TESE DE DOUTORAMENTO

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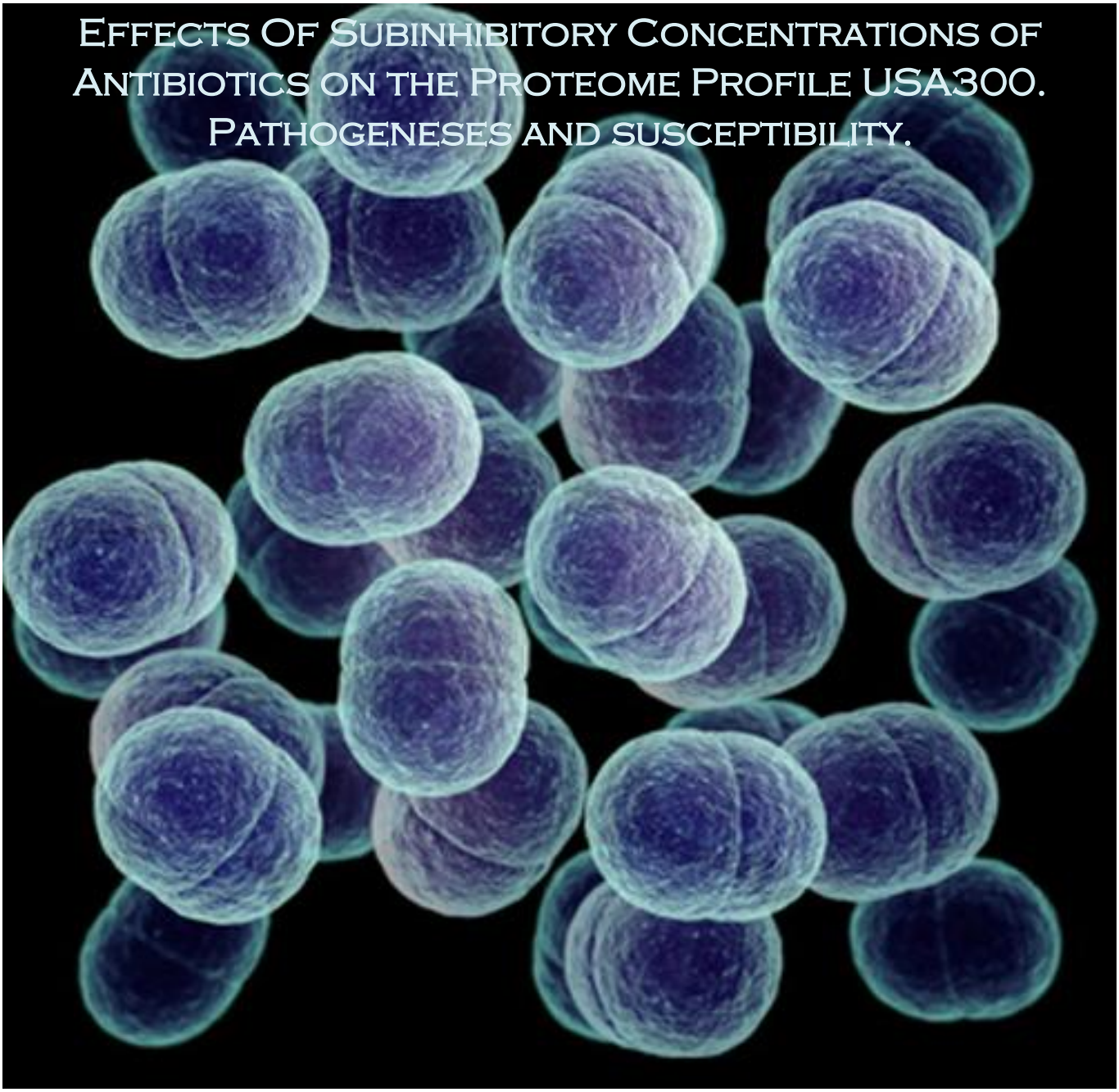

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Eva Torres Sangiao

Departamento de Microbioloxía

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DEPARTAMENTO DE MICROBIOLOXÍA

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COMMUNITY-ACQUIRED METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*:

EFFECTS OF SUBINHIBITORY CONCENTRATIONS OF ANTIBIOTICS ON THE PROTEOME PROFILE USA300. PATHOGENESES AND SUSCEPTIBILITY.

