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Multiple gene contribution to the phenotypic and genotypic Daptomycin non-susceptible (DNS) variants of Methicillin-Resistant *Staphylococcus aureus* isogenic strains

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TESI DI DOTTORATO

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Abstract

Daptomycin (DAP) represents today a valid alternative choice in the treatment of multi-drug resistant (MDR) *Staphylococcus aureus* infections that do not respond to vancomycin therapy. Knowledge of clinical failure associated with DAP non-susceptibility (DNS) have been accounted in the USA and Europe, and the possibility of glycopeptide induction of low levels of DAP efficacy is worsening this scenario.

The phenotypic alterations that characterize DNS *S.aureus* take place in changes in cell-wall arrangement, turnover, membrane structure and potential, and in modifications of cell autolysis and permeabilization.

This study intended to analyze the molecular traits responsible for the phenotypic features of DNS strains, in three sets of isogenic clinical *S.aureus* in which DNS phenotype arise after DAP therapy.

With regard to the genes involved in cell-wall charge, quantitative relative real time RT-PCR data revealed that *mpr*F showed an up-regulation in DNS strains presenting a *mpr*F mutation, whereas a down-regulation in DNS strains which not presented mutations. All DNS isolates had a *dlt*A up-regulation, while both DNS and hDAP strains presented a *cls*2 up-regulation. Considering genes involved in the cell-wall turnover and autolysis, all DNS isolates exhibited high transcription levels of *sce*D and *atl*.

Our data confirm the multi-factorial nature of DNS and suggest that the keystone of this phenotype is the mechanism of electrostatic repulsion and, indirectly, a reduction of autolysin activity due mainly to a *dlt*A over expression-dependent, *cls*2-independent mechanism, as well as to the accumulation of additional secondary factors, such as the presence of mutations correlated with the increased levels of *mpr*F transcription.

Abstract

Daptomicina (DAP), un lipopeptide ciclico, costituisce ad oggi una valida alternativa per il trattamento delle infezioni da *Staphylococcus aureus* multi-resistente (MDR) che non rispondono alla terapia con vancomicina. Case reports di fallimenti terapeutici associati a ridotta sensibilità a DAP sono stati riportati sia negli USA ed in Europa, inoltre, la possibilità che l'esposizione ai glicopeptidi possa condizionare l'efficacia del trattamento con DAP aggrava tale scenario.

I cambiamenti fenotipici alla base della ridotta sensibilità a DAP (DNS) possono riguardare alterazioni del cell-wall, del turnover, del potenziale di membrana, modificazioni dell'attività autolitica e della permeabilità cellulare.

Obiettivo di questo lavoro è stato quello di analizzare i tratti molecolari responsabili delle caratteristiche fenotipiche della DNS in tre gruppi di ceppi clinici isogenici di *S.aureus* nei isolati dopo terapia con DAP.

Per quanto riguarda i geni coinvolti nelle cariche di parete, i dati di real time RT-PCR hanno rivelato una up-regolazione di *mpr*F nei ceppi DNS che presentavano mutazioni in tale gene, mentre una down-regolazione nei ceppi DNS che non presentavano tali mutazioni. Tutti i ceppi DNS presentavano una up-regolazione dei trascritti di *dlt*A, inoltre sia gli isolati DNS che hDAP mostravano una up-regolazione di *cls*2. Considerando invece i geni coinvolti nel turnover del cell-wall e nell'autolisi, tutti i ceppi DNS esibivano elevati livelli di trascrizione di *sce*D e *atl*.

In conclusione, i nostri dati suggeriscono la multi-fattorialità del meccanismo di ridotta sensibilità a DAP e ci permettono di proporre come caratteristica principale di tale fenotipo un meccanismo di repulsione elettrostatica e, indirettamente una riduzione dell'autolisi, *dlt*A-dipendente *cls*2-indipendente, ma anche all'accumulo di fattori secondari aggiuntivi come la presenza di mutazioni correlate con gli alti livelli di trascrizione di *mpr*F.

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1. INTRODUCTION

1.1. Daptomycin Non-susceptible *Staphylococcus aureus*: definition, epidemiology and clinical implications

Daptomycin (DAP) has been the first approved member of a new class of antibiotics - the cyclic lipopeptides - which came to market in 2003 in the USA and in 2006 in Europe, for the treatment of complicated skin and soft-tissue infections caused by Gram-positive bacteria, and of Methicillin-Resistant *Staphylococcus aureus* (MRSA) bacteraemia and endocarditis (1,2). MRSA infections are one of the most frequent causes of both hospital- and community-acquired infections. The Centers for Disease Control and Prevention (CDC) estimate that the number of people developing a serious MRSA infection in 2005 in USA was about 94,360 (3). Worrisomely, these infections are becoming less easily treated with currently available agents, even with prompt diagnosis and aggressive adjunctive therapies.

Nowadays, DAP and linezolid represent a valid alternative choice in the treatment of severe MRSA or multi drug resistant (MDR) *S. aureus* infections that do not respond to vancomycin (VAN) therapy (4,5).

Indeed, the challenge for the medical community is represented by the finding in the clinical setting of *S. aureus* strains that exhibit a diminished susceptibility to DAP during glycopeptides (vancomycin or teicoplanin) therapy. This phenomenon appears to be associated with high bacterial-load infections and with the presence of a biomedical device or dead tissue along with poor blood perfusion (6-8).

Complicating such scenarios, recent studies have suggested that the initial treatment of MRSA infections with vancomycin may provide the base for the subsequent loss of daptomycin susceptibility (6,7).

The current susceptibility cut off minimum inhibitory concentration (MIC) value of *S. aureus* isolates to daptomycin is set at a breakpoint of >1 mg/L, and these strains are defined as resistant or non-susceptible both by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and by the Clinical and Laboratory Standards Institute (CLSI) (9,10).

Different studies have shown that susceptibility to daptomycin in *S. aureus* may be heterogeneous, meaning that a population of organisms contains a sub-population that shows visible growth in media containing an antibiotic concentration above the MIC (11). The accurate detection of this phenotype can be done by determining population analysis profiles area under the curve (PAP/AUC), which can detect ≤1 in 1,000,000 organisms. However, this method is too cumbersome to be practical in a clinical laboratory (12).

As in the case of glycopeptides, the mechanisms leading to an attenuated daptomycin activity against *S.aureus* strains are complex and for this reason is important to examine the clinical features associated with loss of DAP susceptibility.

To date, most cases of clinically acquired daptomycin non-susceptibility (DNS) of *S. aureus* have occurred in a setting of inadequate dosing

and/or high-inoculum and biofilm-related infections, such as infectious endocarditis (IE) or bone infections **(Figure 1)** (13,14).

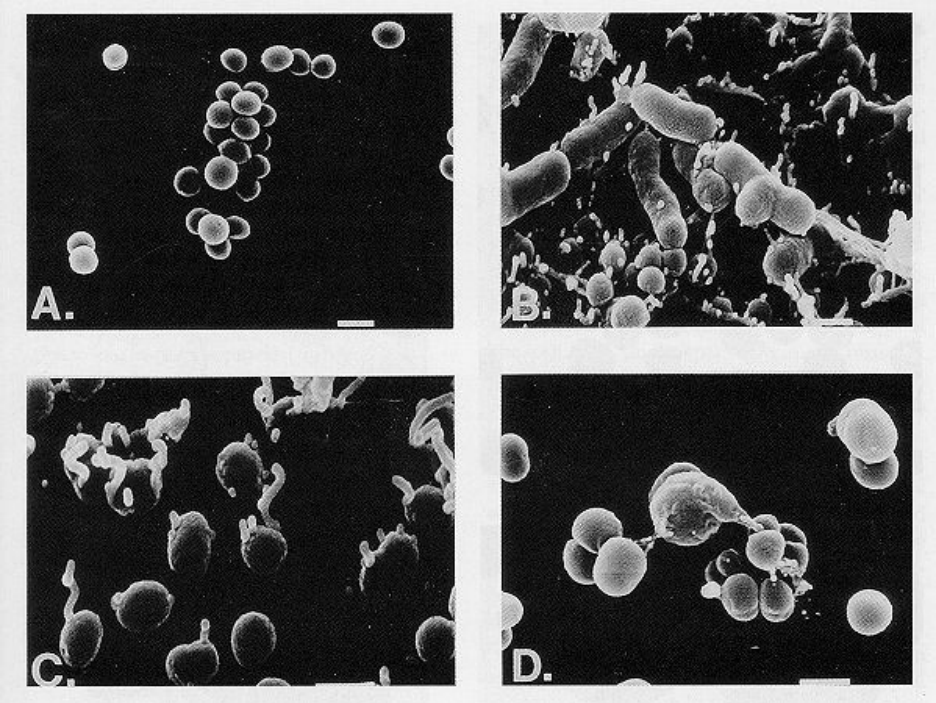


Figure 1. SEM (scanning electron microscope) demonstrating effects of DAP on *S.aureus*. A) Susceptible control with no DAP; B) and C) susceptible strain with lethal concentration of DAP; D) resistant strain grown in sub-lethal concentration of DAP. **Adapted from "The Effects of Daptomycin on Chemical Composition and Morphology of *Staphylococcus aureus*". PROCEEDINGS OF THE OKLAHOMA ACADEMY OF SCIENCE, 1998; 78:15.**

Even if in the clinical setting, the emergence of DAP resistance is low until now, knowledge of clinical failure associated with increasing daptomycin MICs have been accounted in case reports from diverse geographic locations, including the United States and Europe (15,16). These patients had staphylococcal or enterococcal infections associated with septic arthritis, osteomyelitis and/or septic thrombophlebitis (17-19).

