ABSTRACT

In the last decade the increased use of vancomycin and teicoplanin (glycopeptides), as the first-line antibiotics for the therapy of infections due to Methicillin Resistant *Staphylococcus aureus* (MRSA), has led to the emergence of Vancomycin Intermediate *Staphylococcus aureus* i.e. heterogeneous Vancomycin-Intermediate-*Staphylococcus aureus* (hVISA) and homogeneous Vancomycin-Intermediate-*Staphylococcus aureus* (VISA). The genetic basis of reduced vancomycin susceptibility has been the subject of different papers: numerous genes (autolytic, cell-wall turnover and cell-envelope positive charge) and regulatory loci have been reported to be associated with glycopeptide intermediate resistance.

Moreover, some recent papers reported a positive correlation between vancomycin and daptomycin reduced susceptibility but the molecular machinery involved in this cross resistance, and the molecular and phenotypic changes of hVISA and VISA strains are not well understood.

The goal of our study was to investigate: the molecular basis of vancomycin reduced susceptibility, and the activity of vancomycin and daptomycin on the expression of the genes involved.

The study was performed on a sample of strains including VSSA, hVISA and VISA, grown with or without vancomycin or daptomycin,

and carried out by phenotypic assay (autolysis in Triton x-100, δ-haemolysin production on 5% sheep blood agar) and by quantitative relative real time RT-PCR on the genes involved in: autolysis (*atl*, *lyt*M), cell-wall turnover (*sce*D), membrane charges (*mpr*F, *dlt*A) and regulatory mechanisms (*agr locus*, *gra*RS, *wal*RK).

Our results show that hVISA and VISA strains, with respect to VSSA, are characterized by an up-regulation of *sce*D, a down-regulation of *rna*III (agr locus) and an up-regulation of *mpr*F in Mu3 (hVISA) and *dlt*A in Mu50 (VISA). The VISA strain, in addition, is characterized by a down-regulation of *atl* and *lyt*M genes versus hVISA and, obviously, VSSA.

At the light of these results we can conclude that hVISA and VISA strains possess an increased cell-wall turnover and positive charge, but a reduced *agr locus* functionality: these features are responsible for reduced vancomycin susceptibility and this distinguishes hVISA and VISA from VSSA. Furthermore, the VISA phenotype comes out from hVISA when it acquires a reduced both autolysis and net negative cell-envelope charge.

Another outcome is that vancomycin and daptomycin act in the same way, stimulating hVISA to acquire the VISA behaviour and increasing, in VISA, the cell-wall pathway at the basis of the intermediate

vancomycin phenotype. Moreover, daptomycin induces a charge repulsion mechanism in hVISA and VISA due to *mpr*F up-regulation.

ABSTRACT

Negli ultimi dieci anni l'aumentato uso di vancomicina e teicoplanina (glicopeptidi), come antibiotici di prima linea nel trattamento di infezioni causate da *Staphylococcus aureus* meticillino-resistenti (Methicillin Resistant *S.aureus*-MRSA), ha portato all'insorgenza di ceppi con ridotta sensibilità a vancomicina noti come heterogeneous Vancomycin-Intermediate *S.aureus* (hVISA) e Vancomycin-Intermediate *S.aureus* (VISA).

Le basi genetiche della ridotta sensibilità a vancomicina sono state oggetto di numerosi lavori scientifici, che hanno riportato il coinvolgimento di diversi geni (autolitici, implicati nel turnover del cell-wall e nella carica positiva netta di parete) e loci regolatori; ma i cambiamenti molecolari e fenotipici alla base dei fenotipi hVISA e VISA non sono ancora stati del tutto chiariti.

Recenti pubblicazioni, inoltre, hanno mostrato la presenza di una correlazione positiva tra la ridotta sensibilità a vancomicina e quella a daptomicina, ma il macchinario molecolare alla base di tale cross resistenza non è ancora stato spiegato.

Lo scopo del nostro lavoro è stato quello di studiare le basi molecolari della ridotta sensibilità a vancomicina e l'attività di vancomicina e daptomicina sull'espressione di geni coinvolti nel fenotipo di ridotta sensibilità ai glicopeptidi.

Lo studio è stato condotto su un campione di ceppi VSSA, hVISA e VISA, analizzati in presenza ed in assenza di concentrazioni sub-inibenti di vancomicina o daptomicina nel terreno di coltura. Il piano sperimentale ha previsto: saggi fenotipici (saggi di autolisi in Triton x-100, saggi di δ-emolisi su piastre di agar sangue di montone al 5%) e saggi quantitativi relativi di real time RT-PCR sui geni coinvolti nell'autolisi (*atl*, *lyt*M), nel turnover del cell-wall (*sce*D), nelle cariche positive nette di parete (*mpr*F, *dlt*ABCD) e nei meccanismi di regolazione (*agr*, *gra*RS, *wal*RK).

I risultati di real time RT-PCR quantitativa relativa mostravano una up-regolazione di *sce*D e una down-regolazione di *rna*III (*agr locus*) nei ceppi hVISA e VISA, rispetto al VSSA; inoltre, era presente una up-regolazione di *mpr*F in Mu3 (hVISA) e di *dlt*A in Mu50 (VISA). Il ceppo VISA, inoltre, era caratterizzato da una down-regolazione di *atl* e *lyt*M rispetto ad hVISA e VSSA.

Alla luce di questi risultati possiamo concludere che le caratteristiche alla base della ridotta sensibiltà a vancomicina che distinguono i fenotipi hVISA e VISA da quello VSSA sono: un aumento del turnover cellulare e delle cariche positive di parete ed una ridotta funzionalità di *agr locus*. Possiamo aggiungere inoltre che, il fenotipo VISA emerge da quello hVISA quando quest'ultimo acquisisce una ridotta autolisi e una riduzione delle cariche negative nette di parete.

Per quanto riguarda l'attività modulatoria di vancomicina e daptomicina, i nostri saggi di espressione ci portano a concludere che le due molecole antimicrobiche agiscano nella medesima maniera, cioè spingendo l'hVISA ad acquisire un comportamento da VISA, e aumentando nel VISA il pathway del cell-wall che rappresenta il fattore chiave nel fenotipo di ridotta sensibilità ai glicopeptidi. Daptomicina, inoltre, up-regolando *mpr*F in hVISA e VISA induce, in questi fenotipi, un meccanismo di repulsione di carica.

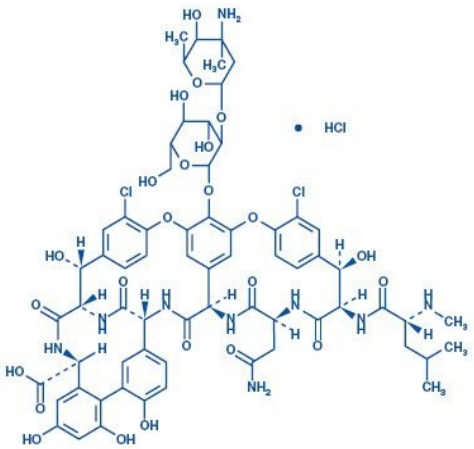
1. INTRODUCTION

Staphylococcus aureus has been recognized as an important cause of human disease for more than 100 years (1), and has become one of the most frequent causes of a wide range of both hospital- and community-acquired infections that range from superficial skin and soft tissue infection to life-threatening toxic shock, pneumonia, endocarditis and septicemia. The outstanding adaptive capacity of this pathogen has resulted in the worldwide emergence and spread of clonal strains that have acquired resistance to the majority of currently available antimicrobial agents (2).

Significant events in the evolution of *S.aureus* have included the development of methicillin resistance, now a problem for many hospitals around the world, and the recent emergence of community strains of methicillin resistant *S.aureus* also harbouring genes associated with increased virulence (3,4,5).

The acronym MRSA (Methicillin Resistant *S.aureus*) is one of the most widely known terms to arise from the infectious disease field in modern times. This pathogen is remarkable not only because of the significant associated morbidity and mortality, breadth of infectious syndromes, and very broad antibacterial resistance profile, but also because of the continuing and truly remarkable increase in the total numbers of infections (6,7). Today vancomycin use and MRSA are

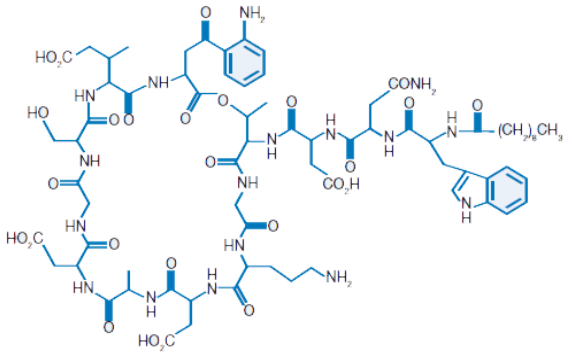
linked. The glycopeptide antibiotic vancomycin (VAN), in fact, used to be the first-line antibiotic for the therapy of infections due to MRSA, which are becoming increasingly common worldwide. For many years there was no indication that vancomycin resistance in *S.aureus* was likely to be a problem. Therefore, initial reports of reduced vancomycin susceptibility in clinical isolates of *S.aureus* from Japan in 1997 generated significant concern in the medical community (8,9).



Chemical structure of vancomycin

Emerging resistance to glycopeptides in Methicillin-Resistant *S.aureus* (MRSA) poses a great threat to antimicrobial chemotherapy worldwide. Together with the recent discovery, in 2002, of the first clinical isolate of fully Vancomycin-Resistant *S.aureus* (VRSA), numerous other isolates of homogeneous Vancomycin-Intermediate *S.aureus* (VISA) or heterogeneous Vancomycin-Intermediate *S.aureus* (hVISA) have been isolated worldwide (10). Several new antibiotics have been developed to counter this threat, and among them,

daptomycin (DAP) is reported to be potentially useful for treatment of infections caused by multidrug-resistant MRSA, including hVISA and VISA (11).