Memoria presentada por

EVA TORRES SANGIAO

para optar o grao de Doctor en Farmacia

Universidade de Santiago de Compostela

Departamento de Microbioloxía

&

Universitas Bergen

Department of Sciences, The Gade Research Group

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Carlos Garcia Riestra, Professor of Microbiology of the School of Medicine of the University of Santiago,

Support that the PhD thesis entitled

"Community-Acquired Methicillin Resistant *Staphylococcus aureus:* Effects Of Subinhibitory Concentrations of Antibiotics on the Proteome Profile USA300. Pathogeneses and susceptibility".

that **Eva Torres Sangiao**, BD in Pharmacy and Clinical Specialist/Consultant Microbiology and Parasitology, presents, it has been conducted under the co-direction and co-supervision of the University of Santiago, is considered completed and is authorized for submission, to be defended in the appropriate court, considering that it meets the requirements in article 34 of the regulation of the doctoral studies, and that as co-director do not incur the causes for abstention laid down in law 30/1992.

Santiago de Compostela, March 2015

Signs

Prof. Carlos García Riestra

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Esta tesis se ha realizado con el apoyo económico de

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Mama, te quiero!.

DEDICATORIA / DEDICATORY

A mi padre,

Por todo lo que sigues enseñándome día a día.

Te quiero, te echo de menos, y no olvides que siempre te querré.

For my dear daddy,

I miss you dad, and wish you can be here. Always I want you.

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ACME: Arginine Catabolic Mobile Element
Agr: Accessory Gene regulator
CA-MRSA: Community acquired- methicillin resistant *Staphylococcus aureus*
CC: Clonal Complex
CFU: Colony Forming Unit
CWA: Cell Wall Anchored
HA-MRSA: Hospital acquired-methicillin resistant *Staphylococcus aureus*
Hla/Hly: α-haemolysin, α-toxin
h-VISA: heterogeneous Vancomycin Intermediate *S. aureus*
LFQ: Label Free Quantification
MGEs: Mobil Genetic Elements
MICs: Minimal Inhibitory Concentration
MLST: Multilocus Sequence Typing
MRSA: Methicillin Resistant *S. aureus*
MSSA: Methicillin Susceptible *S. aureus*
PBPs: Penicillin Binding Protein
PSMs: Phenol-Soluble Modulins
PVL: Panton Valentine Leukocidin
SCC: Staphylococcal cassette chromosome
ST: Sequence Type
Two-CS: Two-Component System
TSST: Toxic Shock Syndrome Toxin
VISA: Vancomycin Intermediate *S. aureus*
VRSA: Vancomycin Resitant *S. aureus*

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Introduction

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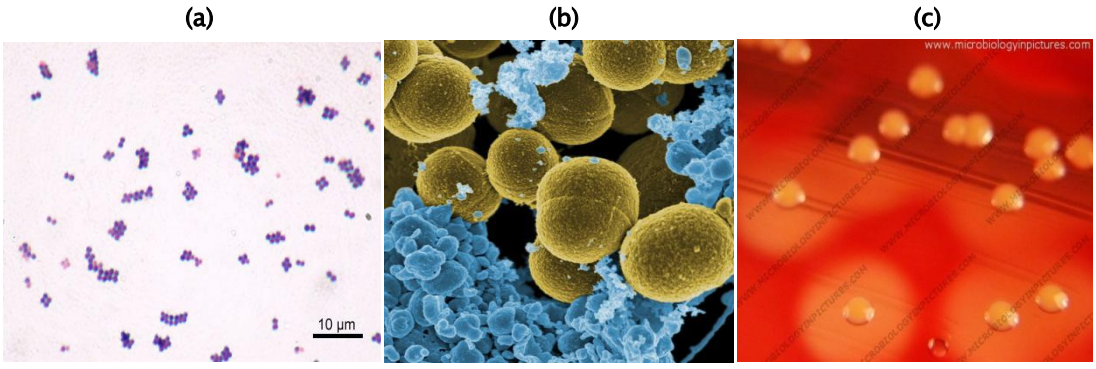
1 *Staphylococcus aureus*: The microorganism.

Staphylococcus aureus belongs to, Phylum 2 of *Bacteria*, class *Bacilli*, of the order *Bacillales*, family *Staphylococcaceae*, genus *Staphylococcus.* The genus includes 46 species, some of which are opportunistic pathogens for humans and animals (http://www.bacterio.cict.fr/s/staphylococcus.html).

The term *Staphylococcus* comes from the Greek *staphyle* = a bunch of grapes and *kokkus* = grains, the name was originally given by Ogston around 1883 although both Koch and Pasteur had also observed.[1] This are gram-positive cocci, non-spore former, low G+C content,[2] of 0.5-1.5µm in diameter, and a feature presentation in irregular clusters similar to grapes because of their ability to divide in three planes (**Figure 1a-b**).