Fowler *et al.* in a randomized study of DAP versus standard therapy (i.e. vancomycin or semi-synthetic anti-staphylococcal penicillin, both

administered with initial low-dose gentamicin) for *S. aureus* bacteraemia and endocarditis, reported the development of increasing DAP MICs in 5.8% of patients and increasing VAN MICs in 13.2% of patients, providing an important prospective dataset of more than 1200 serial clinical *S. aureus* isolates (2).

In another case of MRSA IE and septic thrombophlebitis, the patient's strain developed non-susceptibility to glycopeptides during treatment with glycopeptides, and, after switching therapy to DAP, a daptomycin non-susceptible (DNS) phenotype. Finally the MRSA strain was successfully eradicated from the bloodstream only after therapy with linezolid and fusidic acid (13).

Interestingly, in one case, a patient developed a DNS MRSA infection in the setting of persistent bacteraemia secondary to vertebral osteomyelitis, despite being totally daptomycin-naïve (20).

Taking these information together, it emerges that the microbiological significance of these clinical observations are not clear yet, and that, up till now, glycopeptide exposure alone has to be proven to influence daptomycin non-susceptibility.

1.2. Consequences of glycopeptide exposure and cross-resistance to daptomycin

The possibility of glycopeptide induction of low levels of DAP non-susceptibility and rising of DAP MICs is becoming clearly important in the decision-making process for clinicians who are faced with a patient with a serious MRSA infection. Moreover, data from *in vitro* and animal

studies suggest that daptomycin may have a lower rate of killing of heterogeneous Glycopeptide-Intermediate *S.aureus* (hGISA) and Glycopeptide-Intermediate *S.aureus* (GISA) than against Glycopeptide-Susceptible *S.aureus* (GSSA) (21).

CLSI has established VAN breakpoints as follows: strain are susceptible at a vancomycin broth MIC of ≤2 mg/L, intermediate at a vancomycin MIC of 4 to 8 mg/L, and resistant at a VAN MIC of ≥16 mg/L (9). Hence, the current definition for GISA is a *S.aureus* isolate with a vancomycin MIC of 4 to 8 mg/L, while hGISA isolate is a *S.aureus* isolate with a VAN MIC within the susceptible range when tested by routine methods, but where a portion of the population of cells grow in the vancomycin-intermediate range. Typically, the resistant population is present at a frequency of $\leq 10^{-5}$ to 10^{-6} (22).

Recent studies have found that the reduced susceptibility to VAN in MRSA in which coexists diminished susceptibility to DAP could correlate well with the increased cell-wall thickness typical of hGISA and GISA strains (23-25).

Although no definitive mechanism has been elucidated, the report by Cui *et al*. suggests that thick cell-walls induced by glycopeptides could impair the diffusion of daptomycin, a big size molecule, with a molecular weight of 11600 Da, to target sites in the cell membrane (26).

Consequently, even if appears reasonable that VAN exposure by itself can induce low-level of DNS or daptomycin hetero-resistance (hDAP),

on the other hand, these changes appear to be strain specific and may result unstable (24,27,28).

Such alterations may lead to a weaken activity versus other antimicrobial molecules such as platelet antimicrobial peptides, endogenous molecules, whose bactericidal activity is attenuated when function of the *accessory gene regulator* (*agr*) is decreased and when the organism is exposed to vancomycin *in vivo* (29).

Sakoulas *et al.* described three sets of clinical MRSA isolates, each set of isolates being collected from a separate patient, in which GISA or hGISA phenotypes arose during vancomycin therapy with a simultaneous increases in DAP MICs and DAP heteroresistance (28).

Thus, it appears realistic that the exposure of *S. aureus* to VAN influences its global condition, resulting in significant metabolic and physiologic alterations.

1.3. Staphylococcal cell-wall and mechanism of action of daptomycin and vancomycin

1.3.1. *S.aureus* cell-wall

The staphylococcal cell-wall is a dynamic, semi-rigid structure composed of three components: peptidoglycan, teichoic acids and surface proteins.

Of these three constituents, peptidoglycan is the major component and forms a macromolecular net in which glycan strands are cross-linked by short peptides. The glycan strands are composed of repeating disaccharide units of β-1-4 linked N-acetylglucosamine and N-

acetylmuramic acid. Extending from the carboxyl residue of the β-1-4 linked acetylglucosamine moiety is the stem peptide with the sequence: L-alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine. A series of five L-glycine residues are attached to the l-lysine of the stem peptide which is a characteristic feature of the *S. aureus* cell-wall. The peptidoglycan composition from different *S.aureus* strains is highly conserved suggesting that the composition is species-specific (30).

The staphylococcal cell-wall contains also teichoic acids, which represent up to 50% of the dry weight of the purified staphylococcal cell-wall. Teichoic acid chains are attached to the 6-hydroxyl groups of some of the *N*-acetylmuramic acid residues of the glycan chains and, together with the peptidoglycan, form a multilayered network that surrounds the *S.aureus* cell (30). Typically, the degree of murein cross-linking in the *S.aureus* cell-wall is high, with bridged peptides as a ratio of all peptide ends in the order of 80 to 90% (31). Wall teichoic acids (WTAs) are covalently linked to peptidoglycan and present d-alanine and *N*-acetylglucosamine residues (32). Lipoteichoic acids (LTAs) are glycerol phosphate polymers linked to a glycolipid terminus in the cytoplasmic membrane. The functions of WTAs and LTAs are still being elucidated, but with the recent generation of defined mutants it appears that the complete loss of LTA leads to cell death in *S.aureus* (33). Lipoteichoic acids appear to also be involved in cell division (34). Some data suggest that the role of teichoic acids is to help protect the cell envelope acting as a mechanical barrier to host defence molecules and

antibiotics, and also, the positive charge of d-alanine residues repels positively charged molecules such as defensins (35,36).

1.3.2. Mechanism of action of DAP and VAN

Daptomycin structure consists of an anionic core and a C-10-lipid side-chain, and is produced by a non-ribosomial peptide synthetase (NRPS) mechanism in *Streptomyces roseosporus*. It is composed by 13 D- and L- amino acids of which tend to form a cyclic frame linked by an ester bond between the C-terminus **(Figure 2)** (4).

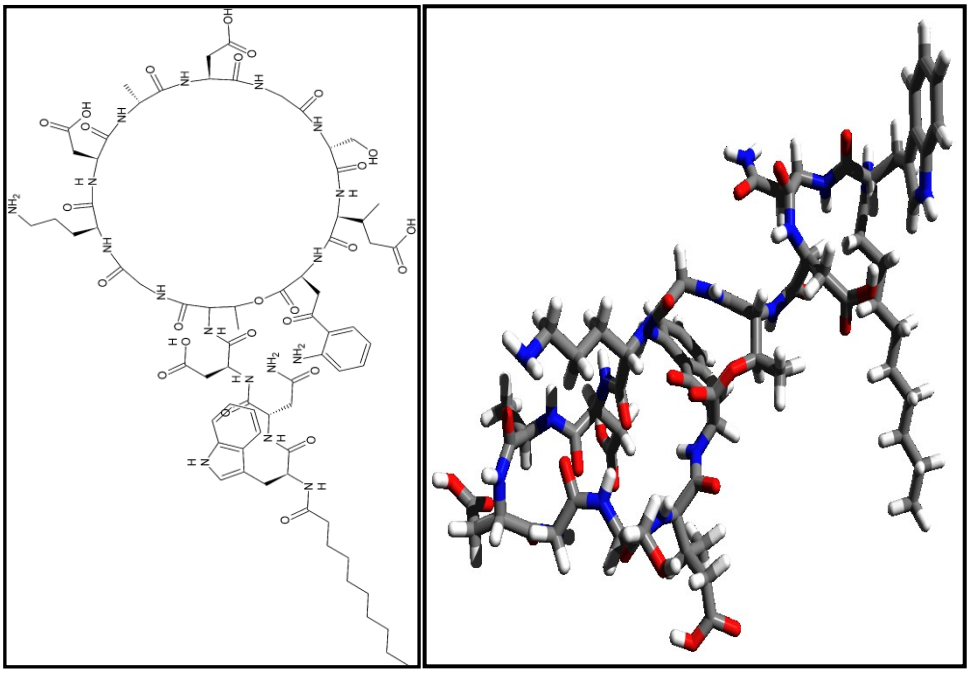


Figure 2. Molecular and 3D structure of Daptomycin molecule

By aggregating in solution in the presence of at least an equimolar ratio of Ca2+, the net charge of the molecule becomes positive and enables electrostatic interactions with negatively charged cell membrane, binding it (37).

It forms thus oligomers and inserting its tail into the bacterial cell membrane subsequently forms channels through which intracellular ions (potassium), leak out of the cell, diminishing cell membrane negative potential and causing failure of the synthesis of DNA, RNA, and proteins, ultimately resulting in a rapid bacterial cell death **(Figure 3)** (38). This initial model was later modified according to the observation that daptomycin forms micelle-like structures in the medium and binds to the membrane in the already oligomerized form (39).

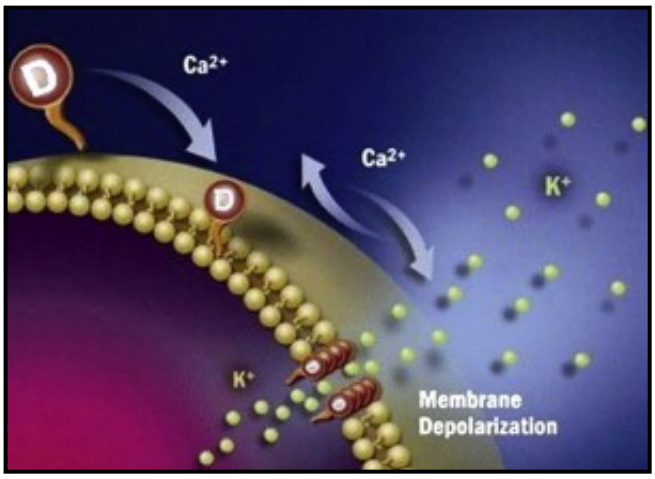


Figure 3. Mechanism of action of Daptomycin, potassium leaks out of the cell diminishing cell membrane negative potential and causing cell death.

