead to the onset of the DAP-R phenotype after antimicrobial exposure. This work analysed the molecular mechanisms of DAP resistance in three sets of clinical isogenic isolates of DAP-R MRSA with respect to the single events already published (mentioned in the text). This study could have a limitation in the relatively small sample size of DAP-R clinical isolates, but it must

be kept in mind that DAP-R strains are still a rare phenomenon in clinical settings.

We collected two other DAP-R strains after the conclusion of this study and preliminary data appear to confirm the role of the targets already highlighted. Moreover, the major role of *dltA* in DAP resistance in MRSA was strengthened by data from another pair of isogenic strains, in which we found *dltA* overexpression only in the DAP-R parental isolate, whilst the DAP-S isolate, isolated afterwards from the same patient, acted as a 'DAP-R lacking phenotype' losing this molecular feature (data not shown). Further studies will be necessary to fully clarify the onset of this complex mechanism of resistance.

In conclusion, DAP resistance is due to a complex network of events in which different aspects can occur. A keystone can be surely represented by a mechanism of electrostatic repulsion and, indirectly, a reduction of autolysin activity due to a *dltA* overexpression-dependent, *cls2*-independent mechanism. The accumulation of adjunctive secondary factors such as the presence of *mprF* mutations related to increased levels of transcription may play a role in the complex phenomenon of becoming resistant.

Acknowledgments

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