Chemical structure of daptomycin

1.1. Vancomycin-Resistant *Staphylococcus aureus* (VRSA), Vancomycin-Intermediate *Staphylococcus aureus* (VISA) and heterogeneous Vancomycin-Intermediate *Staphylococcus aureus* (hVISA)

Glycopeptides resistance in *Staphylococcus* has been attributed to various cell-wall abnormalities evolving in a multi-step fashion, and has emerged by two mechanisms. Highly glycopeptide-resistant *S.aureus* strains (VRSA) acquired the exogeneous multigene *van*A complex carried on transposon Tn1546 from *Enterococcus faecalis* by horizontal gene transfer. However, to date, only nine cases of VRSA have been reported from the United States, with two additional cases, one from India and one from Iran (12-16). This indicates that

although this mechanism of resistance is significant, it is not evolving or spreading rapidly. The second mechanism of resistance, termed endogenous or low-level (hVISA and VISA), arises because spontaneous mutation(s) are thought to confer a selective survival advantage. Endogenous resistance is supposed to occur stepwise: emergence of resistance to low-antibiotic levels must be first acquired to allow growth in progressively higher antibiotic concentrations (17,18). The exact molecular mechanisms leading to endogenous resistance to teicoplanin and vancomycin are unknown. A common resistance pathway has been suggested since, in general, strains with reduced susceptibility to vancomycin also display reduced teicoplanin sensitivity. However, teicoplanin resistance can be acquired without alteration in vancomycin susceptibility (17,19). Endogenous resistance is more often observed and clinical studies have linked clinical failure of glycopeptide with progressive selection of bacterial isolates showing increasing glycopeptide resistance levels. In some reported cases, as little as a two-fold change in MIC (Minimum inhibitory concentration) altered clinical outcome (20,21). Such concerns have resulted in the recent re-evaluation of clinical breakpoints for glycopeptides. The Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS), in fact, defined resistance breakpoints for MIC and disc diffusion testing of vancomycin against

S.aureus over 20 years ago (22). Initial breakpoints were as follows: susceptible at a vancomycin broth MIC of ≤4 mg/L, intermediate at a vancomycin broth MIC of 8 to 16 mg/L, and resistant at a vancomycin broth MIC of ≥32 mg/L. Subsequently, in 2006, the CLSI redefined vancomycin breakpoints as follows: susceptible at a vancomycin broth MIC of ≤2 mg/L, intermediate at a vancomycin broth MIC of 4 to 8 mg/L, and resistant at a vancomycin broth MIC of ≥16 mg/L (23). Hence, the current definition for VISA is an *S.aureus* isolate with a vancomycin broth MIC of 4 to 8 mg/L.

Summary of terminology for strains of *S.aureus* with reduced vancomycin susceptibility referred to in clinical case reports

Glycopeptide susceptibility classification	Broth microdiluition (mg/L)		
	CLSI prior 2006	CLSI after 2006	EUCAST
Susceptible (VSSA)	≤4	≤2	≤2
Intermediate (VISA)	8-16	4-8	-
Resistant (VRSA)	≥32	≥16	≥4

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The rationale for changing breakpoints was an increasing association between a vancomycin MIC of 4 mg/L and vancomycin treatment failure and also the increased detection of heteroresistant strains (22). However, the change in breakpoints will not help detect

heteroresistant strains with a vancomycin broth MIC of ≤2 mg/L. In addition, given the potential for differences in the vancomycin MIC results based on the methodology used (24,25), a vancomycin broth MIC using reference methodology such as CLSI broth microdilution should be used as the resolutive test for the definition of VISA.

The optimal laboratory detection of hVISA remains instead uncertain. Essentially, an hVISA isolate is an *S.aureus* isolate with a vancomycin MIC within the susceptible range when tested by routine methods (previously a vancomycin broth MIC of ≤4 mg/L and now a vancomycin broth MIC of ≤2 mg/L) but where a proportion of the population of cells are in the vancomycin-intermediate range (17). Typically, the resistant population is present at a frequency of $\leq 10^{-5}$ to 10^{-6}, hence the difficulty in the detection of this resistant phenotype using CLSI methods where an inoculum of 5×10^4 CFU per well (broth MIC) or 1×10^4 CFU per spot (agar dilution) is used (23). The relative proportion of the population of cells that is resistant to vancomycin at 4 mg/L can vary from strain to strain so that there is a spectrum from vancomycin-susceptible *S.aureus* (VSSA) to VISA. The accurate detection of this phenotype requires a vancomycin population analysis profile (PAP): in the modified vancomycin PAP described by Wootton *et al*., prototype hVISA strain Mu3 is used as a standard reference for the detection of hVISA isolates (26). By using

PAP as a reference method, the hVISA phenotype can be detected for strains of *S.aureus* with vancomycin MICs as low as 0.5 to 1 mg/L (22,27). In a recent clinical study, the hVISA phenotype was detected in 50% of clinical MRSA isolates with a vancomycin broth MIC of 2 mg/L (28). Therefore, if heteroresistance to vancomycin is clinically important for *S.aureus*, the current CLSI guidelines for the testing of vancomycin against *S.aureus* will not detect potentially important resistance in many isolates (19).

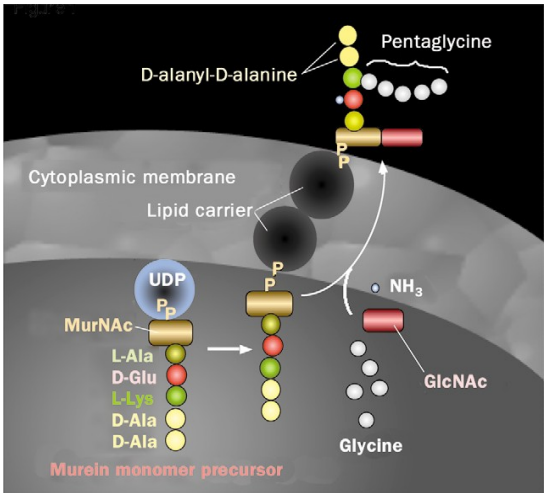
1.2. Staphylococcal cell-wall and mechanism of action of vancomycin and daptomycin

The staphylococcal cell-wall is a dynamic structure important for maintaining cell integrity and in host-pathogen interactions (29). The outermost surface of *S.aureus* is covered by a polysaccharide capsule. Under this lies the cell-wall, a structure composed of highly cross-linked peptidoglycan (PG) (a complex structure composed of sugars and amino acids, also called murein), teichoic acids, and cell-wall associated proteins (30). The peptidoglycan is composed of glycan chains made up of the alternating amino sugars *N*-acetylglucosamine and *N*-acetylmuramic acid. Stem pentapeptides (l-Ala-d-iso-Gln-l-Lys-d-Ala-d-Ala) are attached to the carboxyl group of each *N*-acetylmuramic acid, and interpeptide bridges (pentaglycines, made

up of glycine residues) connect the lysine component of one stem peptide to the penultimate d-alanine of a neighboring stem peptide (29). 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 (29,31). 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% (32). The peptidoglycan composition from different *S.aureus* strains is highly conserved suggesting that the composition is species specific (29).

Synthesis of the murein monomer

The murein monomer is composed of two amino sugars (N-acetyl muramic acid (MurNAc) and N-acetyl glucosamine (GlcNAc)) and ten aminoacids. The murein monomer precursor is composed of MurNAc and stem peptides (L-alanine, D-glutaminc acid, L-lysine, and two D-alanines). It is synthesised in the cytoplasm and attaches to a lipid carrier in the cytoplasmic membrane. Then, during its transfer to the outer surface of the cytoplasmic membrane, GlcNAc and five glycines are added, and its isoglutamic acid is amidated to become a mature murein monomer.

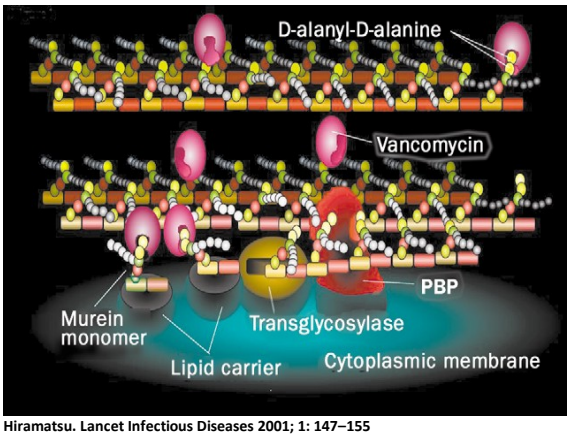


Hiramatsu. Lancet Infectious Diseases 2001; 1: 147–155

The staphylococcal cell-wall also contains teichoic acids, which represent up to 50% of the dry weight of the purified staphylococcal cell-wall. Ribitol teichoic acids [or wall teichoic acids (WTAs)] are covalently linked to peptidoglycan and present d-alanine and *N*-acetylglucosamine residues (33). 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* (34). Lipoteichoic acids appear to also be involved in cell division (33). Some data suggest that the role of teichoic acids is to help protect the cell envelope acting as a mechanical barrier to host defense molecules and antibiotics, and also, the positive charge of d-alanine residues repels positively charged molecules such as defensins (35,36). Wall teichoic acids also contribute to lysozyme resistance in *S.aureus* by preventing lysozyme binding to peptidoglycan (37).

Vancomycin 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 precursor for cell-wall synthesis (38). Vancomycin inhibits late-stage peptidoglycan

biosynthesis and acts outside the cytoplasmic membrane, which results in the intracellular accumulation of UDP-linked MurNAc-pentapeptide precursors (39,40). The vancomycin complex involves a number of hydrogen bonds between the peptide component of vancomycin and the d-Ala-d-Ala residues (41). Any process that interferes with vancomycin binding to d-Ala-d-Ala residues in the cell-wall will decrease the potency of the drug. The addition of "false" binding sites (e.g., a d-Ala-d-Ala-containing ligand) to a bacterial culture containing vancomycin leads to competition between binding sites and to a reduction of vancomycin activity. 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



Hiramatsu. Lancet Infectious Diseases 2001; 1: 147–155

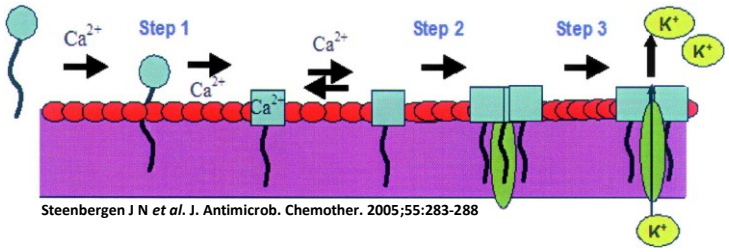
Vancomycin mechanism of action

Drug binds to D-alanyl-D-alanine residues of the murein monomer. The murein monomer, bound by vancomycin, does not serve as a substrate for glycosyltransferase.

this location, and the distance of this diffusion varies depending on the cell cycle, where a longer septum exists later in the cycle (42).

Daptomycin, a cyclic polypeptide, is a semi-synthetic lipopeptide antibiotic derived from *Streptomyces roseosporus*. It exhibits its bactericidal function by penetrating the bacterial cell-wall to bind cytoplasmic membranes, causing their rapid depolarization, resulting in the loss of membrane potential. This leads to inhibition of protein, DNA, and RNA synthesis, and finally bacterial cell death (11).

Daptomycin antibacterial activity requires Ca^{++}, which facilitates penetration into the membrane. Although daptomycin has one basic and four acidic amino acids, Ca^{++}-bound daptomycin acts as a cationic peptide and binds acidic lipids by an electrostatic interaction (43). Due to its unique mechanism of action, it has been generally assumed that daptomycin-resistant organisms are difficult to generate (11).



Daptomycin mechanism of action

Hypothetical steps: step 1, daptomycin binds to the cytoplasmic membrane in a calcium-dependent manner; step 2, daptomycin oligomerizes, disrupting the membrane; step 3, the release of intracellular ions and rapid cell death.