In fact, Hobbs *et al.* found that changes in dissipation of membrane potential and leakage of intracellular material occur rather late, after cell death, and with no significant alterations in membrane integrity (40).

Researches to date indicate that DAP acts on cell membrane and up-regulate cell-wall stress stimulon (CWSS), a group of genes that are commonly induced upon treatment with cell-wall active antibiotics, as it is vancomycin (41). Among these genes have been recognized e.g.: *fmt*, *htr*A, *prs*A, *sgt*B, *mur*Z, *lyt*R, *tca*A (42).

The pharmacology of DAP is well known, it has a rapid bactericidal activity and shows a concentration-dependant killing (43).

The glycopeptide antibiotic vancomycin (VAN), is an inhibitor of cell-wall synthesis in *S.aureus* and other gram-positive organisms, in fact, it binds to the C-terminal d-Ala-d-Ala residue of the peptidoglycan precursor and forms a stable, non-covalent complex, which prevents the use of the precursors for cell-wall synthesis **(Figures 4-5)** (44).

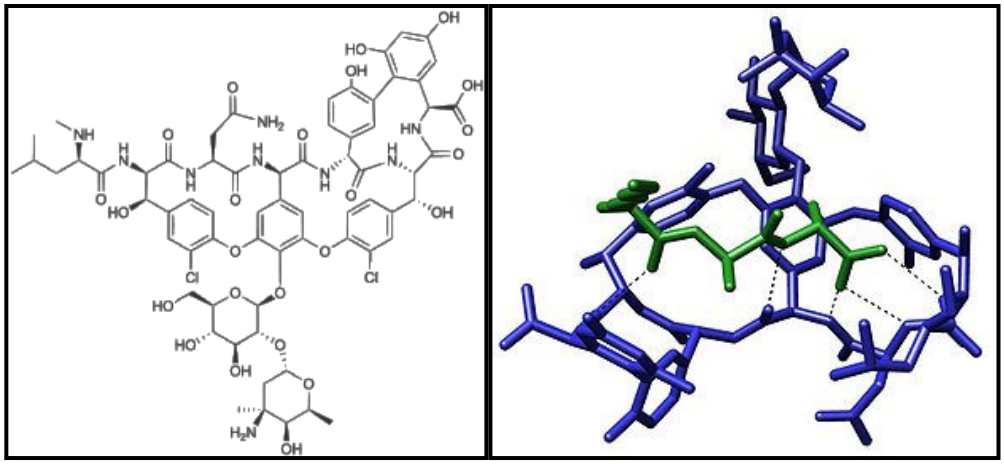


Figure 4. Molecular and 3D structure of Vancomycin molecule

VAN inhibits late-stage peptidoglycan biosynthesis and acts outside the cytoplasmic membrane, which results in the intracellular accumulation of UDP-linked MurNAc-pentapeptide precursors. The VAN complex involves a number of hydrogen bonds between the peptide component of vancomycin and the d-Ala-d-Ala residues (45,46).

The main location for cell-wall synthesis in *S.aureus* is the division septum and not the whole-cell membrane. This means that vancomycin has to diffuse to the tip of the division septum to bind to peptidoglycan precursors at this location, and the distance of this diffusion varies

depending on the cell cycle, where a longer septum exists later in the cycle (47).

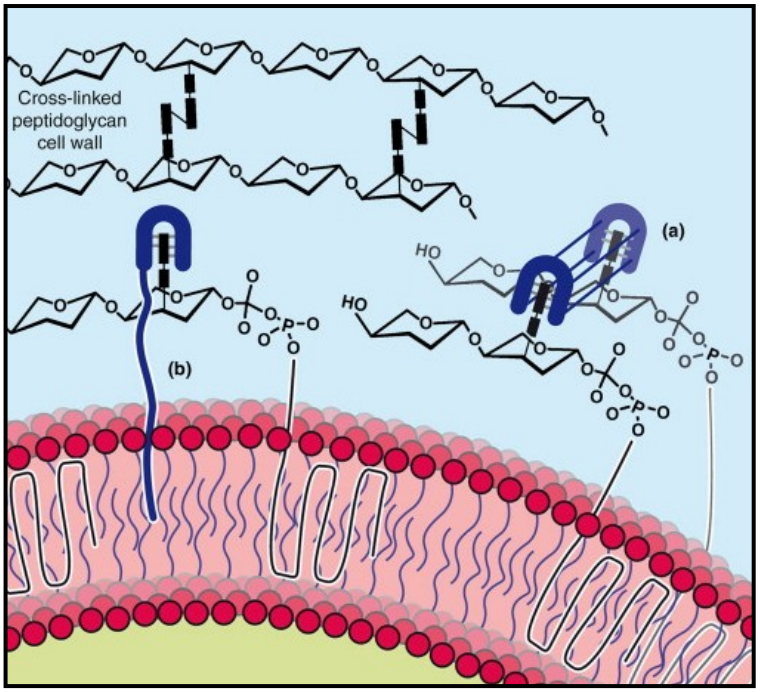


Figure 5. Mechanism of action of glycopeptides (a) VAN activity is based on dimerization, which enhances binding to the target peptide through both cooperative and allosteric effects. **(b)** Lipoglycopeptides (e.g.teicoplanin) have fatty acyl chains anchored in the phospholipid bilayer that enhance the binding affinity. **Adapted from Trends in Biotechnology. Volume 28, Issue 12, December 2010, Pages 596–604**

1.4. Phenotypic features of DNS and hGISA/GISA strains

The role of cell-wall targeting in the mechanism of action of DAP has been generally accepted, but the single contributions of the targets and the mechanism of resistance have not been fully elucidated because of genetic inconsistencies between non-susceptible isolates.

Therefore, despite daptomycin resistance is uncommon, and frequency of spontaneous daptomycin resistance in *S.aureus* is low (10^{-10}), it is important to identify phenotypic and molecular features of this

phenotype, that could be then recognized by clinicians and microbiologist (48).

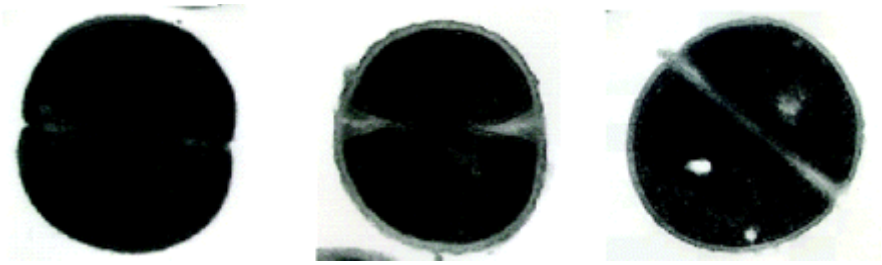
The phenotypic alterations that may occur can be grouped into:

1) changes in cell-wall arrangement and turnover;

2) changes in membrane composition, structure, and potential;

3) modifications in sensitivity to autolysis and permeabilization.

This common resistance pathway has been suggested for GISA and hGISA since, in general, are involved in the shift of a strain towards a non-susceptible phenotype, changes in the global cell-wall arrangement (29). The first explanation for this correlation may be that the thickened cell-wall that acts as a common obstacle to DAP and VAN penetration. Even though daptomycin does not bind peptidoglycan, it might be hard for DAP to smoothly penetrate the cell-wall when it becomes as thick as that of GISA **(Figure 6).**



A. MRSA DAP susceptible 26,65 nm | **B.** DNS 41,81 nm | **C.** Revertant susceptible strain 26,65 nm

Figure 6. Transmission electron micrographs of cell wall thicknesses of DNS strains. The values given under each panel are the means (nm) of the cell wall thickness at a magnification of ×25,000. **Adapted from Antimicrob. Agents Chemother. 2008 .52(12):4289-4299.**

A detailed study analysed if changing in the cell membrane (CM) structure correlate with in vitro DAP resistance in a strain set from an endocarditis patient failing DAP therapy. The different membrane

structures identified sequential phenotypes that distinguished the daptomycin-susceptible from the DNS strains. In fact, the CM of the DNS isolates resulted significantly more fluid than those of the susceptible parental strain and such enhanced membrane fluidity has been also correlated with *in vitro* resistance to a wide range of cationic antimicrobial molecules (49,50).

Jones *et al*. found out that DNS *S. aureus* membranes further than having an enhanced membrane fluidity had an increased translocation of the positively charged phospholipid lysyl-phosphatidylglycerol (LPG) to the outer membrane leaflet, and an increased net positive surface charge (50). DNS *S. aureus* strains, have showed, beside an increased synthesis of lysyl-phosphatidylglycerol (LPG), also an increased translocation of this component to the outer membrane, and hence modifications in electrostatic potential (51).

Kaatz *et al.* evaluated a clinical DNS isolate from a patient with MRSA endocarditis and noted not only a reduced DAP binding to the whole cell, but also a reduced CM potential and loss of an 81-kDa protein recognized, by the authors, as a putative DAP chaperone (52).

Reduced autolytic activity is another widespread feature of hGISA/GISA and DNS strains and is a common early phenotypic change in serial isolates obtained during persistent infection, although this has not been demonstrated for all isolates (53-55).

1.5. Molecular features of DNS and hGISA/GISA strains

Several genes, such as *dlt*ABCD (alanylation of wall teichoic acids), *mpr*F (lysylation of phosphotidylglycerol), *sce*D, *atl*, and *lyt*M (autolysins) as well as Two-Component Systems (TCSs), such as *wal*RK, *gra*RS, *vra*SR, and *agr* locus (29,56,57), have been supposed to be implicated in *S. aureus* non-susceptibility to DAP and glycopeptides; however, the exact molecular mechanism in the generation of these phenotypes has not been determined yet.