The type specie, *Staphylococcus aureus* or "golden staphylococci" (**Figure 1c**), to produce carotenoids during growth, and have all the typical features of the genre. It is aerobes or facultative anaerobes, stationary, mesophilic and amino acids and vitamins are needed to grow. It is able to ferment glucose and mannitol with acid production, and tolerates highly variable environmental conditions. Thus, it can grow in any temperature between 6-46ºC (optimum 30-37ºC), pH between the values of 4.0-9.8 (optimum setting to neutrality). It is also tolerant to salt concentrations resisting up to 20% NaCl, and this is a property used to create selective culture conditions for its isolation and propagation.[3] This characteristic allows it to grow in foods with very low water activity. It is also quite resistant to drying, freezing and heat, but not as much as the endospores of spore-forming bacteria.

Figure 1. ***S. aureus*** **morphology**. (**a**) Gram stain of gram-positive coco grouped in clusters. (**b**) Scanning micrograph of *S. aureus*. (**c**) Growth in blood-agar-plate of *S. aureus´* s golden colonies.



S. aureus is the most virulent and pathogenic specie for human, due to its potential to cause a wide spectrum of infections and to be implicated in major outbreaks or hospital

and community epidemics. However it can be also found colonizing the skin and mucous membranes.[2]

1.1 Habitat and epidemiology.

Staphylococcus is ubiquitous colonizers of the skin and mucosa of all animals including mammals and birds. Some species have their preferential ecological niche as indicated by their names, i.e., *S. epidermidis* and *S. capitis* are constantly colonizing the skin, and hair & scarp, respectively.[2]

S. aureus is widespread among primates, but not restricted to them. In humans, the primary reservoir of *S. aureus* is the anterior nostrils, mainly in adults, though it can be isolated from multiple locations. Approximately 20% of healthy people are chronic nasal carriers of *S. aureus*, 30% intermittent carriers and 50% non-carriers. Colonization by *S. aureus* is more common in hospitals, especially in immunodeficient patients (undergoing dialysis, insulin-dependent diabetics, HIV seropositive), or intravenous drug users and patients with skin lesions.[4]

Methicillin-resistant *S. aureus* (MRSA) colonization has been increasing during the last decade. Factors associated with MRSA carriage include, prolonged hospitalization, prior use of antibiotics, surgery, intensive care units (ICUs) stay, living-in a nurse-home and close coexistence with a carrier of MRSA or infected patient.[5] Obviously, MRSA nasal carriers have an increased risk of infection with this microorganism, and have a higher morbidity and mortality compared with patients infected with methicillin sensible (MSSA).[6] Hence, to determine the clonality of MRSA in these patients, it is important to differentiate sporadic cases of MRSA and epidemic situation by a single clone. In addition, though community acquire- (CA-)MRSA has increased, the prevalence rate of MRSA among people without risk factors is relatively low, and most colonization and infection by MRSA, still develops in hospitals and/or health-care associated settings and in close contact with carriers.[2]

1.2 Microbiological diagnosis: Culture and Identification.

S. aureus grows well on non-selective culture media such as trypticase-soy agar supplemented with 5-10% sheep-blood (blood-agar-plate) or poor liquid medium such as trypticase-soy broth. In cultures from clinical samples, which may have gram-negative bacteria, it is advisable to use a selective medium to isolate *S. aureus*. For diagnosis from normally sterile samples, besides solid-agar medium, enrichment and thioglycolate broths should be used.[7]

The selective media used in most clinical laboratories to isolate *S. aureus* is the mannitol-salt agar medium (Chapman medium), which allows a presumptive identification based on the acquirement of a characteristic yellow coloration for colonies, due to mannitol fermentation with acid production, as a result the medium turns from pale pink to yellow (Figure 1.2.A).

In recent years, it has been developed culture media incorporating a chromogenic substrate and so allowing direct identification of *S. aureus,* even MRSA. In the presence of specific enzymes and substrates, these chromogens are modified to colored colonies (Figure 1.2.B). Although the cost of these resources is high, they let you generate the isolation and identification of *S. aureus* and MRSA directly.[8-10]

The identification of *S. aureus* can be performed by a few conventional biochemical tests. The workflow, from my point of view, could be, catalase detection to differentiate the genus *Staphylococcus* (catalase-positive) from the general *Streptococcus* and *Enterococcus* (catalase-negative), fermentation of glucose to differentiate among the genus *Micrococcus* (non-ferment anaerobically) and *Staphylococcus* (yes-ferment),[7] and coagulase, which remains the most commonly test used for identification of *S. aureus*. The coagulase test differentiates *S. aureus* (coagulase-positive) from the rest of staphylococci (coagulase-negative, CoNS) and it is based on the ability of *S. aureus* to produce the extracellular enzyme, which coagulates the plasma.