1.3. Phenotypic features of VISA and hVISA strains

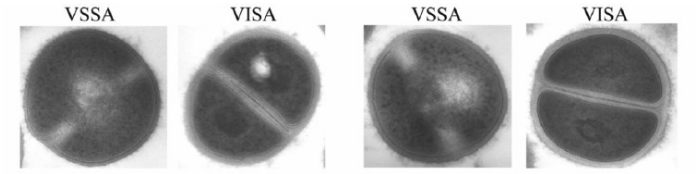
Common biochemical and morphological changes can be found in many hVISA and VISA, either clinical or laboratory induced strains; however, when observed in detail, the cell-wall rearrangements that occur in VISA strains can vary between strains (44). A consistent feature is cell-wall thickening, that may be associated with activated cell-wall synthesis (45,46,47). The cell-wall thickening is reduced when isolates are serially passaged and resistance levels drop (48). Other common features include an increased level of production of abnormal muropeptides; increased levels of d-Ala-d-Ala residues; reduced levels of peptidoglycan cross-linking in most isolates studied; a reduced growth rate; and reduced whole-cell lysostaphin susceptibility (49,50,51). The thickened cell-wall appears to be the most consistent feature, and although the exact mechanisms leading to thickening have not been determined, the thickened cell-wall is thought to prevent the diffusion of vancomycin to its active site in the cytoplasmic membrane in the division septum (51). Recent studies using fluorescent vancomycin and fluorescent ratio imaging microscopy demonstrated that the vancomycin binding capacity was increased in resistant strains, with evidence of a delayed access of vancomycin to the active site in the septum in resistant strains (42).

Recent papers report a strong positive correlation between reduced susceptibilities of VISA strains to daptomycin and vancomycin, and the correlation is just related to cell-wall thickening.

In fact, a thickened cell-wall is a common characteristic of VISA strains, serving as a physical barrier against the penetration of vancomycin molecules and resulting in vancomycin intermediate resistance. Since daptomycin has a quite big molecular size (molecular weight, 1,620.67), comparable to vancomycin (molecular weight, 1,485.7), it is possible that daptomycin might not be able to penetrate the cell-wall smoothly if the bacterial cell-wall becomes as thick as that of VISA strains. If this is the case, daptomycin might be blocked by the thickened cell-wall before reaching the cytoplasmic membrane, resulting in ineffective bactericidal function on the target cells (11).

Reduced autolytic activity is another widespread feature of hVISA and VISA strains and is a common early phenotypic change in serial isolates obtained during persistent infection (45,46,52,53), although this has not been demonstrated for all isolates (47). Studies on VISA strain Mu50 reported reduced whole-cell autolytic activity, the same as in other VISA strains (54). Some data suggest a possible role for wall teichoic acids of VISA strains suppressing peptidoglycan degradation by autolytic enzymes (51), while other studies suggest

that a reduction in the autolytic activity and altered peptidoglycan hydrolase activity of VISA autolysin extracts are responsible for the reduced autolytic activity (53). It was proposed that vancomycin directly blocks the activity of a peptidoglycan hydrolase by binding in the staphylococcal cell-wall and this explains the reduced autolytic activity demonstrated in the presence of vancomycin (55); however, this does not explain the reduced autolytic activity of hVISA and VISA strains in the absence of vancomycin (47).



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The panel demonstrates significant cell-wall thickening in VISA strains compared to VSSA strains (Transmission electron microscopy)

1.4. Molecular features of VISA and hVISA strains.

The genetic basis for vancomycin resistance in VISA remains largely unknown. Ever since the emergence of VISA in 1996, a number of investigations have been carried out to discover the molecular mechanism in the generation of this phenotype. More than a dozen genes have been reported to be associated with intermediate

glycopeptide resistance, including *pbp*B (56), *pbp*D (57), *sig*B (58,59), *tca*A, *mgr*A, *rsb*U, *spa* (60), *dlt*ABCD (61), *mpr*F (62), *sce*D (63), *atl*, and *lyt*M (11,46) as well as Two-Component Systems (TCSs, integrative signal transduction regulatory pathways composed of a sensing module, a histidine kinase, and its cognate transcription regulator) such as *wal*RK (2), *gra*RS (64), *vra*SR (19), and *agr locus* (65,66). Most of these reports have provided evidence of a correlation between gene transcriptional levels and altered glycopeptide susceptibilities of tested cells. Several sets of up- and down-regulated genes associated with glycopeptide resistance, including global regulators, were also reported (58,67,68,69).

However, except for the mutation in *vra*SR (I5N mutation confers heterogeneous vancomycin resistance when it was introduced into a VSSA-MRSA strain) and *gra*RS (N197S mutation converts hVISA phenotype in VISA), none of these reports identified the mutation responsible for altered vancomycin susceptibility (2).

1.4.1. The role of accessory gene regulator locus in vancomycin resistance

The accessory gene regulator locus (agr), a very well-characterized quorum-sensing two-component regulatory system, is a principal global regulator within the overall staphylococcal virulon (70). It

consists of a ~3-kb locus with divergent transcription units, which are driven by two promoters, P2 and P3 (71). The *agr locus* consists partly of 4 genes (*agr*BDCA), controlled by the P2 promoter, that act like a two-component regulatory system with its auto-inducing ligand. This transcript is called RNAII. The auto-inducing peptide (AIP) is encoded by *agrD* and is processed and secreted by the transmembrane protein AgrB (72). AgrC acts as the sensor histidine kinase that is phosphorylated in response to the AIP (73), while AgrA is the response regulator that is thought to bind and activate the two *agr* promoters, P2 and P3, thus completing the auto-inducing circuit; however, this has been difficult to demonstrate definitively (71).

The *agr* locus is conserved throughout staphylococci, with a hyper-variable region found between the 3' end of *agrB* and the 5' region of *agrC* (and including *agrD*) leading to the designation of four *agr* specificity groups or types (75). The ability of an auto-inducing peptide to activate its receptor is highly sequence specific, and a single amino acid change can alter group specificity. Therefore, functional variants of the *agr* locus would lead to cross-group inhibition rather than cooperative communication (71). The *agr* types appear to map to different clonal complexes by multilocus sequence typing (75), suggesting that *agr* may have evolved early in the staphylococcal evolutionary process (76).

An RNA molecule, RNAIII, (~0.5 kb) is produced by the divergent *agr* transcript promoted by P3 and overlaps the *hld* gene, which encodes δ-haemolysin (77). RNAIII is the intracellular effector molecule of the *agr* locus and is responsible for the direct activation or inhibition of other loci. RNAIII transcription increases during growth and is maximal in the post-exponential growth phase (78). It has a long half-life of approximately 15 min. RNAIII has a complex secondary structure consisting of 14 hairpins, many of which are conserved in all staphylococcal species (79). RNAIII up-regulates the transcription of genes encoding most of the extracellular proteins and down-regulates the transcription of genes encoding many surface proteins. RNAIII was also shown to act at the level of translation to control the expression of α-haemolysin and protein A (80, 77). Although *hld* is co-transcribed with RNAIII, it does not have any regulatory effect, as the deletion of the genomic region encoding this small 26-amino-acid haemolysin did not lead to an *agr*-negative phenotype (81).

A number of studies have linked alterations in *agr* activation or function with vancomycin tolerance, an increased tendency to develop vancomycin resistance, and the presence of the hVISA or VISA phenotype. In a number of these studies, determinations of *agr* activity were performed by the analysis of δ-haemolysin production on sheep blood agar and by using microarray transcriptional analysis

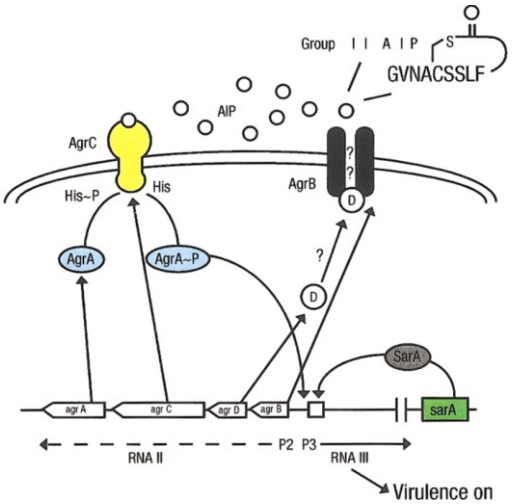
or real time quantitative PCR to measure RNAIII transcripts in paired isolates. Although mutations have been found in the *agr* locus in a small number of isolates, reduced levels of *agr* expression occur without mutations in the locus (47,82,65,66). The mechanisms of reduced levels of *agr* expression in these isolates are not completely understood. Initially, it appeared that *agr* group II isolates were over represented in VISA strains from around the world; however, a subsequent study from Europe demonstrated that many of their VISA and hVISA isolates were of *agr* group I (65,66,83). In addition, it was demonstrated that *S.aureus* from *agr* groups I to IV all develop intermediate vancomycin resistance upon *in vitro* exposure to the drug (84). It was demonstrated that when hVISA or VISA develops from VSSA, there is a reduction in levels of *agr* expression in the resistant isolates (47,82). Given the global regulatory role that *agr* plays in virulence factor expression, in particular the production of exotoxins and biofilm, it is likely that strains of hVISA and VISA that have reduced *agr* activity produce fewer exotoxins than parental strains. This has not been tested specifically (19,85).

In a number of *in vitro* studies, reduced *agr* function has been shown to favour the development of vancomycin resistance (65,66,84); however, an association between *agr* function and teicoplanin resistance development was not demonstrated (86). Assuming that

the relationship between *agr* function and the development of vancomycin resistance exists, it is worth noting that in one paper, 48% of hospital MRSA strains were found to have reduced *agr* function, compared to 3.5% of community-associated MRSA strains (87), suggesting a possible higher tendency for hospital MRSA strains to develop into hVISA or VISA strains. A clinical study also linked *agr* group II polymorphisms with poor responses to vancomycin therapy for patients with MRSA infections (88). In addition, a loss of *agr* function was associated with a reduced autolysis, a common feature of hVISA and VISA strains (19,65,66).

Structure and function of the accessory gene regulator (agr) locus of *S.aureus.*

Transcription at the P2 promoter results in the production of RNAII, which encodes 4 gene products: AgrB, AgrD, AgrC, and AgrA. AgrC is the receptor histidine kinase, and AgrA is the response regulator of a 2-component signal transduction pathway. This pathway is induced by a post-translationally modified cyclic octapeptide (AIP) that is processed from the AgrD gene product and secreted into the extracellular space via the 26-kDa transmembrane protein, AgrB. Activation of the circuit by the binding of AIP to AgrC leads to phosphorylation of AgrA, which, with the assistance of SarA, results in the production of RNAIII, the effector molecule that modulates transcription of numerous genes, including virulence factors. RNAIII is also translated into δ-hemolysin; therefore, δ-haemolysin activity can serve as a marker for production of RNAIII.



Sakoulas G *et al*. Clin Infect Dis. 2006;42:S40-S50

1.4.2. The role of *gra*RS in vancomycin resistance.

GraRS is a two-component regulatory system, so named for its glycopeptide resistance association (it has also been called *aps* for its more general antimicrobial-peptide-sensing capacity).

It is among the genes that have been observed to be upregulated in VISA strains (89). The over-expression of *gra*R or *gra*S results in a slight increase in the MIC of vancomycin, and a knockout mutation results in hypersensitivity (64,73). However, in two different instances, point mutations have resulted in increased resistance to vancomycin, presumably by modifying the activity of the proteins (90). The GraRS two-component regulatory system has been shown to control the expression of a large number of genes, including many genes involved in cell-wall synthesis. Interestingly, among the genes up-regulated in a knockout mutant, there are the genes involved in purine biosynthesis. The GraRS two-component regulatory system also positively regulates *rot* and *mgrA*, which encode global regulatory proteins that in turn control the expression of many genes encoding virulence determinants and others where the gene product is yet another regulatory protein (91).