Several recent studies have identified the differences in expression and the mutations in clinical and laboratory-derived DNS strains, and found out that a combination of both these genetic alterations seems to determine an additive effect on the DAP MIC value.

Of interest, one of these studies used whole-genome sequencing to detect mutations in *mpr*F*, cls2* and *pgs*A genes in a collection of isogenic, clinical *S.aureus* strains that had been exposed to DAP. Point mutations at these loci, encoding for membrane phospholipid biosynthesis, have, as a result, been associated with the development of daptomycin non-susceptibility (58).

A recent study analyzed by whole-genome sequencing and by gene-replacement a *S.aureus* strain that was generated in vitro by serial daptomycin selection, and found in the *rpo*B gene (RNA β-polymerase subunit) a non-synonymous single nucleotide polymorphism (SNP) that was associated with the DAP resistance of the strain and was

accompanied by a thickened cell-wall and reduction of the cell surface negative charge (59).

Our recent findings suggest that both VAN and DAP stimulate hVISA strains to acquire the VISA phenotype by an up-regulation of autolysin *sce*D, a down-regulation of the *rna*III (*agr* locus) and an up-regulation of *mpr*F, and increase, in VISA, the cell-wall pathway by an up-regulation of *dlt*A and a down-regulation of the autolysins *atl* and *lyt*M (60).

Of note, hGISA or GISA strains seem to be induced to resistance only after exposure to glycopeptides, so the mechanism that leads to the development of these phenotypes is directly connected with the target of these antibiotics (61).

Thus, DAP and glycopetide non-susceptible phenotypes are likely the end result of a common pathway that consists of multiple accumulations of genetic mutation and transcriptional regulation.

1.5.1. The role of *mpr*F

Genetic and biochemical evidence have shown that MprF (multiple peptide resistance factor) is involved in the biosynthesis of lysyl-phosphatidylglycerol (Lys-PG), a basic phospholipid in which L-lysine from lysyl-tRNA is transferred to phosphatidylglycerol (PG) (62). Moreover, has been postulated its role in facilitating bacterial resistance to cationic antimicrobial peptides by reducing the net negative charge of the cell membrane (57).

*mpr*F codes a large membrane protein that contains 14 trans-membrane domains (TMDs) and has two key functions: the addition of

positively charged lysine residues on PG to form LPG, and the translocation of this LPG to the outer leaflet of the cytoplasmatic domain (57).

There have been conflicting reports concerning the role of MprF in *S. aureus* diminished susceptibility to VAN and DAP.

With reference to VAN Nishi *et al.* noted that the *mpr*F inactivation in vancomycin-susceptible *S. aureus* resulted in a decreased VAN susceptibility, while Ruzin *et al.* reported that the inactivation of *mpr*F allowed for more binding of vancomycin to *S. aureus* cell membranes and resulted in increased susceptibility to vancomycin among GISA strains (63,64).

As regards DAP, expression of *mpr*F-specific antisense RNA was recently shown to re-establish susceptibility to DAP in DNS strains (65). Furthermore, from different studies appears that some DNS *S.aureus* strains have accumulated point mutations in MprF that appear to lead to a gain-in-function of the protein, but it remains to be elucidated if these point mutations lead to increased Lys-PG production or to increased Lys-PG flipping to the outer leaflet of the CM (66,67).

Friedman *et al*. recently demonstrated that, for three *S. aureus* isolates with reduced daptomycin susceptibilities, selected by *in vitro* exposure to serially increasing concentrations of DAP, the initial increase in DAP MICs was associated with amino acid substitutions in MprF, and specifically P314L, T345I and T345A (68).

Julian *et al.* analyzed more DNS clinical isolates and concluded that some, but not all, non susceptible strains had this SNP in MprF (68).

On the other hand, Pillai *et al.* found that there were no mutations resulting in amino acid substitutions in MprF of three DNS GISA isolates, which had never been exposed to DAP. As a control, they sequenced also the *mpr*F gene of the DNS MRSA isolate that did not demonstrate VAN heteroresistance and had been recovered from a patient who had been treated with DAP (69).

Peleg *et al.* discovered in 21 isogenic clinical strains that had been exposed to DAP, different point mutation (T345I, S295L, I420N, S337L), particularly one (G61V) was found within the N-terminal translocation domain of MprF, and one (L826F) was mapped in the C-terminal lysinylation domain, hypothesizing that these two predicted amino acid changes may either directly enhance translocation of LPG to the bacterial surface by increasing translocase activity of MprF, or enhance lysinylation of PGs (58). Both these changes would intensify the reduced net negative charge of the bacterial surface, contributing to daptomycin non-susceptibility.

Finally, Boyle-Vavra *et al.* found in a DNS MRSA isolate obtained from an isogenic susceptible strain, after initiation of DAP therapy, a C-to-T base substitution at nucleotide 1010 in the *mpr*F gene that resulted in a Serine-to-Leucine amino acid substitution (S337L) in the MprF protein. This was mapped within the tenth putative transmembrane domain of the protein (70).

1.5.2. The role of *dlt*ABCD

The *dlt*ABCD operon controls the alanylation of wall teichoic acids (WTA) in response to antimicrobial challenge and is under the control of *gra*RS. The *dlt*ABCD 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 (36).

This greater net positive surface charge, as mediated by *mpr*F, would theoretically reduce the overall access of calcium-decorated DAP to its membrane target (71).

*dlt*ABCD regulates also indirectly the activity of the autolytic system, since a decrease of the cell-wall positive charge accelerates autolysin activity (36).

There has been made associations concerning the role of *dlt*A in hVISA/VISA and DNS *S.aureus*.

Cafiso *et al.* found an increased expression of *dlt*A in VISA isolates and this consequently enhanced D-ala teichoic acid synthesis determining a greater amount of these components that could be related not only with a greater positive net charge of the CW, but also with the reduced autolysis ratio that characterizes VISA phenotype (60).

Bayer *et al.* investigated the transcriptional expression of *dlt*ABCD in DNS strains and demonstrated that enhanced *dlt*ABCD expression is related, also in this phenotype, to an increased positive charge of the CW and to a reduced daptomycin binding. Furthermore, analyses of the *dlt*ABCD promoter region, in the same study, showed no difference in

sequence between the DNS and susceptible strains indicating that the increased expression of the operon, in the DNS strains, likely results from the influence of regulatory factors other than a cis-acting element (56). In agreement, Boyle-Vavra *et al*. did not find any polymorphism in the *dlt* operon in their study on DNS isogenic strains (70).

Of note, in one study, even though the increased expression profiles of *dlt*ABCD and phenotypic gain-in-function of the sample, the net surface charge paradoxically became less positive during in vitro Dap^r selection (72). This founding suggested the concept that other surface factors may contribute to the net envelope charge. One of the principal factors, for these authors, would be the extent of cell-wall glutamate amidation, which has been linked to glycopeptides intermediate resistance in VISA (72).

1.5.3. The role of *cls2*

Cardiolipin-synthase2 gene (*cls*2) is involved in the synthesis of cardiolipin (CL), from the complete conversion of two molecules of phosphatidylglycerol (PG) to one molecule of CL and one molecule of glycerol.

CL is a phospholipid that possesses four acyl groups and has a negatively charged (-2) head group; in the stationary phase or under conditions of stress, such as unfavourable growth conditions or cell-wall acting antibiotics, CL compose the 30% of the *S. aureus* CM (73,74).

CL plays an important role in the preservation of osmotic stability and has been described as a proton reservoir, thus, its presence can

decrease the membrane potential. Cls2 is predicted to contain two TMDs across residues 13-35 and 45-67, as well as two putative cardiolipin-synthase domains across residues 229-256 and 407-434 (73).

A recent study identified four SNPs within *cls*2, residing exclusively within the two putative TMDs at the N-terminus of the protein and hypothesized that mutations within the TMDs impair membrane localization and function of Cls2, resulting in altered cardiolipin synthesis (58).

1.5.4. The role of autolysins: *atl* and *sce*D

The major autolysis gene (*atl*) of *S.aureus* yields a 63-kDa amidase and a 54-kDa glucosaminidase after processing, *sce*D gene (SAV 2095 *sce*D-like gene) encodes instead a lytic transglycosylase (75).

These genes play a key role in controlling cell-wall expansion, remodelling, and daughter cell separation, but primarily participate in peptidoglycan turnover. In particular, autolysins cleave the cell-wall in a tightly controlled manner to maintain cell-wall integrity during cell division (76).

A proteomic study reported that the SceD protein was increased in abundance in the cell envelope fraction of strains with reduced vancomycin susceptibility, and its expression and activity changes were responsible for an altered cell-wall turnover rate and an altered peptidoglycan structure (77).

DNS strains, in a recent study, showed lower rates of autolysis than the parental susceptible strains and, since the autolytic pathway in *S.aureus* appears to contribute to full execution of the lethal pathway of cationic antimicrobial peptides, it is not surprising that DAP non-susceptibility is also associated with defective autolysis (49,50).

Our findings, in another study, suggested that a reduced expression of *atl* in GISA with respect to hGISA contributed to the characteristic low autolysis ratio of GISA strains and that both VAN and DAP induced, also in the susceptible strain, the synthesis of enzymes involved in the cell-wall architecture, responsible for the mechanisms of glycopeptide reduced susceptibility. While in the same study was found that the *sce*D over-expression was a constitutive feature of the hVISA and VISA phenotype, in agreement with results obtained by other authors (60, 78).