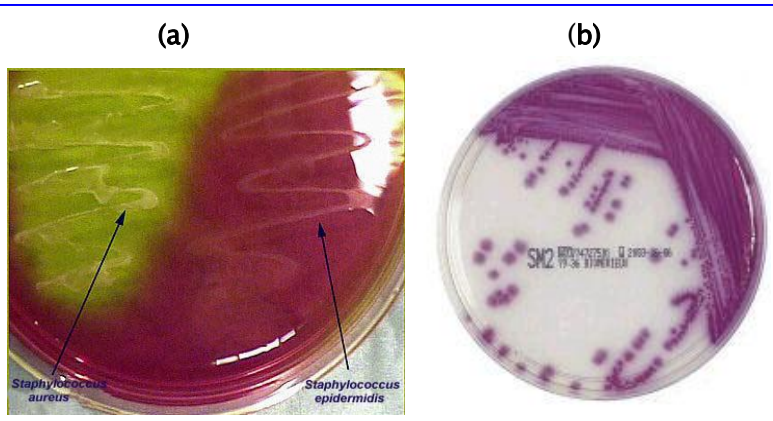


Figure 2.1. Culture and identification. (**a**) *S. aureus* on mannitol-salt agar medium. (**b**) MRSA on selective-differential medium.

In addition, small colony morphology variants (SCVs) of *S. aureus* have been described growing on blood-agar-plate as colonies approx. 1/10 of the usual size morphotype. These colonies are not pigmented and non-haemolytic, and require at least 48 hours of incubation to develop. They are mutants in the respiratory chain with low membrane potential, auxotrophic for haemin, use fewer carbohydrates and are resistant to aminoglycosides. In culture medium, these SCVs may occur alone or together with the

usual morphotype, giving the impression of a mixed culture. After subculturing, they may be stable or revert to the wild-morphotype, especially if the medium are supplemented with haemin or thymidine and incubated in a CO_2 atmosphere.[11,12] SCVs were recovered from the sputa of up to 20% of patients with cystic fibrosis carrying *S. aureus* or osteomyelitis infections, and were associated with prior treatment with trimethoprim-sulfamethoxazole[13] and aminoglycosides.

Clinical laboratories generally have commercial phenotypic identification systems. These manual or automatic systems use various dried substrates which allow the identification of different staphylococci spp. with a reliability ranging from 70 to over 90% depending on the system.[14] Currently some clinical laboratories have mass spectrometry technology, such as MALDI-TOF (Matrix Assisted Laser Desorption / Ionization- Time of Flight), allowing identification scores > 2.

1.3 Molecular diagnosis.

Although laborious and very costly, molecular diagnosis plays an increasing role in rapid detection of microbial pathogens and identification of drug-resistance determinants so that results can be obtained within a few hours. Identification techniques based on molecular probing[15] fluorescent detection of 16S rRNA with a peptide nucleic acid probe *(peptide nucleic acid fluorescence in situ hybridization,* PNA-FISH). It identifies *S. aureus* in positive blood cultures in less than 3 hours with $\geq$ 95% sensitivity and specificity.[16] The method allowed rapid discrimination between *S. aureus* and potential contaminant coagulase-negative, thus improving therapeutic decision-making.[17] Another technique developed to quantify organisms directly in clinical sample has been *multiplex real-time PCR,* which amplified simultaneously genes of species and resistance mechanism.[18,19] A wealth of molecular techniques is being developed and proposed for routine use. Further high-throughput techniques, including RNomics and proteomics, might provide a comprehensive picture of the "good- coagulase-negative" and the "bad-aureus" staphylococci and help to decide which of them must be considered for therapeutic intervention.

1.3.1 Molecular Typing.

Molecular typing systems are one of the great microbiological diffusion contributions in recent years. These systems consist of a variety of techniques, which are intended to compare the composition of the nucleic acids of two or more microorganisms, thereby can be recognized the relationship between epidemiologically linked isolates, and therefore a recent derived from a common precursor microorganism. In addition,

techniques must be able to differentiate unrelated isolates, irrespective of their belonging to the same microbial species or taxon.[20,21]

The application of these techniques is essential in the study of nosocomial infections, especially in hospitals where there are available ICUs, neonatal units, burn units, hematology or oncology, wherever inpatients are more capable of acquiring nosocomial severe infections, particularly MRSA (59% for ICUs patients, 55% in non-ICU patients, and 48% in outpatients),[22] which usually appears to be highly clonal.