Neoh *et al*. also used comparative genomics to investigate the evolution of VISA from hVISA by comparing the genomes of the hVISA strain Mu3 with a related VISA strain isolated from a different

patient, strain Mu50. Sixteen point mutations were observed, including a predicted amino acid substitution (N197S) in the GraR of Mu50, the response regulator of the above-mentioned GraRS two-component regulatory system. The introduction of GraR N197S from Mu50 into Mu3 converted this hVISA strain into a full VISA strain, accompanied by cell-wall thickening and decreased autolysis (92). The introduction of GraR N197S into N315 (VSSA) had no effect upon vancomycin susceptibility, thus indicating that additional genomic changes in N315 were required to develop the VISA phenotype. Microarray studies of Mu3 expressing GraR N197S suggested that at least 14 genes were specifically up-regulated by GraR, including the genes encoding the ABC transporters *vra*FG and *vra*DE. These experiments also showed that GraR N197S expression in Mu3 led to a down-regulation of *spa*. The reduced level of protein A expression is a known VISA phenomenon. In contrast, a *graRS* knockout in Mu3 did not lead to the VISA phenotype or any of the associated cell-wall changes. These data suggest that perhaps the N197S amino acid substitution resulted in GraR remaining in an activated "on" state (90). Another comparative genomics study investigated the evolution of VISA and also implicated the *graRS* locus in resistance. An isogenic pair of bloodstream VSSA and VISA isolates, obtained from a patient before and after 42 days of vancomycin therapy, were fully

sequenced and compared. Six mutations were detected, and one of these mutations was in *graS*, resulting in an amino acid substitution (T136I). This mutation was confirmed to be a key contributor to an increase in vancomycin resistance by the replacement of *graS* in the VSSA strain JKD6009 with *gra*S (T136I) from JKD6008. Significantly, however, the allele swap did not restore the full VISA phenotype and so, presumably, one or more of the remaining five mutations detected in JKD6008 are also required to generate the full VISA phenotype (19,93).

Different papers have also shown regulation of *mpr*F and *dlt*ABCD by *gra*RS but these studies did not assess the phenotypic consequences of this expression pathway in detail. However, a recent paper showed that the expression of *mpr*F and *dlt*ABCD were dependent on the co-transcription of both *gra*RS and *vra*G, and that reduced expression of *mpr*F and *dlt*A genes in *gra*R mutant was phenotypically associated with reduced surface positive charge (94,95).

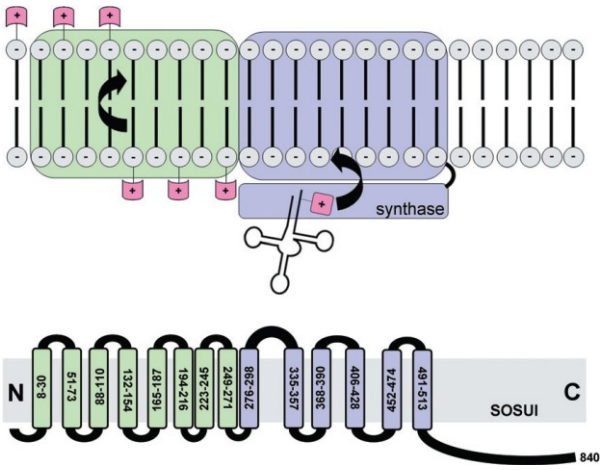
1.4.3. The role of *wal*RK (*vic*RK or *yycFG*) in vancomycin resistance

The highly conserved WalK/WalR (also known as YycG/YycF) two-component system is specific to low-G/C Gram positive bacteria. While this system is essential for cell viability, both the nature of its regulon and its physiological role have remained mostly

uncharacterized. Recently, Dubrac *et al.* (96) underlined the importance of the system in cell-wall metabolism in *S.aureus*; two reports, instead, have described the correlation of the *wal*RK system with the phenotype of vancomycin intermediate resistance in *S.aureus*. In particular, Jansen *et al.* reported that *wal*RK was highly up-regulated due to an insertion mutation in the *wal*RK promoter in a VISA clinical isolate (97,98) and *Mwangi et al.*, found a mutation in the *yyc*H gene in a clinical VISA strain (99). Thus both studies have suggested that the increment of vancomycin resistance was mediated by activation of the *wal*RK system. On the contrary, in a recent paper, Hiramatsu *et al*. reports a deletion mutation in *wal*RK and a truncating mutation in a proteoliytic regulatory gene, *clp*C, responsible for the raised vancomycin resistance in a laboratory derivate strain but they did not find any significant changes in the expression of *wal*RK in any of the resistant mutants studied (2). Moreover, Delaune *et al.* recently reported on the effect of *wal*RK on cell morphology, showing that *wal*RK depletion could raise the cell-wall thickness of *S.aureus*, but this regulatory pathway toward cell-wall thickening remains to be studied (97). The cause of raised resistance due to the *wal*RK mutation still remains unknown (2).

1.4.4. The role of *mprF* in vancomycin resistance

MprF proteins are integral membrane proteins with a characteristic structure. A large hydrophobic domain is found at the N-terminus. It consists of 14 transmembrane domains (TMDs) in *S.aureus* and many other bacteria but may contain as few as six TMDs in actinobacteria or may even lack TMDs entirely in some exceptional cases (100). A well-conserved hydrophilic cytoplasmic domain forms the C-terminus of most MprF proteins.



Proposed domain structure (top) and topology (bottom) of the *S.aureus* MprF.
The Lys-PG synthase domain is shown in blue. The exact extension of the Lys-PG flippase has not been elucidated yet.

Ernst *et al*. Molecular Microbiology (2011) 80(2), 290–299

The enzyme MprF catalyses Lysyl-phosphatidylglycerol (Lys-PG) biosynthesis. MprF recently turned out to be a bifunctional protein with two unprecedented functions in lipid lysinylation and in translocation of the resulting Lys-PG. MprF was identified when its inactivation rendered a *S.aureus* transposon mutant susceptible to a wide range of cationic antimicrobial peptides (CAMPs) leading to the name 'multiple peptide resistance factor' (MprF) (101). The cationic properties of CAMPs impart strong affinities to the negatively charged bacterial 'standard' lipids phosphatidylglycerol (PG) and cardiolipin (CL, also known as diphosphatidylglycerol), however, lysinylation of PG leads to a lipid with positive net charge, which reduces the bacterial affinity for CAMPs and renders bacteria more tolerant to CAMPs (102,103). MprF and Lys-PG are known to affect the susceptibility of *S.aureus* to cationic antibiotics such as the glycopeptides vancomycin and the aminoglycoside gentamicin (104). Shlaes *et al.* demonstrated that the inactivation of *mprF* by the insertion of Tn917 caused a significant increase in the binding of vancomycin to the cell membranes, in fact, vancomycin molecules contain NH_3^+ group and the inactivation of *mpr*F might promote vancomycin binding to the cell membrane by an electrostatic mechanism. This observation serves as a likely mechanism of the increased vancomycin susceptibility associated with *mprF*

inactivation (62). Similar observations were recently made for the lipopeptide antibiotic daptomycin (approved as an antibiotic of last resort against methicillin- and vancomycin-resistant *S.aureus*), which shares cationic properties and a membrane associated target (currently unknown) with CAMPs upon binding of calcium ions (105, 106). However, a number of reports on spontaneously daptomycin-resistant *S.aureus* strains that occurred during therapy have raised the spectre of spreading *S.aureus* clones with resistance to almost all available antibiotics (43,107). Most of the spontaneously daptomycin-resistant *S.aureus* clones turned out to have point mutations in a particular domain of MprF, which brought this protein into the focus of extensive research activities. Of note, MprF cannot be the target for daptomycin as *mpr*F deletion mutants are hyper-susceptible to daptomycin. On the contrary, all the reported mutations appear to lead to a gain of function of the protein that interferes with the ability of daptomycin to inactivate the bacteria. Expression of *mpr*F-specific antisense RNA was recently shown to re-establish susceptibility to daptomycin in daptomycin resistant strains thereby supporting the important role of MprF (108,109). Certain point mutations were repeatedly found in independent studies with clinical isolates (61,107) or with strains cultivated in broth with increasing daptomycin concentrations. Most of them accumulated in

a specific region of the N-terminal, the hydrophobic part at the junction of the Lys-PG synthase and flippase domains. It remains to be elucidated if these point mutations lead to increased Lys-PG production, to increased Lys-PG flipping to the outer leaflet of the cytoplasmic membrane or to another change in membrane properties. Some recent studies presented conflicting data that favour either of the possibilities indicating that MprF-mediated daptomycin resistance is a complex and maybe multifactorial process. Thus, it remains to be investigated in more detail how MprF leads to daptomycin and vancomycin resistance (61,109).

1.4.5. The role of the *dlt*ABCD operon in vancomycin resistance

The *dlt*ABCD operon controls the alanylation of wall teichoic acids (WTA) in response to antimicrobial challenge, indicating that the structure of teichoic acids can change in response to challenges, and is under the control of *gra*RS (*aps*) (89,91,94). The *aps* and *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 (35,36). There is also evidence of a link between the D-alanylation state of teichoic acids and vancomycin susceptibility in *S.aureus*, where a *dlt* mutant strain lacking in D-alanine was shown to have increased vancomycin

susceptibility compared to that of the wild-type strain (36). Wall teichoic acids also have a role in attachment to host cells, with studies demonstrating reduced nasal colonization and reduced binding to endothelial cells in strains deficient in WTA and in strains with a reduced *dlt*ABCD-mediated alanylation of teichoic acids (110, 111,112). It is therefore possible that for isolates of hVISA or VISA where the development of resistance is associated with an increased level of expression of *gra*RS or mutations in the locus, alterations in susceptibility to antimicrobial peptides are likely to occur, favouring resistance to these agents. There has been an association between hVISA and VISA and reduced susceptibility to daptomycin, in fact, Bayer *et al.* investigated the transcriptional expression of *dlt*ABCD genes also in daptomycin non-susceptible strains and demonstrated that enhanced *dlt* expression is related to increased positive charge and reduced daptomycin binding. Further work is required to clearly define the interplay between hVISA, VISA, and susceptibility to antimicrobial peptides in *S.aureus* (61).