Thus, while there is a growing consensus that decreased autolysis of GISA strains may, directly or indirectly, contribute to their reduced susceptibility to glycopeptides, the molecular pathways involved in the defective autolysis in DNS *S.aureus* are still undefined (79,80).

1.5.5. The role of global regulators: GraRS and WalKR

GraRS also called ApsRS is a two-component regulatory system (TCS), so named because its up-regulation has been found to be associated with glycopeptide resistance (81). The system has been shown to control the expression of several genes, including some involved in cell-wall synthesis or global regulatory proteins (82).

Knockout mutations in GraRS have resulted in hypersensitivity to VAN, while its over-expression, maybe due to presence of SNP, in a slight increase in the VAN MIC (83,84). These mutations might lead the regulons to remain in an activated “locked-on” or modified state. The consequence of this modification includes cell-wall thickening, decreased autolysis, reduced protein A production, increased capsule expression and increased D-alanylation of teichoic acids (30).

A recent study compared the genomes of two prototype strains, i.e. the hVISA Mu3 and the VISA Mu50, and observed sixteen point mutations, including an amino acid substitution (N197S) in the GraR of Mu50, the response regulator of the TCS. The introduction of the N197S into Mu3 converted the strain in a full VISA, accompanied by cell-wall thickening and decreased autolysis. The introduction of N197S into a VSSA had instead no effect on VAN susceptibility, indicating that additional genomic changes were required to develop the VISA phenotype. Another comparative genomic study detected six mutations and one of these mutations was in *gra*S, resulting in an amino acid substitution (T136I) in a VISA isolate obtained from a patient after 42 days of vancomycin therapy (85).

Different papers have also shown regulation of *mpr*F and *dlt*ABCD by GraRS, in particular, a recent study showed that the expression of these genes was dependent on the co-transcription of GraRS (86,87).

The WalKR (also known as YycGF) TCS is essential for cell viability, but its physiological role have remained mostly uncharacterized.

Recently, two reports have described the correlation of the WalKR system with the phenotype of vancomycin intermediate resistance in *S.aureus*. In particular, Jansen *et al.* reported that WalRK was highly up-regulated due to an insertion mutation in the promoter of a VISA clinical isolate (88,89) and Mwangi *et al*., found a mutation in the TCS in a clinical VISA strain (90).

On the contrary, in a recent paper, Hiramatsu *et al.* reported a deletion mutation in WalKR, but they did not find any significant changes in the expression of the system (91).

In one study the WalKR TCS was altered by a SNP in two out of three of the serial-passage strains with DAP and one of the DNS clinical isolates, but since the system is essential, has been predicted that the alterations resulting in daptomycin resistance do not shut down completely the protein's function (92).

2. AIM OF THE STUDY

To define the molecular mechanism at the base of daptomycin non susceptibility we investigated three groups of isogenic strains, each set of isolates being collected from a separate patient, and isolated after antibiotic therapy with daptomycin, in three different Italian Hospitals, for:

- adhesin and toxin gene content by conventional and multiplex PCR;
- biofilm production by a spectrophotometric quantitative assay;
- δ-hemolysin production by assay on 5% sheep blood agar plates;
- *mprF*, *cls2*, *graR* and *walK* sequencing;
- the expression profile of some genes involved in autolysis (*atl*), cell-wall turnover (*sceD*), membrane charges (*mprF*, *dltABCD*, *cls2*) by relative quantitative real time RT-PCR.

3. MATERIALS AND METHODS

3.1. Strains

S.aureus strains used in this study were three groups of multiple strains of the same patient at multiple time points, isolated after antibiotic therapy with different antimicrobials including teicolpanin and daptomycin in three different Italian Hospitals, as shown in **Table 1**.

In detail, in group 1, strain 1A was isolated after topic gentamicin and cotrimoxazole administration, strain 1B after piperacillin/tazobactam, teicoplanin and gentamicin and 1C after daptomycin therapy.

Group 2 strains were all been isolated after management with daptomycin/linezolid; while in group 3, strain 3A was isolated after teicoplanin exposure and strains 3B and 3C after daptomycin.

MIC, Macro Etest (MET) MIC determination for glycopeptides and daptomycin and PAP/AUC for both glycopeptides and daptomycin were conducted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (9,93). Molecular characterization (MultiLocus Sequence Typing, Staphylococcal Cassette Chromosome *mec*, PFGE) was performed as previously published (94). Mu3 (hVISA), Mu50 (VISA) and ATCC 29213 (VSSA) were used as control strains. All these data were supplied by Dr F. Campanile research group.

In particular as shown in **Table 2,** the three sets of isogenic strains were MRSA, as defined by oxacillin MICs. All strains belonging to group 1 were ST398, SCC*mec* IVa and had an identical *Apa*I/PFGE profile, α1. The group 2, instead, was ST5, SCC*mec*II and showed a *Sma*I/PFGE

profile of USA100. The group 3 was ST22, SCC*mec*IV and belonged to the three subtypes of the same *Sma*I/PFGE profile, G.

The strains have been also categorized as hGISA, hTISA or hVISA (hetero Teicopanin or Vancomycin Intermediate *S.aureus*), namely strains that exhibited a PAP/AUC profile of hetero resistance versus teicoplanin or versus both teicoplanin and vancomycin (hVISA), and hDAP (hetero Daptomycin *S.aureus*) respectively, according to vancomycin/teicoplanin and daptomycin PAP/AUC analysis data.

DNS *S.aureus* strains were established by DAP MIC values according the EUCAST guidelines (10).

Taking this into account, the group 1 was composed by one VSSA/DSSA strain (1A), one hTISA/DSSA (1B) and one hVISA/DNS (1C) strain; whereas group 2 included one hTISA/ DSSA (2A), two hTISA/hDAP (2B-D) and one hVISA/DNS (2C). The group 3 was constituted by one hVISA/hDAP (3A), and two qVISA/DNS (3B-C), where qVISA indicates a strain that shows a population analysis profile of VAN and TEC as Mu50, but with a VAN MIC value <8 mg/L.

3.2. Molecular characterization

agr-typing was performed by PCR amplification of the hyper-variable domain of the agr locus using oligonucleotide primers specific for each of the four major specificity groups as described by Shopsin B *et al.* (95).

3.3. Pathotype and Biofilm production

The virulence gene content, including adhesion genes (*spa*, *cna, fnb*A, *sdr*C/E *clf*A/*f*B, *atl,* and *ica*A) and toxin genes (*hl*-s, *se*-s, *eta*, *luk*S/F, and *tst*), was determined by using both conventional PCR and multiplex PCR as previously published (96). Both methods were performed using primers designed on the deposited gene sequences shown in **Table 3.**

The Multiplex PCR comprised Mix1: *fnb*A, *ica*A, and *sdr*E; Mix2: *sej, and atl*; Mix3: *hlg*, *hla*, *hld*, *sea,* and *eta*; and Mix4: *cna*, *clf*A, and *clf*B. The identification of the capsular polysaccharide types 5 and 8 was performed with specifically designed primers on the variable segments of the deposited gene sequences of the *cap* locus. Amplification mixes contained the following final concentrations: 10mM Tris-HCl pH 8.8, 1.5mM $MgCl_2$, 150mM KCl and 0.1% Triton X-100, 1U of Taq-polymerase (DyNAzymeTMII DNA polymerase, Finnzymes, Oy), 200mM each deoxynucleoside triphosphate and 100ng of genomic DNA. PCR and Multiplex PCR conditions were an initial 5 min denaturation step at 94°C, followed by 30 cycles of 1 min at 93°C, 1 min at annealing temperature, and 1 min at 68°C, with a final annealing step of 10 min at 68°C. All PCR products were run on 1% agarose gels and stained with SYBR green (0.4 µg/mL).

Biofilm production ability was tested by a spectrophotometric quantitative assay. Each strain was grown in Tryptone Soy broth (Oxoid), with the addition of 0.25% glucose (TSBG). These assays were performed in microtitre plates as described previously (97,98). Each

reported value was the average of 12 measurements at 490nm (±SD). Values ≥0.12 were regarded as biofilm positive and values of >0.4 were considered strong producers, 0.4–0.2 were medium producers and <0.2 were considered weak producers. These absorbance values were a species-related modification of previously published values (99).

3.4. Screening of δ-hemolysin activity on 5% sheep blood agar plates

The functionality of the *agr*-operon was measured by delta-hemolysin production, testing the strain by cross-streaking perpendicularly to RN4220, which produces only β-haemolysin, on 5% sheep-blood-agar (SBA) plate with 6 mg/L vancomycin (VAN) as previously published (100). This test can usually identify the three staphylococcal haemolysins active on SBA – α, β, and δ – due to the interactions between them: β-haemolysin enhances lysis by δ-haemolysin, but inhibits lysis by α-haemolysin. Delta-hemolysin produced by a test strain results in a zone of enhanced hemolysis in areas where this lysis overlaps with the beta-hemolysin zone of RN4420 (101).