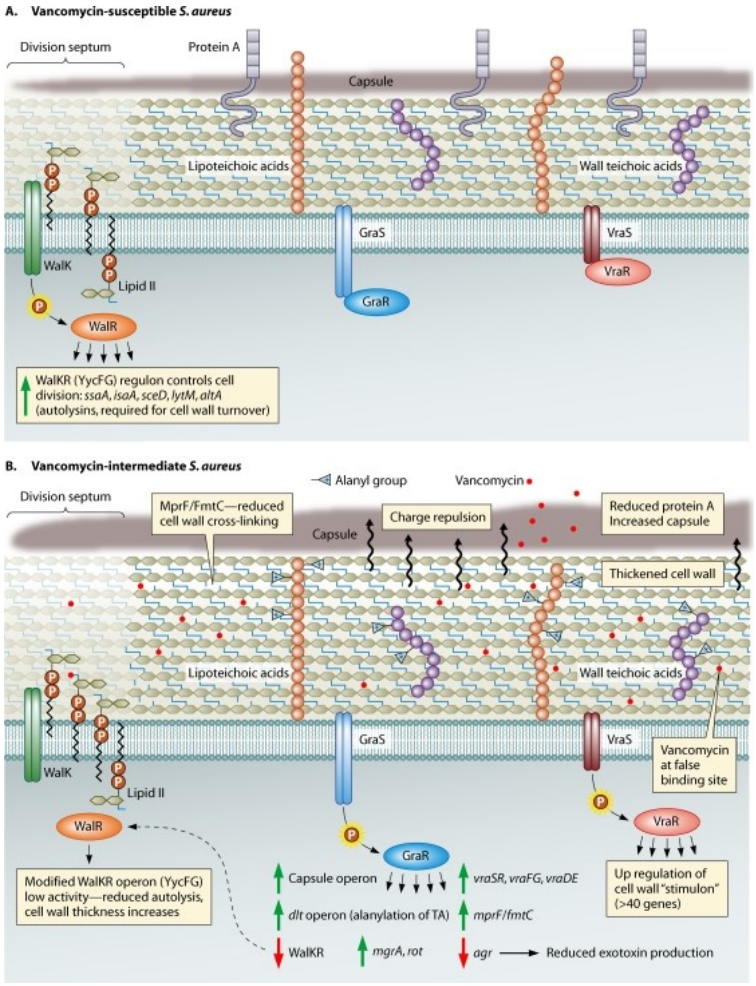
1.4.6. The role of *atl*A, *lyt*M and *sce*D in vancomycin resistance

Among factors controlling cell-wall expansion, remodelling, and daughter cell separation, peptidoglycan hydrolases, referred to as *autolysins*, which participate in peptidoglycan turnover playing an

essential role. Autolysins that cleave the cell-wall in a tightly controlled manner to maintain cell-wall integrity during cell division are classified according to their specific cleavage types. The major autolysis gene (*atl*) of *S.aureus* yields a 63-kDa amidase and a 54-kDa glucosaminidase after processing (113). Other autolytic genes include *sle*1, which encodes an additional *N*-acetylmuramyl-l-alanine amidase that is distinct from *atl* (114); *lytM*, which encodes glycylglycine endopeptidase (115,116); and *sce*D gene (SAV 2095 *sce*D-like gene) encoding a lytic transglycosylase (117,118). The decreased autolytic activity of a substantial proportion of clinical or laboratory GISA isolates has been previously reported (36,54,119,120). Two alternative mechanisms were proposed for explaining the autolysis-deficient phenotypes of GISA, one being the reduced expression of *atl* and the other the occurrence of cell-wall alterations that would decrease GISA susceptibility to autolysis (113). Recently, a proteomic paper reported that hydrolase LytM and the SceD protein, a putative transglycosylase, were increased in abundance in the cell envelope fraction of strains with reduced vancomycin susceptible, whereas the enzyme D-Ala-D-Ala ligase was decreased in its cytosol fraction. LytM, PBP2 and D-Ala-D-Ala ligase catalyze reactions in the biosynthesis or the metabolism of cell-wall peptidoglycan. This paper proposed that expression and activity changes of these enzymes in strains with

reduced vancomycin susceptible are responsible for an altered cell-wall turnover rate, and an altered peptidoglycan structure, which has yet to be elucidated for this highly vancomycin-resistant strain (63). 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 linking defective autolysis to glycopeptide resistance are still undefined (54,55,113).

Overview of some general cell-wall characteristics of VSSA and VISA strains showing the key regulatory elements linked to intermediate-level vancomycin resistance, as uncovered by comparative genomics and genetic studies



(A) VSSA strain in the absence of vancomycin showing a normal peptidoglycan (PG) layer, with the production of protein A and normal capsular polysaccharide expression. Also shown is the division septum, where cell-wall growth occurs. The lipid II-linked PG precursors assemble at the division septa, and the dipeptide moiety of lipid II is the target of vancomycin. (B) VISA strain with mutations in either the *graRS*, *vraSR*, or *walKR* operon (or all) that might lead to their respective regulons remaining in an activated "locked-on" or otherwise modified state. The consequence of this modification includes cell-wall thickening, decreased autolysis, reduced protein A production, increased capsule expression, increased d-alanylation of teichoic acids, and reduced *agr* activity.

Howden P.B. *et al.* CLINICAL MICROBIOLOGY REVIEWS, Jan. 2010, p. 99–139

2. AIM OF THE STUDY

To define the molecular mechanism at the base of glycopeptide intermediate resistance and the activity of vancomycin and daptomycin on the expression of genes involved in the mechanisms of cross resistance, we investigated a sample of VSSA, hVISA and VISA isolates for:

- autolysis ratio by Triton X-100 assay
- δ-hemolysin production by assay on 5% sheep blood agar plates
- *gra*R and *wal*K mutations
- the expression profile of some genes involved in autolysis (*atl*, *lyt*M), cell-wall turnover (*sce*D), membrane charges (*mpr*F and *dlt*ABCD) and global regulation (*agr*, *wal*RK), in drug-free conditions and with vancomycin or daptomycin in growth media, by relative quantitative real time RT-PCR.

3. MATERIALS AND METHODS

3.1. Bacterial strains

The *S.aureus* strains used in this study were NRS149 (VSSA), Mu3 (hVISA), and Mu50 (VISA) kindly supplied by NARSA (Network on Antimicrobial Resistance in *Staphylococcus aureus*) (www.narsa.net), considered as prototypes and controls of VSSA, hVISA and VISA phenotypes both in phenotypic and molecular experiments, and three clinical isolates collected in our laboratory CZ1, SS33, and 004/210. The source and relevant characteristics of the bacterial strains studied are listed in table 1.

3.2. MICs and Macro Etest

MIC (Minimum Inhibitory Concentration) determination for glycopeptides and daptomycin was performed according to CLSI guidelines (121).

The Macro Etest (MET) procedure was performed growing all clinical isolates overnight to a 2.0 McFarland standard in Müller-Hinton broth. A 100 µL sample was streaked onto BHI agar and vancomycin and teicoplanin Etest strips were applied (AB BIODISK, Solna, Sweden). Plates were incubated for 48 h at 37°C and were then evaluated for growth following the manufacturer's instructions

(EAS003; AB BIODISK) (122). Mu3 (hVISA), Mu50 (VISA) and ATCC 29213 (VSSA) were used as control strains.

3.3. Population analysis profile/area under the curve analysis (PAP/AUC)

The PAP/AUC procedure was performed as previously published (122). Briefly, colonies from cultures grown overnight on Tryptic Soy Agar were inoculated into Tryptic Soy broth. Following incubation for 24 h, dilutions of 10^{-3} (10^5 CFU/mL) and 10^{-6} (10^2 CFU/mL) were prepared in saline and 50 µL were inoculated onto different BHI agar plates containing 4.0, 6.0, 8.0, 12.0, and 16.0 mg/L vancomycin, and 4.0, 8.0, 16.0 and 32.0 mg/L teicoplanin. After 48 h of incubation at 37°C, colonies were counted and the log CFU/mL was plotted against the vancomycin concentration using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). The ratio of AUC of the test isolates to the AUC of *S.aureus* Mu3 was calculated and was interpreted as described previously (26). Mu3 (hVISA), Mu50 (VISA) and ATCC 29213 (VSSA) were used as control strains.

3.4. Molecular characterization

Molecular characterization of MRSA strains included in the study was conducted by MultiLocus Sequence Typing (MLST), Staphylococcal

Cassette Chromosome *mec* (SCC*mec*) typing and accessory gene regulator (*agr*) typing. All techniques were performed as previously published (123,124).

3.5. Autolysis ratio by Triton X-100 assay

Triton X-100 autolysis was assessed as described previously (45). Briefly, mid-logarithmic phase cultures grown in brain heart infusion broth (BHI) were assayed by rinsing bacterial cell pellets twice in ice-cold water followed by re-suspension in lysis buffer [0.05 M Tris–HCl pH 7.2, 0.05% Triton X-100 (Sigma)]. The decrease in absorbance (A_{620}) was monitored at 30 min intervals for 5h. The experiments were performed in triplicate and the results shown as the mean ± standard deviation of the autolysis ratio calculated as: O.D. T_0/T_5.

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

The functionality of the *agr*-operon was measured by δ-haemolysin production testing the strain by cross-streaking perpendicularly to RN4220, which produces only β-haemolysin (125,126), on sheep blood agar (SBA) plates. 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 (127). δ-haemolysin produced by a test strain results in a zone of enhanced haemolysis in areas where this lysis overlaps with the β-haemolysin zone of RN4420.

3.7. 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 3h) in the presence of VAN or Ca^{2+}-DAP, at a concentration equal to two dilutions below the initial MIC value, or in their absence, called "free condition" (F). Cells were then harvested by centrifugation, and the bacterial pellet was stored at -80°C until use. The cultures were re-suspended in 200 μl 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 10,000g 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 12,000g 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 determined by analysis of the $A_{260/280}$ ratio and analysis of the rRNA bands on agarose gels. RNA concentration was determined spectrophotometrically. Each extracted RNA sample was also used as a template in the PCR assay to confirm the absence of DNA contamination.

Retro-transcription and cDNA synthesis were 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 10^2 to 10^6 target copies per reaction (10^4-10^8 target copies per sample, standard curve), and only oligonucleotides giving PCR cycles which generated 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. *gyrB* was used as a normalizer (internal control) as previously published (128). All the primers used for the real time RT-PCR study are listed in table 2.

The expression of the studied genes is represented as the increment/decrement (fold changes) of: i) hVISA and VISA isolates (Mu3-CZ1-SS33-Mu50-004/210) versus the VSSA isolate (NRS149), in drug-free conditions (F) (shown as a histogram or by fold-change values); ii) each strain in culture with VAN or DAP with respect to the drug-free condition (shown as a histogram or by fold-change values); iii) each strain versus all the others both in drug-free conditions and in media with the addition of VAN or DAP (shown as a relationship of the relative amount of transcripts of all strains).

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 ($E = 10^{[-1/slope]}$) (129,130).

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 $P(H_1)$ 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 2,000 random reallocations of samples and controls between the groups. Statistical differences are significant when $p<0.05$.

3.8. Sequencing and sequence analysis

All amplification products were purified using the QIAquick PCR gel extraction Kit (Qiagen) and sequenced with a LI-COR DNA 4000L sequencer. The DNA sequence was analyzed by the gapped blast software (131).

4. RESULTS

4.1. Phenotype and molecular characteristics of the strains

Results of the macro Etest (MET), MIC and molecular characterization of strains in study are shown in table 1.

The sample, tested by Macro Etest and PAP/AUC analysis, presented a range of vancomycin resistance from susceptible (NRS149) to heterogeneous vancomycin-intermediate (Mu3, CZ1, SS33, 004/210) to homogeneous vancomycin- intermediate (Mu50).