3.5. RNA extraction, retro-transcription and quantitative real Time RT-PCR

An aliquot of an overnight culture was diluted 1:50 and bacteria cells were grown in BHI at the exponential phase (OD_{600}= 0.4 at 3 h). Cells were then harvested by centrifugation, and the cultures were re-suspended in 200µl of diethylpyrocarbonate (DEPC) treated H_2O, 1ml

of Trizol-reagent (GibcoBRL, Paisley, UK) was added and incubation continued for a further 5 min. Following incubation, 200 µl of chloroform was added, mixed by agitation; the mixture was incubated for 5 min, and centrifuged at 10,000 g for 15 min. After centrifugation, 1 ml of cold-isopropanol was added and the mix was maintained at -20°C for 60 min, followed by centrifugation at 12,000 g for 15 min and re-suspension of the pellet in 50 µl of DEPC-H_2O and storage at -20°C. Genomic DNA was removed by treatment with Rnase-free-DnaseI (Ambion, Austin, TX, USA). RNA quality was established by analysis of the A260/280 ratio and analysis of the rRNA bands on agarose gels. RNA concentration was determined spectrophotometrically. Each extracted RNA sample was also checked for the presence of DNA contamination as a template in the PCR assay to confirm the absence of DNA contamination. Retro-transcription, cDNA synthesis, was carried out by using the hexanucleotide primers 'ImProm-II Reverse Transcriptase Kit' (Promega) according to the manufacturer's instructions. Quantitative real time RT-PCR was performed in a MX 3000P Instrument (Stratagene) with a 2.5 µl template (cDNA), the Brilliant SYBR Green QPCR Master mix (Stratagene), and 30 pmol of primers in a final volume of 25 µl. PCR reaction efficiency was verified by using serial dilutions of cDNA ranging from 102 to 106 target copies per reaction (104–108 target copies per sample, standard curve), and only oligonucleotides giving PCR cycles which generate a linear fit with a slope between -3.1 and -3.6 and amplification efficiency value (Rsq)

of 90–110% were chosen. All real time RT-PCR were performed in triplicate at an initial denaturation of 95°C for 10 min, followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final cycle at 95°C for 1 min, 55°C for 30 sec and 95°C for 30 sec. Primers for quantification were selected to amplify a fragment of less than 300bp. *gyr*B was used as a normalizer, internal control, as previously published (102). All the primers used for the real time RT-PCR study are in **Table 4**. The expression of the studied genes was represented as the increment/decrement (fold changes) of each strain versus the DSSA isolate within each set of isolates. For each analysis, three to five distinct biological replicates were carried out. Expression analyses were performed using the relative expression software tool REST2009 (Relative Expression Software Tool). REST applies the efficiency-corrected comparative CP method and performs randomization tests to estimate a sample's expression ratio and the likelihood of up or down-regulation, taking into account reference genes and the individual amplification efficiency of each gene. The Excel-based relative expression software tool, REST 2009, was applied for group wise comparison and statistical analysis of the qPCR data as described in Pfaffl *et al.* (http://rest.gene-quantification.info/). The relative expression ratios were calculated by a mathematical model, which included an efficiency correction for real-time PCR efficiency of the individual transcripts, as follows: Ratio=$(E_{target})^{\Delta CP}$ $target^{(control-sample)}$/ $(E_{ref})^{\Delta CP}$ $ref^{(control-sample)}$. The relative expression ratio of a target gene was

computed based on its real time PCR efficiencies (E) and the crossing point difference (ΔCP) for an unknown sample versus a control. For each gene, cDNA dilution curves were generated and used to calculate the individual real time PCR efficiencies ($E=10^{[-1/slope]}$) (103,104). In particular, the purpose of this test was to determine whether there was a significant difference between samples and controls, while taking into account issues of reaction efficiency and reference gene normalization. The test uses the hypothesis test P(H1) that represents the probability of the alternate hypothesis that the difference between sample and control groups is due only to chance. The hypothesis test performs at least 2000 random reallocations of samples and controls between the groups. Statistical differences were given as significant when $p<0.05$.

3.6. Sequencing and sequence analysis

*mpr*F, *cls*2, *gra*R and *wal*K sequencing amplification products were purified using the QIAquick PCR gel extraction Kit (Qiagen) and sequenced with a LICOR DNA 4000L sequencer. The DNA sequence was analyzed by the gapped blast software (105).

4. RESULTS

4.1. *agr*-group and δ-hemolysis

Our results showed that the strains 1A-B-C and 3A-B-C were agr-I whereas the 2A-B-C were agr-II. All strains in study were negative for δ-hemolysin production as shown in **Table 2.**

4.2. Pathotype and Biofilm production

All isogenic strains had *spa*, *atl* and *ica*A adhesin genes, but not all of them were biofilm producers. In particular, all strains of the group 2 were biofilm producers, as strain 3A and strain 3C, whereas group 1 and strain 3B were not biofilm producer. Moreover, all strains possessed *clf*A/B, *sdr*C/E and *cna* as shown in **Table 5.**

All strains in study presented the *cap*5 gene encoding the Capsular Antigen of type 5. *hl*-s genes were found in the entire sample, with *hlb* without the phi2819 insertion. As concern the 16 tested enterotoxin genes, the group 1 was lacking, whereas both the group 2 and the 3 presented *sed* and *sej*, and only the group 2 had *seg, seo, sem, sei* and *sen*. The entire sample was lacking of *tst* and PVL gene (*luk*S/F) as shown **in Table 6.**

4.3. Sequencing and sequence analysis

Sequence analysis of the regulator gene *gra*R excluded the presence of the mutation changing the 197th Asn in Ser, related to vancomycin resistance in all strains.

walK gene sequencing, also a gene part of a TCS regulatory system, revealed the absence of the truncating mutation of 3 nucleotides (CAA) from the position 1111 to 1113, in all tested strains.

The sequencing of *mprF* gene evidenced the mutation giving the S295L amino acid substitution only in the 1C strain, but was also found the mutation giving the T345I amino acid substitution in 2C strain, whereas no mutations were found in the *cls2* gene in all DNS isolates as shown in **Table 7.**

4.4. Study of Expression

Figure 7 shows the relative quantitative expression of autolytic, cell-wall turnover and cell-wall charge genes considered in the study, as fold changes of DNS versus DAP susceptible strains within the distinct groups of multiple isolates studied.

With regard to the three genes involved in cell-wall charge, *mprF* expression levels showed an up-regulation of its transcription in 1C, 2C-D, whereas a down-regulation in 3B-C isolates.

All DNS isolates had a *dltA* up-regulation, but it was evident that the group 3 showed a greater transcription level than the others DNS strains. No significant differences were found in the *dltA* mRNA transcripts of the hDAP strains of the group 2.

Moreover, all DNS and hDAP isolates presented a *cls2* up-regulation.

Considering genes involved in the cell-wall turnover and autolysis, all DNS exhibited high transcription levels of *sceD* and *atl*.

5. DISCUSSIONS

Daptomycin, vancomycin and linezolid are the first-line anti-staphylococcal agents of use in the treatment of multidrug resistant *S.aureus* (MRSA as well as MSSA) infections, therefore the recent emergence of strains with resistance or reduced susceptibility to these antimicrobials represents a serious problem of public health.

To further complicate the management of these severe infections, glycopeptide and daptomycin cross-resistance occurs, leading in some cases to the therapeutic failure (2,6,8). Recent studies have hypothesized that diminished vancomycin susceptibility of *S.aureus* is associated with the development of diminished daptomycin susceptibility (14,18,23,24), as it was also demonstrated, at molecular level, by our recent findings (60).

The phenotypic alterations in non-susceptible daptomycin *S.aureus* can be categorized into: i) cell wall structure changes; ii) changes in membrane composition, structure and potential; iii) modifications in depolarization, autolysis and permeabilization (1).

Numerous genetic loci have been involved in this complex mechanism such as the *mpr*F, encoding the bifunctional membrane protein mediating both the lysinylation of PG and its translocation to the outer leaflet of the membrane (56). Point mutations in *mpr*F, causing a gain-in-function, would accelerate membrane translocation of Lysyl-PG, resulting in a reduced net-negative membrane charge that can electrostatically repel calcium-complexed daptomycin (57).

Mutations in *walK*, encoding a sensor histidine kinase that regulates cell wall metabolism and virulence, and a point mutation in *rpoB* and/or *rpoC*, (subunits of RNA polymerase) has been further associated with daptomycin reduced susceptibility (67).

Moreover, an increased expression of the *dltABCD* operon, responsible for D-alanylating wall teichoic acids and involved in the net-positive surface charge, has been shown to be associated with reduced susceptibility to daptomycin (72).

It has been shown that negatively charged lipids, such as phosphatidylglycerol (PG) and cardiolipin, in the presence of calcium, allow daptomycin to insert and perturb bilayer membranes (106,57).

Thus, even if the phenotypic and molecular changes of DNS strains have been the topic of numerous recent papers, the molecular mechanisms responsible for daptomycin reduced susceptibility are still not clearly understood.

This study has the aim to investigates the mechanisms of daptomycin non susceptibility, on an accurately selected series of isogenic isolates, which include the initial daptomycin susceptible infecting strain and each subsequent DNS strain, isolated after exposure to different antibiotics, including teicoplanin and daptomycin (Table 1-2).

In detail, in the group 1 the isolation of the hTISA (1B) from the previous susceptible strain was subsequent to teicoplanin exposure, as the hVISA-DNS (1C) was subsequent to daptomycin exposure.

In the group 3, the treatment with teicoplanin determined the isolation of an hVISA isolate (3A), whereas the management with daptomycin led to the isolation of the DNS strains, shifting from hVISA to qVISA-DNS (3B-C). In the group 2, the DNS isolates was already altered in the susceptibility versus glycopeptides (in this case only teicoplanin), the subsequent isolation of the second hTISA strain (2B) was successive to teicoplanin exposure, whereas the isolation of DNS (2C) and hVISA (2D) occurred after the daptomycin and linezolid treatment.

From these observations, it would seem that the three phenotypes of reduced susceptibility (hTISA, hVISA, DNS) can emerge after the consecutive administration of teicoplanin firstly and then daptomycin respectively.

Moreover, it is important to highlight that all DNS strain were also hGISA, namely strains that exhibit a PAP/AUC analysis profile of heteroresistance versus teicoplanin (hTISA) or versus vancomycin and teicoplanin (hVISA) (Table 2).

Furthermore, it is interesting to note that all daptomycin susceptible of each group of strains, even if susceptible to glycopeptides, were delta-haemolysis negative, inducing, as hypothesized, a predisposition of the strain to acquire these new characters under antibiotic pressure.