Moreover, PAP/AUC analyses (gold standard method) results showed that CZ1, SS33, 004/210 have a PAP/AUC ratio, calculated with respect to Mu3, of 1.06, 1.07 and 1.22 respectively.

Results of daptomycin susceptibility assay revealed that all the strains were DAP susceptible with the only exception of Mu50 that had a MIC value of 2 mg/L (borderline phenotype).

The molecular characterization of strains revealed that all isolates belonged to agr-group II and to the clonal complex 5 (CC5) in particular: the prototype strains (NRS149, Mu3 and Mu50) were sequence type 5 (ST), while clinical isolates (CZ1, SS33 and 004/210) were ST228 (nosocomial ST). All the strains in study were methicillin resistant and possessed the staphylococcal cassette chromosome *mec*I (SCC*mec*I) and *mec*II.

4.2. Total autolysis ratio and δ-haemolysin production

The total autolysis assays, in drug-free condition, revealed a lower autolysis ratio in VISA strains, Mu50 (49%) and 004/210 (60%), and a large autolysis ratio (75-76%), similar to the VSSA one, in hVISA strains (Mu3, CZ1, SS33).

δ-haemolysin production assay results show: no δ-haemolysis activity in Mu50 and 004/210; a low activity in Mu3, CZ1 and SS33; and a strong activity in NRS149 (Tab.3 and Fig.1).

4.3. *gra*R and *wal*K mutations

Sequence analysis of *gra*R in CZ1, SS33, and 004/210, excluded the presence of the A1197S mutation that is related to vancomycin resistance. *wal*K sequencing revealed the absence of the truncating mutation of 3 nucleotides (CAA) from the position 1111 to 1113 in all tested strains.

4.4. Relative quantitative expression of autolytic, cell-wall charge and virulence regulator genes in drug-free conditions

The results of relative quantitative expression, in drug-free conditions, of the studied genes involved in autolysis, cell-wall charge and global regulators, presented as a ratio of transcripts of hVISA and VISA versus VSSA (calibrator), are shown in table 4 and figure 2A-B.

In particular, *rna*III, virulence regulator, was statistically down-regulated in Mu3, CZ1, SS33, 004/210, and more so in Mu50, with respect to NRS149, reflecting the results obtained in haemolysis assays (Tab.3), whereas the *gra*RS expression profile was, in all isolates in study, similar to that of NRS149 (data not shown). Regarding the expression results of *wal*RK, it showed an up–regulation in Mu3, CZ1, SS33 with respect to NRS149; no significant differences, instead, were found between Mu50 and 004/210 versus NRS149.

The expression study of the *atl* gene revealed only a down-regulation of this gene in Mu50 compared with NRS149; no significant differences were found in the other strains. The behaviour of *lyt*M transcripts was similar to the *atl* one, with the addition of a down-regulation of this gene also in 004/210.

The investigation of *sce*D transcripts showed an up-regulation in all isolates in study versus NRS149.

Concerning the analysis of *mpr*F expression, it showed an up-regulation in Mu3, CZ1, SS33 and 004/210 versus NRS149, while no significant difference was found in Mu50 with respect to NRS149. The study of *dlt*A transcripts only revealed its up-regulation in Mu50 versus NRS149.

4.5. Relative quantitative expression of autolytic, cell-wall charge and virulence regulator genes in the presence of VAN or DAP

Table 5 and figure 3 show the effect of sub-inhibitory concentrations of VAN and DAP on the expression profile of the studied genes in NRS149, Mu3 and Mu50.

In particular, the analysis of *rna*III and *gra*RS expression revealed no significant differences, and so no effect of VAN and DAP in all strains investigated (data not shown). *wal*K, instead, showed a down-regulation in Mu50 in the presence of both antimicrobials with respect to the drug-free condition (Tab.5).

Regarding the *atl* gene, in the presence of VAN and DAP, it was down-regulated in Mu3 and Mu50 with respect to the same strains in drug-free conditions. Comparing *atl* expression between Mu3 and Mu50, both with VAN and DAP, a lower amount of transcripts were found in Mu50.

The *lyt*M expression profile, with both antimicrobials, was similar to that of *atl* both in Mu3 and Mu50, but no significant differences were found between the *lyt*M transcripts of Mu50 with VAN with respect to the same in drug-free condition.

Even though the two antimicrobials have different effects on the *lyt*M expression of Mu3 and Mu50, a similar ratio of *lyt*M transcripts was found between these isolates in the presence of VAN and DAP.

*sce*D expression investigation showed a down-regulation in the presence of DAP, and more with VAN, in Mu3 and Mu50; comparing Mu3 with Mu50, in the presence of both antimicrobials, lower amounts of Mu3 transcripts with respect to Mu50 were revealed.

An up-regulation of *mpr*F in Mu3, and more in Mu50, was found in the presence of DAP; with VAN, on the contrary, *mpr*F was down-regulated in both strains.

Comparing Mu3 and Mu50, with both antimicrobials, no statistically significant differences were found in *mpr*F expression.

*dlt*A expression profile showed a down-regulation in Mu50 with both antimicrobials and in Mu3 with DAP, but no significant differences of *sce*D expression were found between Mu3 with VAN and the same in the drug-free condition. Comparing Mu3 and Mu50 in the presence of DAP, a higher *dlt*A transcription was found in the latter with respect to the former; in the presence of VAN, instead, no significant difference was revealed.

The analysis expression profiles of NRS149 (VSSA) in presence of VAN and DAP with respect to the drug-free condition showed: in the presence of VAN, a down-regulation of *lyt*M and *dlt*A, and an up-regulation of *atl*; in the presence of DAP, a down-regulation of *atl*, *sce*D and *dlt*A (Tab.5 and Fig.3).

5. DISCUSSION

The emergence of glycopeptide resistance in *S.aureus* is considered to be a serious menace around the world since vancomycin used to be the only first-line antibiotic for the therapy of MRSA. The acquisition of a vancomycin reduced susceptible phenotype is a multifactorial event and it has been linked to several abnormal physiological properties, including a thickened cell-wall, reduced cell-wall turnover and autolysis, and increased cell-wall synthesis that lead to a decreased rate of diffusion of vancomycin molecules to their active site, the division septum (19,132).

The genetic changes related with these physiological properties are far from clear despite this being the focus of numerous investigations.

On the basis of previous papers on proteomics and gene expression, we selected and studied the transcription profile of the following genes involved in regulatory mechanisms, cell-wall turnover, cell separation, cell-wall and cell envelope charge: i) agr locus, functionality and haemolytic activity; ii) *gra*RS and *wal*RK, two-component regulatory systems; iii) atl, encoding the most predominant *S.aureus* peptidoglycan hydrolase; iv) lytM, encoding Gly-Gly endopeptidase; and v) the *sce*D gene (SAV 2095 *sce*D-like gene) encoding a lytic transglycosylase (65,66,133).

Numerous studies have linked alteration in agr locus activation or function, performed by the analysis of δ-haemolysin on sheep blood agar, with reduced vancomycin susceptibility (19,134).

Our δ-haemolysin assay results revealed a low production of δ-haemolysin in hVISA and no production in VISA, most likely due to the loss of agr functionality (in agreement with previously published data) given that *hld* gene and its promoter are intact in prototype strains (Mu3 and Mu50). The expression study performed on *hld* correlated perfectly with the δ-haemolysin assay: we, in fact, observed a reduction of the amount of *hld* transcripts moving from VSSA to hVISA, and from hVISA to VISA. The agr locus plays a crucial role as a global regulator of virulence factor expression. It is possible that hVISA and VISA strains that have reduced agr-activity produce few exotoxins and thus these strains could be less toxigenic with respect to VSSA (19).

These results (presented at ECCMID 2011 Congress, Milan, by our research group) support the use of the δ-haemolysin assay as a rapid, easy and cheap screening method to identify hVISA and VISA isolates.

With regard to the two-component regulatory system GraRS, this appears to be involved in the reduced susceptible phenotype in different ways. The GraR N197S mutation, in fact, was shown to convert the hVISA phenotype (Mu3) into VISA, and a knockout

mutation results in the enhancement of autolysis and the reduction of net positive surface charge resulting in consequent hypersensitivity (92).

Our *gra*R sequence analysis excluded the presence of drug resistance associated mutations and the relative quantitative expression studies, conducted on our sample in all conditions (drug-free and in the presence of both drugs), revealed neither statistically relevant *gra*R up-regulation nor its involvement in *mpr*F and *dlt*A expression. These data are in contrast with other data previously published (92,109).

Concerning the analysis in drug-free conditions of the regulator WalRK, our results show an up-regulation and an *atl*/*lyt*M expression similar to VSSA in hVISA a *wal*RK: these transcription profiles correlate well to a high Triton X-100 autolysis. Expression analysis in VISA, instead, revealed a not statistically relevant increment in *wal*RK and a decrement of *atl*/*lyt*M correlating with a low Triton X-100 autolysis.

With this in mind, we conclude that the low *wal*RK expression in VISA can be correlated to the reduction of major autolysin activity; moreover, the presence of VAN and DAP can increase these molecular features in the VISA phenotype.

Our analysis of autolytic genes revealed that, in the drug-free condition, VISA strains showed a down-regulation of *atl* and *lyt*M with respect to hVISA and VSSA strains. These data suggest that a low Triton X-100 autolysis ratio is a VISA constitutive feature due to a reduction of *atl*/l*yt*M expression and represent a key characteristic in reduced vancomycin susceptibility.

The *atl*/*lyt*M expression profile and autolysis ratio of hVISA, instead, were similar to that of VSSA, indicating that cell lysis is not a key feature in the hVISA phenotype.

The expression study of *atl*/*lyt*M in drug-induced condition leads us to conclude that VAN and DAP show the same modulating activity on these genes in hVISA and VISA strains.

VAN and DAP, in fact, down regulate *atl* strongly in Mu3 (hVISA) and Mu50 (VISA), and help hVISA to acquire a behaviour similar to VISA; thus, we conclude that the reduction of *atl* expression is “drug-induced” in hVISA and “drug-enhanced” in VISA. A comparable conclusion can be made for the activity of both antimicrobials on *lyt*M in hVISA (“drug-induced” effect). In addition, in VSSA strains, VAN down-regulates *atl* as does DAP on *lyt*M, stressing that the presence of any of the two antimicrobials can induce VSSA to acquire the molecular characteristic traits of reduced susceptibility phenotypes.

Our *sce*D expression study, in drug-free conditions, shows a large quantity of *sce*D transcripts in hVISA and VISA, this data correlate with an increased cell-wall turnover and an altered cell-wall structure that are particular traits of these phenotypes as shown also in other papers (135).

The effect of VAN and DAP on *sce*D expression is similar, in fact, both down-regulated *sce*D in hVISA and VISA, in contrast with results of other authors (63). Our conclusions on these results are that *sce*D over-expression represents an innate character of the reduced susceptible phenotype.

With regard to *mpr*F and *dlt*A genes (involved in positive net charge respectively of cell-wall and cell envelope) they are associated with VAN and DAP reduced susceptibility, as has been confirmed in numerous papers.

The physiological role of *mpr*F is the synthesis of Lys-PG and the translocation of positively charged phospholipids to the outer membrane leaflet (62). The *dlt*ABCD operon, instead, controls D-alanylation of wall teichoic acids in response to antimicrobial challenge increasing the positive charge of the cell-wall, and in some way accelerates the autolysin activity (36,122,136,137).

Our investigations evidenced, in drug-free conditions, an up-regulation of *mpr*F in hVISA and *dlt*A in VISA. These results can be

translated into a major Lys-PG synthesis in hVISA and into a major D-alanylation of teichoic acids in VISA. In the light of these data, we conclude that the charge repulsion mechanism at the basis of reduced binding of cationic compounds (including VAN and DAP) is due to two different mechanisms in hVISA and VISA. In particular, the synthesis of Lys-PG plays a key role in hVISA increased positive cell-wall charge and thus in reduced susceptibility of this phenotype; while the synthesis of D-ala teichoic acid is crucial in VISA enhanced positive charge of the cell envelope, and then in the establishment of the vancomycin intermediate phenotype. The *dltA* up-regulation in VISA can be associated also with the reduced autolysis ratio specific to this phenotype and our conclusions are supported by the high autolytic activity of hVISA, where no *dlt*A up-regulation was found. Regarding the analysis of VAN and DAP effects on *dlt*A expression of hVISA and VISA, no inducing activity was found.

The expression results of *mpr*F show, instead, an up-regulation of *mpr*F in hVISA and VISA in the presence of DAP (not VAN), in particular the DAP effect is “drug-enhanced” on hVISA and “drug-induced” on VISA, demonstrating an *mpr*F implication in DAP reduced susceptibility.

Different studies showed that point mutations in the N-terminal region of *mpr*F are repeatedly found in DAP resistant strains, and all

reported mutations appear to lead to a gain of function of the MprF that interferes with the ability of DAP to inactivate the bacteria. In addition, some recent studies demonstrated that the DAP resistant strain possesses the S295L mutation and shows an *mpr*F up- or down-regulation associated with an up-regulation of *dlt*A (61,109,138). Our results show an *mpr*F down-regulation and a *dlt*A up-regulation in Mu50 (borderline phenotype with DAP MIC 2 mg/L) which does not possess the S295L mutation.

In conclusion, our study reveals in hVISA and VISA common constitutive characters that are closely related with reduced susceptibility phenotype, and that distinguish them from the VSSA phenotype.

These common features are: i) an increased cell-wall turnover, ii) an increased positive net cell-wall charge, and iii) a reduced agr-functionality.

In particular, the hVISA phenotype is due to an increased cell-wall turnover via *sce*D up-expression and a positive charge repulsion mechanism caused by *mpr*F up-expression; the VISA phenotype, instead, shows a reduced autolysis ratio for down-regulation of *atl*/*lyt*M and a reduction of negative cell envelope charge due to *dlt*A up-regulation. We can thus conclude that VISA emerges from hVISA

when it acquires a reduced autolysis and a reduced net negative charge via *dlt*A (Fig.4).

As regards the modulatory effect of the antimicrobials in study, VAN and DAP can influence their cross-resistance mechanisms and both, but particularly DAP, act on hVISA to acquire a VISA behaviour and on VISA to enhance its cell-wall molecular mechanism characteristic of vancomycin reduced susceptibility. In addition, DAP induces a repulsion electrostatic mechanism up-regulating *mpr*F in hVISA and VISA.

The acquisition of reduced glycopeptide susceptibility represents a significant event in *S.aureus* evolution; despite this having been a common theme in the staphylococcal literature, significant controversy still exists. A resolution of this controversy requires more molecular investigations and a full understanding of the physiology and molecular pathway of *S.aureus*.

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7. TABLES

Tab. 1: Phenotypic and molecular characteristics of the strains included in the study

Name	Origin	Source	Oxacillin Resistance	Glycopeptide phenotype	MIC (mg/L)			Macro ETest (mg/L)		MLST/*sscmec* characterization	agr-group
					V	T	D	V	T		
NRS149	NARSA	Nares of nurse	MSSA	VSSA	1	0.5	0.5	1	0.5	ST5	II
Mu3	NARSA	Purulent sputum	MRSA	hVISA	0.5	8	1	6	24	ST-5 *scc-mec*II	II
Mu50	NARSA	Wound Skin/Soft tissue	MRSA	VISA	8	16	2	12	12	ST-5 *scc-mec*II	II
CZ1	Clinical Isolate	Air pipe	MRSA	hVISA	1	0.5	0.5	4	16	ST-228 *scc-mec*I	II
SS33	Clinical Isolate	Blood	MRSA	hVISA	0.5	0.5	0.5	4	16	ST-228 *scc-mec*I	II
004/210	Clinical Isolate	Abscess/pus	MRSA	hVISA	1	0.5	0.5	6	12	ST-228 *scc-mec*I	II

Legend: V=Vancomycin, T=Teicoplanin, D=Daptomycin, S=Susceptible, R=Resistant

Tab. 2: Primer sequences of the studied genes

Primer	Gene	Sequence	Amplicon (bp)	Reference
CV102	***gyr*B**	5′ CAACTATGAAACATTACAGCAGCGT 3′	256	This study
CV103		5′ TGTGGCATATCCTGAGTTATATTGAAT3′		
CV110	***atl***	5′ GCTGTATCAGAATTTGGTGTTACATAG3′	279	This study
CV66		5′ CGGCTTATCAATGGTTCCTTG 3′		
CV122	***lyt*M**	5′ CTATACATTCGTAGATGCTCAAGGAC 3′	228	This study
CV123		5′ CGCTTGGTTGTTGTTACTATGTG 3′		
CV120	***sce*D**	5′ CACCTGATGTTGGATTTACAGCA 3′	202	This study
CV121		5′ CAATCACAAGAAGTTGAAGCACCA 3′		
CV130	***mpr*F**	5′ GAACCACCGTTTTCAACTGAA 3′	244	This study
CV131		5′GTAAATCTAACTCTGGCAACCATC 3′		
CV79b	***dlt*A**	5′ ATGTTTAGCATCAGGCGGTAC 3′	247	This study
CV80		5′ACTTGGGAAACGGCTCACTAA 3′		
CV25	***rna*III**	5′CTGAGTCCAAGGAAACTAACTCTAC 3′	235	Cafiso *et al.*2007
CV55		5′ TGATTTCAATGGCACAAGAT 3′		
CV114	***gra*R**	5′GTTGCTGGTATTGAAGATTTCG3′	202	This study
CV115		5′CGCCAAGTTCCATACTCATCAC3′		
CV116	***gra*S**	5′CACCTGTGACAGCCATGAAATTA3′	186	This study
CV117		5′CATCAATGACCATGCGTTTAAGTGACA3′		
CV142	***wal*KR**	5′AAACAACTACAATCCCTTCATACTAA3′	250	This study
CV147		5′CTTGACGGTTGGCATACTCACTTAA3′		

Tab. 3: δ-haemolysis, Autolysis Ratio, Percentage of Triton X-100 autolysis *vs.* VSSA

Strain	δ-haemolysin production*	Autolysis Ratio (means ± SD)**	Percentage of Triton X-100 autolysis *vs.* VSSA
NRS149	++	2.68 ± 0.05	-
Mu3	+/-	2.04 ± 0.07	76%
Mu50	-	1.33 ± 0.06	49%
CZ1	+/-	2.00 ± 0.04	76%
SS33	+/-	2.02 ± 0.07	75%
004/210	-	1.62 ± 0.07	60%

Legend:
***** (++) large δ-haemolysis zone; (-/+) low δ-haemolysis zone; (-) no δ-hemolysis zone
****** measured as the T_0/T_5 O.D. ratio ± SD (Standard Deviation)

Tab. 4: Relative quantitative expression of some autolytic, cell-wall charge and regulator genes in drug-free conditions

Gene	Product	Ratio of transcripts between strains of indicated combination as Mean of Fold Changes (standard error) - (in bold significant values p<0.05)					
Autolytic genes		**CZ1 *vs* NRS149**	**SS33 *vs* NRS149**	**Mu3 *vs* NRS149**	**004/210 *vs* NRS149**	**Mu50 *vs* NRS149**	**Relative amt of transcripts (p<0.05) ***
atl	N-acetyl muramoyl-L-alanine amidase	0.73 (0.62-0.98)	0.67 (0.57-0.93)	0.70 (0.60-0.96)	1.53 (1.19-2.06)	**0.59** (0.48-0.79)	**NRS149=Mu3=SS33=CZ1=004/210>Mu50**
***lyt*M**	Peptidoglycan hydrolase	1.09 (0.97-1.26)	1.04 (0.91-1.21)	1.07 (0.95-1.24)	**0.15** (0.09-0.28)	**0.36** (0.27-0.51)	**NRS149=Mu3=SS33=CZ1>004/210=Mu50**
***sce*D**	Trans-glycosylase	**1.85** (1.73-2)	**1.82** (1.70-1.97)	**24.25** (20-29.6)	**5.49** (3.51-9.51)	**19.33** (15.2-23.5)	**Mu50=Mu3>004/210>SS33=CZ1>NRS149**
Cell-wall charge gene							
***mpr*F**	Phosphatidyl- glycerol lysyltransferase	**3.28** (3.08-3.65)	**3.25** (3.05-3.62)	**5.96** (4.87-7.34)	**5.92** (4.69-7.48)	2.08 (1.65-2.59)	**004/210=Mu3>SS33=CZ1>NRS149=Mu50**
***dlt*A**	D-alanine-D-alanyl ligase	**0.34** (0.26-0.42)	**0.31** (0.230.39)	**0.30** (0.22-0.39)	**0.62** (0.46-0.75)	**2.23** (1.66-2.98)	**Mu50>NRS149>004/210>Mu3=SS33=CZ1**
Regulatory systems							
***rna*III**	agr-locus effector	**0.045** (0.03-0.05)	**0.042** (0.03-0.05)	**0.041** (0.03-0.04)	**0.05** (0.03-0.07)	**0.026** (0.02-0.03)	**NRS149>Mu3=SS33=CZ1=004/210≥Mu50**
***wal*KR**	Two component regulatory system	**2.00** (1.97-2.10)	**2.17** (2.00-2.30)	**2.28** (2.17-2.46)	1.12 (0.8-1.2)	0.76 (0.46-1.10)	**Mu3=SS33=CZ1>NRS149=004/210=Mu50**

* The relative amount of transcripts was obtained statistically evaluating gene expression levels of each strain versus all the others

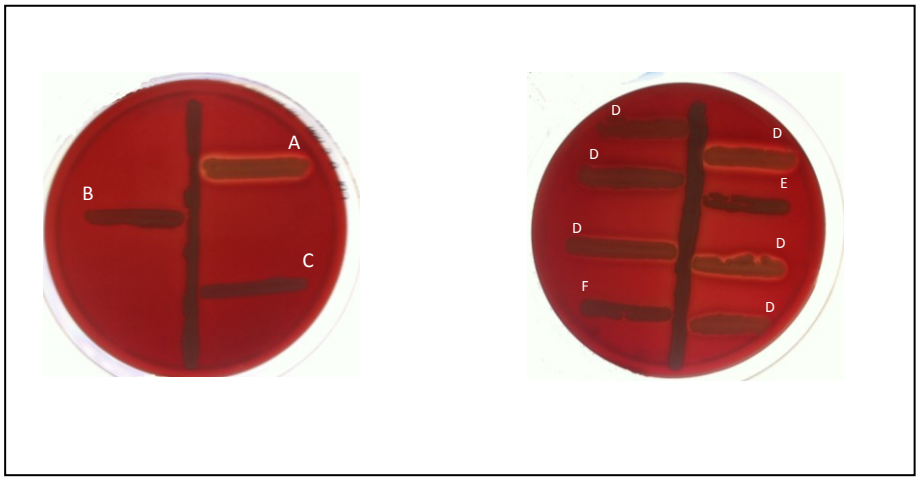
Tab. 5: Relative quantitative expression of some autolytic, cell-wall charge and virulence regulator genes with VAN (V) or Ca^{2+}-DAP (D) versus drug-free conditions (F)