All the sets of isogenic strains were MRSA of diverse genotypes as demonstrated by the membership to different STs, PFGE profiles, SCC*mec*, agr-group and capsular antigen type as follow: group 1 of ST398, SCC*mec*IVa, *Apa*I/α1 PFGE profile, agr-I and Cap5; group 2 of

ST5, SCC*mec*II, *Sma*I/USA100 PFGE profile, agr-II and Cap5; group 3 of ST22, SCC*mec*IV, *Sma*I/G PFGE profile, agr-I and Cap5.

Observing the virulence, it is observed that the group 2 is an hyper-virulent set, as demonstrated not only from the adhesion properties conferred by the ability to form biofilm and by an high adhesin gene content, but also from an high toxigenicity due to the presence of all hemolysins and of seven enterotoxin encoding genes, despite the PVL and *tst* lack.

A considerable virulence it is also found in the set 3 with the same distribution of adhesin genes and biofilm formation ability of the group 2, but with two instead of seven accessory enterotoxin genes.

A different situation was found in the group 1 that shows a less virulence, as the isolates, despite the presence of good adhesin gene content, were not biofilm producers and have a poor toxigenicity in terms of lacking of accessory toxin genes.

Furthermore, all isolates in study have a non-functional *agr*-locus although they belong to different *agr*-groups, evidencing that this is a predictive key feature for the emergence and development of both glycopeptide and daptomycin reduced susceptibility (100).

Looking at the molecular basis of the daptomycin non-susceptibility, this study was focused on the analysis of genes responsible for the phenotypic features of DNS strains such as genes related to the cell-envelope charge (*mpr*F, *dlt*A, *cls*2), genes involved in cell-wall turnover, growth, and cell separation (*sce*D-*,atl*), regulatory genes (*wal*k-*gra*R),

investigating both the presence of well-known mutations and their expression levels.

Focusing our analysis on cell-envelope charge genes results demonstrate that the hVISA-DNS strain in group 1, having the S295L in *mpr*F and no mutation in *cls*2, *wal*k and *gra*R, showed an up-regulation of *mpr*f, *dlt*A and *cls*2 cell-envelope charge genes as well as in the *sce*D and *atl* cell-wall structure related genes versus the VSSA-DSSA.

In group 2, the hTISA-DNS strain had the T345I amino acid substitution in *mpr*F, no mutation in *cls*2, *wal*k and *gra*R. This strain showed an up-regulation of all genes in study versus the hTISA-DSSA, as the strain hVISA-DNS of the group 1 (1C).

A different situation was found in the two qVISA/DNS of the group 3; in fact, they did not have mutations in none of the analyzed gene and showed a down-regulation of *mpr*F and an up-regulation of *dlt*A, *cls*2, *sce*D and *atl* versus the hVISA/DSSA strain (3A).

To analyze the complexity of the molecular mechanisms of daptomycin non susceptibility and to better understand the meaning of these data, it must be remembered that DNS strains have always reduced susceptibility to glycopeptides, so it is necessary to take into account that some molecular traits can be also due to the concurrent glycopeptide-resistance.

Not all DNS strains in the study presented *mpr*F mutations, the DNS strain of the set 1 has the S295L, the one of set 2 the T345I amino-acidic substitution, whereas those of set 3 do not contain any mutation,

but is important to underline that in the two sets containing mutations it is shown an *mpr*F up-regulation.

Since, this latter confers an higher cell envelope positive net charge responsible for a Ca^{2+}-DAP electrostatic repulsion mechanism, the presence of the above mentioned mutations and the *mpr*F up-regulation could be an addictive secondary trait, but not a keystone of DNS phenotype. Furthermore, the actual correlation between the *mpr*F mutations and its up-regulation cannot be closely established. It is known that also hVISA/DAP susceptible strains, as Mu3, present *mpr*F up-regulation despite they do not contain the S295L or other well known mutations in this gene (60).

Thus, it could be speculated, especially for the S295L, that it contributes to enhance *mpr*F up-regulation found in the groups 1 and 2.

This observation is further supported by the *mpr*F down-regulation recovered in the qVISA-DNS of the set 3, having no *mpr*F mutations and showing a VISA PAP/AUC behaviour characterized by an own *mpr*F down-regulation versus a VSSA phenotype, in agreement with our precedent findings (60).

Regarding the *dlt*A gene, it was found that the all DNS strains in study showed an up-regulation of *dlt*A gene. This gene is responsible for the d-alanylation of WTA that confers an increased net positive charge involved in the electrostatic repulsion mechanism of daptomycin resistance and indirectly in the regulation of the autolytic system.

On this basis, it can be hypothesized that the *dlt*A up-regulation is the feature that join all DNS. This feature is also independent from glycopeptide reduced susceptibility, because it is not present in hVISA whereas it is up-regulated only in VISA, as recently published by our group (60).

hGISA/DNS isolates of this study, in fact, presented a high level of *dlt*A expression whereas the qVISA/DNS ones showed a huge expression level, most likely caused by the accumulation of contributions due to both vancomycin and daptomycin reduced susceptibility. The *dlt*A up-regulation, thus, would play a key role in the mechanism of daptomycin non susceptibility representing the actual keystone for this phenotype.

Of note, the strains with an hDAP phenotypes, on the contrary, do not present a significant *dlt*a up-regulation, further supporting the key role of *dlt*A up-regulation exclusively in the DAP non susceptibility and not in mechanisms of DAP heteroresistance.

Regarding the *cls*2 gene, encoding the CL synthetase 2, the DNS strains in this study do not contain known mutations in this gene and all show *cls*2 up-regulation. However, this expression profile is not related to a repulsion mechanism, because *cls*2 is responsible for the net negative charge, thus an up-regulation of this gene increase and do not diminish the net negative charge of cell-envelope.

Shifting our attention to genes involved in cell-wall architecture, these data show that all DNS strains exhibit a *sce*D up-regulation, indicating a high cell-wall turnover activity, common characteristic of hVISA and

VISA strain, and an up-regulation of *atl* indicating a normal autolytic activity. These two observations are in agreement with the characteristic of glycopeptide-reduced susceptible strains, in which it was found a high level of turn-over and a reduced autolytic activity, by means of *atl* down-regulation only in VISA, Mu50, where is related to a full VISA phenotype and a vancomycin MIC value of 8 mg/L, never found in the DNS strains analyzed in this study (60).

In conclusion, our data highlight that daptomycin non susceptibility is due to a complex network in which different aspects can occur.

The foundation is surely represented by a mechanism of electrostatic repulsion, and indirectly a reduction of autolysin activity, due mainly to a *dlt*A over expression-dependent, *cls*2-independent mechanism, but also to the accumulation of additional secondary factors, such as the presence of mutations correlated with the increased levels of *mpr*F transcription, evident in the case in which the strain is both hGISA and DNS. Hence, some aspects remain linked to the daptomycin and glycopeptide cross-resistance, such as the presence of a high cell-wall turn-over, due to *sce*D up-regulation, that contribute to the thickness of the cell-wall and thus to its barrier effect against the penetration of the antimicrobials.

In conclusion, daptomycin non susceptibility shows a multifactor nature demonstrated by the involvement of multiple molecular routes at the basis of the acquisition of different phenotypic changes, enhanced by antibiotic exposure.

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7. TABLES

Table 1. Dates, Hospitals and conditions in which strains included in the study have been isolated

Strain	Date of Isolation	Hospital	Ward	Source	Antibiotic Therapy
1A	10.1.2010	Ospedale civico, Di Cristina, Benefratelli, Palermo (IT)	Vascolar surgery unit	Skin wound	Topic Gentamicin, Cotrimoxazole
1B	5.3.2010	Ospedale civico, Di Cristina, Benefratelli, Palermo (IT)	Vascolar surgery unit	Skin wound	Cotrimoxazole, Piperacillin/Tazobactam, Claritromycin, Teicoplanin, Gentamicin
1C	20.5.2010	Ospedale civico Di Cristina, Benefratelli, Palermo (IT)	Vascolar surgery unit	Skin wound	Daptomycin
2A	29.11.2010	Santa Maria della Misericordia, Udine (IT)	Infectious disease	blood	Daptomycin, Linezolid
2B	30.11.2010	Santa Maria della Misericordia, Udine (IT)	Infectious disease	pus wound	Daptomycin , Linezolid
2C	29.12.2010	Santa Maria della Misericordia, Udine (IT)	Infectious disease	blood	Daptomycin , Linezolid
2D	1.3.2011	Santa Maria della Misericordia, Udine (IT)	Infectious disease	blood	Daptomycin, Linezolid
3A	11.8.2010	Ospedali riuniti di Bergamo (IT)	Infectious disease	pus	Teicoplanin
3B	10.22.2011	Ospedali riuniti di Bergamo (IT)	Infectious disease	pus	Daptomycin
3C	10.2.2011	Ospedali riuniti di Bergamo (IT)	Infectious disease	pus	Daptomycin

Table 2. Phenotypic and molecular characteristics of the strains included in the study

Strain	OXA	MIC OXA (mg/L)	MET OXA (mg/L)	MIC (mg/L)			MET (mg/L)		VAN PAP/AUC	DAP PAP/AUC	ST	*ssc*mec	PFGE	*agr*-type	δ-hemolysin production
				VA	TEC	DAP	VA	TEC							
1A	R	32	256	1	≤0,25	≤0,25	4	4	VSSA	DSSA			ApaI/α1		-
1B	R	64	128	2	≤0,25	≤0,25	16	6	hTISA	DSSA	398	IVa	ApaI/α1	I	-
1C	R	64	256	2	2	4	16	4	hVISA	DNS			ApaI/α1		-
2A	R	256	256	1	2	0,5	16	6	hTISA	DSSA			USA100		-
2B	R	256	192	1	1	≤0,25	12	4	hTISA	hDAP	5	II	USA100	II	-
2C	R	256	128	2	2	2	12	6	hTISA	DNS			USA100		-
2D	R	512	>256	1	0,5	1	12	4	hVISA	hDAP			USA100		-
3A	R	2	4	1	16	0,5	6	>32	hVISA	hDAP			G1		-
3B	R	16	32	2	2	4	8	16	qVISA	DNS	22	IV	G2	I	-
3C	R	4	6/8	2	1	2	12	12	qVISA	DNS			G3		-