		Ratio of transcripts between strains of indicated combination as Mean of Fold Changes (standard error)- (in bold significant values p<0.05)											
Gene	**Product**	**Mu50D *vs* Mu50F**	**Mu50V *vs* Mu50F**	**Mu50D *vs* Mu50V**	**Mu3D *vs* Mu3F**	**Mu3V *vs* Mu3F**	**Mu3D *vs* Mu3V**	**Mu3D *vs* Mu50D**	**Mu50V *vs* Mu3V**	**NRS149D *vs* NRS149F**	**NRS149V *vs* NRS149F**	**Relative amt of transcripts (p<0.05) ***	
Autolytic genes													
atl	N-acetyl muramoyl-L-alanine amidase	**0.3** (0.24-0.34)	**0.32** (0.26-0.36)	0.92 (0.80-1.10)	**0.053** (0.04-0.06)	**0.053** (0.04-0.06)	1.01 (0.94-1.08)	**7.27** (6.55-8.09)	**0.20** (0.16-0.26)	**0.57** (0.43-0.69)	**1.78** (1.42-2.34)	**Mu3F>Mu3D=Mu3V Mu50F>Mu50D=Mu50V**	**Mu3D>Mu50D Mu3V>Mu50V**
*lyt*M	Peptidoglycan hydrolase	**0.27** (0.19-0.47)	0.76 (0.57-1)	**0.35** (0.33-0.37)	**0.03** (0.02-0.04)	**0.02** (0.01-0.03)	1.09 (1.01-1.18)	0.36 (0.32-0.38)	0.89 (0.68-1.15)	1.05 (0.90-1.20)	**0.31** (0.19-0.61)	**Mu3F>Mu3D=Mu3V Mu50V=Mu50F>Mu50D**	**Mu50D=Mu3D Mu3V=Mu50V**
*sce*D	Trans-glycosylase	**0.64** (0.57-0.70)	**0.35** (0.32-0.38)	**1.8** (1.00-2.00)	**0.22** (0.18-0.54)	**0.08** (0.07-0.10)	**2.67** (1.05-6.88)	**2.9** (2.33-3.00)	**13.5** (10.2-17.1)	**0.238** (0.18-0.30)	0.75 (0.55-0.93)	**Mu3F>Mu3D>Mu3V Mu50F> Mu50D>Mu50V**	**Mu50D>Mu3D Mu50V>Mu3V**
Cell-wall charge gene													
mprf	Phosphatidyl-glycerol lysyltransferase	**11.03** (9.96-12.45)	**0.31** (0.26-0.35)	**34.37** (29.81-40.7)	**1.61** (1.47-1.7)	**0.34** (0.32-0.36)	**4.79** (4.37-5.20)	1.16 (1.04-1.32)	1.03 (0.88-1.28)	0.33 (0.29-0.38)	0.40 (0.34-0.47)	**Mu3D>Mu3F>Mu3V Mu50D>Mu50F>Mu50V**	**Mu50D=Mu3D Mu50V=Mu3V**
*dlt*A	D-alanine-D-alanyl ligase	**0.09** (0.07-0.12)	**0.058** (0.054-0.06)	**1.88** (1.4-2.3)	**0.37** (0.30-0.43)	0.88 (0.73-1.04)	**0.42** (0.37-0.48)	**0.22** (0.17-0.3)	0.98 (0.83-1.15)	**0.18** (0.16-0.20)	**0.37** (0.33-0.42)	**Mu3V=Mu3F>Mu3D Mu50F>Mu50D>Mu50V**	**Mu50D>Mu3D Mu50V=Mu3V**
Regulatory systems													
walK	Two component regulatory system	**0.26** (0.15-0.51)	**0.37** (0.27-0.60)	0.70 (0.65-0.72)	1.37 (1.28-1.4)	0.56 (0.48-0.62)	2.44 (2.2-2.6)	**15.81** (10.81-29.0)	0.66 (0.62-0.68)	1.01 (0.88-1.24)	1.21 (1.16-1.26)	**Mu3V=Mu3F=Mu3D Mu50F>Mu50D=Mu50V**	**Mu3D>Mu50D Mu50V=Mu3V**

* The relative amount of transcripts was obtained statistically evaluating gene expression levels of each strain versus all the others

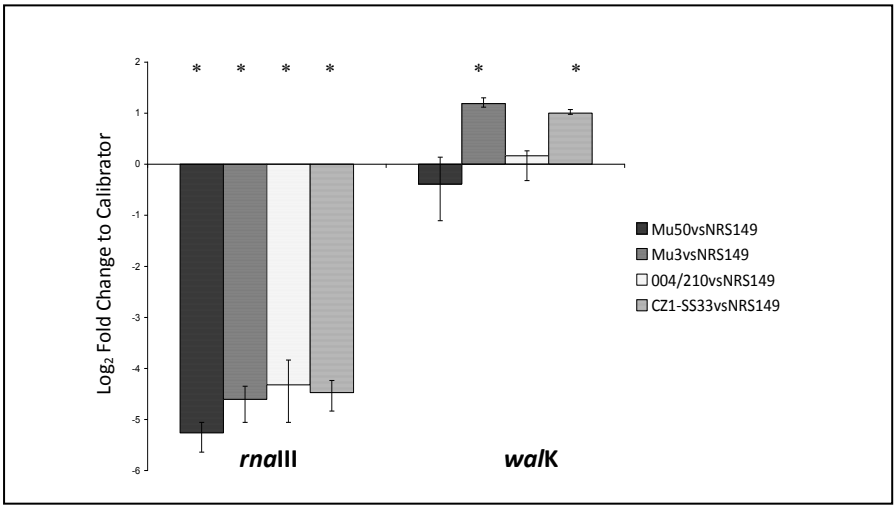
8. FIGURES

Fig. 1: δ-haemolysis assays on 5% Columbia sheep blood agar



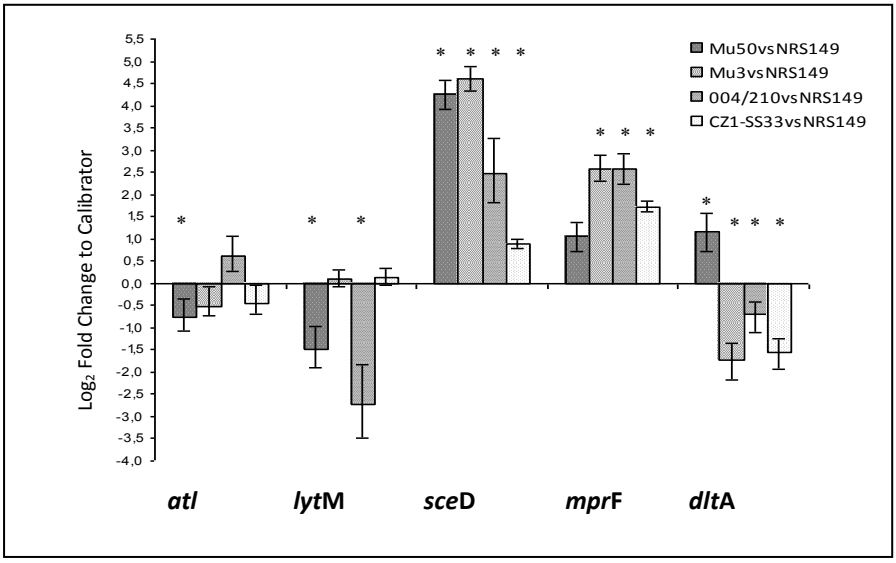
Legend: A) NRS149 (VSSA prototype); B) Mu3 (hVISA prototype); C) Mu50 (VISA prototype); D) VSSA clinical isolate; E) hVISA clinical isolate; F) VISA clinical isolate

Fig. 2A: Relative quantitative expression of the *rna*III and *wal*K regulator genes in hVISA and VISA in drug-free conditions



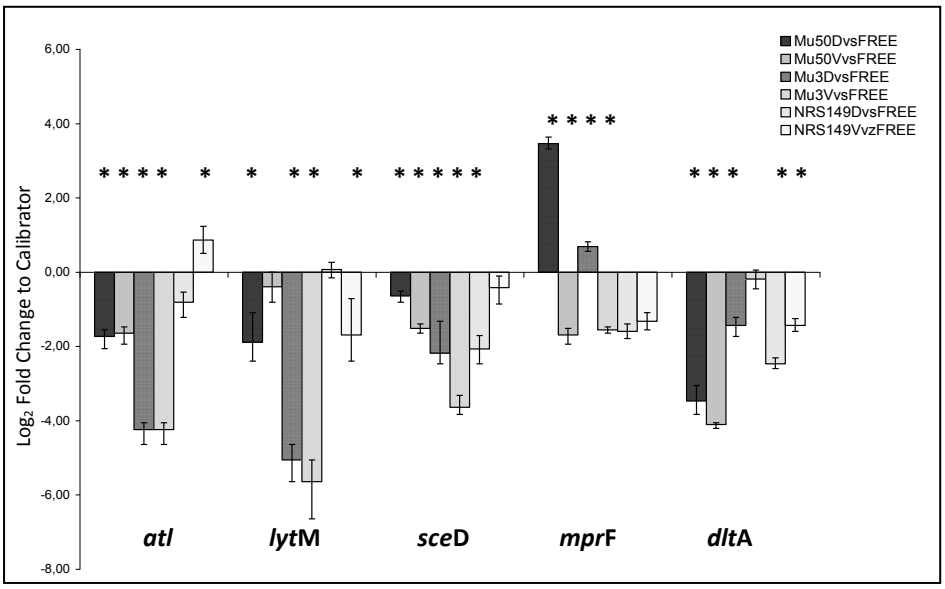
Legend: Statistically significant difference between sample *vs*. VSSA, $p<0.05$, are indicated with ∗

Fig. 2B: Relative quantitative expression of some autolytic, cell-wall turnover and cell envelope charge genes of hVISA and VISA in drug-free conditions



Legend: Statistically significant difference between sample *vs*. VSSA, $p<0.05$, are indicated with ∗

Fig. 3: Relative quantitative expression of some autolytic, cell-wall turnover and cell envelope genes of VSSA, hVISA and VISA with VAN (V) and Ca^{2+}-DAP (D) *vs* free-drug conditions (FREE)



Legend: Statistically significant difference between sample *vs*. VSSA, $p<0.05$, are indicated with ∗

Fig. 4: Trait progression involved in the development of the different vancomycin reduced-susceptible phenotypes