Table 3. Primer sequences used for pathotype determination

Primer	Sequence	Length (bp)	Annealing T°	Concentration (µM	Control Strains	Reference
Multiplex PCR						
hld up	5'-CTG AGT CCA AGG AAA CTA ACT CTA C-3'	220	55	1.2	NCTC 8325	Stefani et *al*. (2009)
hld dw	5'-TGA TTT CAA TGG CAC AAG AT-3'			1.2		
hlg up	5'-GCC AAT CCG TTA TTA GAA AAT GC-3'	938	55	0.4	NCTC 8325	Lina et *al*. (1999)
hlg dw	5'-CCA TAG ATG TAG CAA CGG AT-3'			0.4		
hla up	5'-AGG TTC CAT ATT GAT GAA TCC TG-3'	737	55	0.8	NCTC 8325	Stefani et *al*. (2009)
hla dw	5'-CCA TAG TAA TAA CTG TAG CGA AGT CTG-3'			0.8		
sea up	5'-GAA AAA AGT CTG AAT TGC AGG GAA CA-3'	560	55	0.8	FRI 913	Jarraud et al. (2002)
sea dw	5'-CAA ATA AAT CGT AAT TAA CCG AAG GTT C-3'			0.8		
cna dw	5'-GGT AAG GAA GAA GTG AAC GGG-3'	508	55	0.4	MW2	Stefani et *al*. (2009
cna up	5'-TCT CTG CTT TGT CTA CTG GTG TTG-3'			0.4		
sdrE up	5'-CAG TAA ATG TGT CAA AAG A-3'	767	50	0.8	COL	Peacock et *al*. (2002)
sdrE dw	5'-TTG ACT ACC AGC TAT ATC-3'			0.8		
icaA up	5'-CAT TGA ACA AGA AGC CTG ACA-3'	1060	50	0.4	NCTC 8325	Stefani et *al*. (2009)
icaA dw	5'-ATG CGA CAA GAA CTA CTG CTG C-3'			0.4		
atl up	5'-TAT TTG AGC AAC TTG GTT TAA TGT C-3'	470	50	1.2	NCTC 8325	Stefani et *al*. (2009)
atl dw	5'-CGG CTT ATC AAT GGT TCC TTG-3'			1.2		
fnbA up	5'-CAC AAC CAG CAA ATA TAG-3'	1362	50	0.8	NCTC 8325	Peacock et *al*. (2002)
fnbA dw	5'-CTG TGT GGT AAT CAA TGT C-3'			0.8		
clfA up	5'-CGA CAC AAT CAT CAT CAA CAA ATG-3'	476	55	1.2	NCTC 8325	Stefani et *al*. (2009)
clfA dw	5'-ATA ACC TGC TTG GTG CGG ATA-3'			1.2		
clfB up	5'-GCA ATA CCA CTA CAA CAG AGC CAG-3'	317	55	1.2	NCTC 8325	Stefani et *al*. (2009)
clfB dw	5'-AGC ATC AGC AGC ATT TAC TAC GC-3'			1.2		
sej up	5'-CTC CCT GAC GTT AAC ACT ACT AAT AA-3'	641	50	0.4	NRS 156	Becker et *al*. (2003)
sej dw	5'-TTG TCT GGA TAT TGA CCT ATA ACA TT-3'			0.4		
Conventional PCR						
spa up	5'-GGTACATTACTTATATCTGGTGGCG-3'	1142	56	0.4	NCTC 8325	This work
spa dw	5'-TTCTTATCAACAACAAGTTCTTGACC-3'			0.4		
lukS up	5'-ATC ATTAGGTAAAATGTCTGGACATGATCCA-3'	433	50	0.4	ATCC 49775	Lina et *al*. (1999)
lukF dw	5'-GCA TCA ACT GTA TTG GAT AGA AAA GC-3'			0.4		
tst up	5'-GCC CTT TGT TGC TTG CGA C-3'	548	50	0.4	NRS 111	Stefani et *al*. (2009)
tst dw	5'-TTT CCA ATA ACC ACC CGT TT-3'			0.4		
cap5 up	5'-ATG ACG ATG AGG ATA GCG ATA-3'	838	50	0.4	NCTC 8325	Stefani et *al*. (2009)
cap5 dw	5'-TTC TCG GAT AAC ACC TGT TGC-3'			0.4		
cap8 up	5'-GCA ATA CCA CTA CAA CAG AGC CAG-3'	1100	50	0.4	NRS 103	Stefani et *al*. (2009)
cap8 dw	5'-TTC CAA TTA CAT CAT TTC TAT AAG C-3'			0.4		
sdrC up	5'-CGAACATTGATATTGCGATTGAT-3'	187	55	0.4	NRS 1	Stefani et *al*. (2009)
sdrC dw	5'-GGTAATCTTACAGAACCTGGACGG-3'			0.4		
sec up	5'-CTCAAGAACTAGACATAAAAGCTAGG-3'	271	55	0.4	NRS 1	Stefani et *al*. (2009)
sec dw	5'-TCAAAATCGGATTAACATTATCC-3'			0.4		
sed up	5'-CTAGTTTGGTAATATCTCCTTTAAACG-3'	319	55	0.4	NRS 1	Stefani et *al*. (2009)
sed dw	5'-TTAATGCTATATCTTATAGGGTAAACATC-3'			0.4		
sek/q up	5'-TTAAGTGTCTCAAATAGTGCCAGCG -3'	449	55	0.4	USA300	This work
sek/q dw	5'-ATAGTGTTTTGCTTACCATTGACCC-3'			0.4		

Table 4. Real time primer sequences of the studied genes

Primer	Gene	Sequence	Amplicon (bp)	Reference
CV102	***gyr*B**	5'CAACTATGAAACATTACAGCAGCGT3'	256	Cafiso *et al*. 2012
CV103		5'TGTGGCATATCCTGAGTTATATTGAAT3'		
CV110	***atl***	5'GCTGTATCAGAATTTGGTGTTACATAG3'	279	Cafiso *et al.* 2012
CV66		5'CGGCTTATCAATGGTTCCTTG3'		
CV120	***sce*D**	5'CACCTGATGTTGGATTTACAGCA3'	202	Cafiso *et al.* 2012
CV121		5'CAATCACAAGAAGTTGAAGCACCA3'		
CV130	***mpr*F**	5'GAACCACCGTTTTCAACTGAA3'	244	Cafiso *et al.* 2012
CV131		5'GTAAATCTAACTCTGGCAACCATC3'		
CV79b	***dlt*A**	5'ATGTTTAGCATCAGGCGGTAC3'	247	Cafiso *et al.* 2012
CV80		5'ACTTGGGAAACGGCTCACTAA3'		
CV158	***cls*2**	5'ATTGAGTTAATCGTTGATGAGCAAT'3	225	This study
CV159		5'TTACGGATGTCTTGTTATTAGGTCAT'3		

Table 5. Capsular antigen type and toxin gene content of the entire sample

	Capsular antigen		Toxins																						
Strain	*cap5*	*cap8*	*hla*	*hld*	*hlg*	*hlb*	*splB*	*lukS/F*	*tst*	*sea*	*sec*	*sed*	*sej*	*sek*	*seq*	*seb*	*seg*	*seo*	*sel*	*sem*	*seh*	*sei*	*sen*	*see*	*sep*
1A	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1B	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1C	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2A	+	-	+	+	+	+	+	-	-	-	-	+	+	-	-	-	+	+	-	+	-	+	+	-	-
2B	+	-	+	+	+	+	+	-	-	-	-	+	+	-	-	-	+	+	-	+	-	+	+	-	-
2C	+	-	+	+	+	+	+	-	-	-	-	+	+	-	-	-	+	+	-	+	-	+	+	-	-
2D	+	-	+	+	+	+	+	-	-	-	-	+	+	-	-	-	+	+	-	+	-	+	+	-	-
3A	+	-	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
3B	+	-	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
3C	+	-	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-

Table 6. Adhesion properties of the sample: adhesin content and biofilm production

	Adhesins								
Strain	***icaA***	***sarA***	***spa***	***atl***	***clfA/B***	***sdrC/E***	***fnbA***	***cna***	**Biofilm production**
1A	+	+	+	+	+	+	-	+	-
1B	+	+	+	+	+	+	-	+	-
1C	+	+	+	+	+	+	-	+	-
2A	+	+	+	+	+	+	+	-	+
2B	+	+	+	+	+	+	+	-	+
2C	+	+	+	+	+	+	+	-	+
2D	+	+	+	+	+	+	+	-	+
3A	+	+	+	+	+	+	+	-	+++
3B	+	+	+	+	+	+	+	-	-
3C	+	+	+	+	+	+	+	-	+

Table 7. Presence of amino acid substitution in the studied strains

	Mutation (amino acid substitution)			
Strain	***gra*R**	***wal*K**	***mpr*F**	***cls2***
1A	-	-	-	-
1B	-	-	-	-
1C	-	-	**S295L**	-
2A	-	-	-	-
2B	-	-	-	-
2C	-	-	**T345I**	-
2D	-	-	-	-
3A	-	-	-	-
3B	-	-	-	-
3C	-	-	-	-

8. Figures

Figure 7. Relative quantitative expression of all the studied genes in each group of strains. Statistically significant difference ($p<0.05$) between sample and control are indicated with *.