The main typing methods underlying this comprehension are briefly presented subsequently.

1.3.1.1 *Pulsed Field Gel Electrophoresis (PFGE).*

Pulsed field gel electrophoresis (**PFGE**) is a highly discriminative molecular typing technique that is used in epidemiological studies worldwide. Actually it is the most widely used method and is extremely useful for following given clones, although it does not provide accurate information on the genealogy of the organism.

PFGE is a restriction-fragment length technique to separate large chromosomal fragments generated by digestion with the low-frequency cutting enzyme Sma1 for *S. aureus*. The generated fragments are separated, yielding banding patterns specific for particular clones. Banding comparison has allowed identification of the major epidemic clones, which represented 70% of more than 3000 MRSA isolates recovered worldwide.[22] However, the length of chromosomal fragments, and thus the clone-specific banding, may be modified with acquisition or loss of mobile DNA (MGEs) such as transposons, prophages, or pathogenicity islands. The new banding pattern may identify a different clone, which is in fact the same bacterium that has gained or lost MGEs. If the new organism has acquired properties important for successful spread, it may indeed behave as a new clone with its proper behaviour. Nevertheless, the phylogenetic relation between the new clone and the parent persists.

1.3.1.2 *Secuenciación: "Multilocus Sequence Typing" (MLST).*

Multilocus sequence typing (**MLST**) is a technique designed to track clones and/or clonal lines and is a good molecular marker of long-term epidemiology. However, its discriminatory power is lower than other techniques such as PFGE, and it is a demanding technique in terms of methodology and expensive. MLST is a sequence-based method that allows the unambiguous assignment of the ancestral phylogeny of the staphylococcal population.[39] The technique consists of sequencing a total of seven housekeeping genes:

- *arcC:* carbamate kinase.
- *aroE:* shikimato dehydrogenase.
- *gmk:* guanylate kinase.
- *pta:* phosphate acetyltransferase
- *tpi:* triosephosphate isomerase
- *yqiY:* acetyl coenzyme A acetyltransferase.
- *glpF:* glycerol kinase.

and submitting the sequences to a central database (www.mls.net) where they are checked and matched. The classification is based on allelic profile diversity based on approximately 300–700–bp internal gene fragments. Thousands of sequences have been submitted, generating numerous sequence types (STs). Organisms that share all seven alleles are defined as *clones,* those that share five of seven identical alleles are defined as *clonal complexes* (CC), and those that share less than seven alleles are defined as *unrelated.*

Since the implementation of this technique in *S. aureus*, over 2000 year, it has been found that MRSA strains have a clonal structure highly well conserved compared with MSSA, and that a small number of clones are able to spread.[23,24] Also the data obtained by MLST, indicate that *S. aureus* has a low level of genetic recombination and clonal diversity is more frequently caused by point mutations than genetic exchange processes.

1.3.1.3 *"Spa typing" y "double locus spa-clfb typing".*

Spa typing and ***double-locus spa-clfb typing***, are sequencing-methods, based on PCR amplification of strain-specific regions of hypervariable segments of the *spa* (protein A) or *clfB* (clumping factor B) genes, respectively.[25] The variable regions are made of 24 nucleotides repeats in *spa* (http://spa.ridom.de/) and serine-aspartate repeats in *clfB*, the length of which may vary from duplication or accidental loss of DNA material. Although less discriminative, these simpler methods generate unambiguous data sets that can be compared in multicenter studies.

Typing has become an important part of the comprehension of the *S. aureus* epidemiology. However, no specific types can be attributed to disease producing *versus* colonizing strains, as yet.[25]

2 *Staphylococcus aureus*: Pathogenesis.

S. aureus can cause a wide range of infections, from superficial skin infections to deep-seated infections, from which it spread through the blood stream. The wide range of infections caused, is due to expression of several proteins, which may be surface-associated or secreted. Many of these proteins are involved in colonization of host tissues, lysis of host cell membranes, promotion of bacterial spread within host tissues and survival in phagocytes. These accessory proteins are expressed coordinately during growth, and are controlled by several regulatory systems, such as two component regulatory systems (two-CS) (e.g., *agr*, *arlRS, saeRS, srrAB, vraRS*) and transcriptional regulatory systems (e.g., *sarA* family, *sigB*).[26]

Currently, thirty-five complete *S. aureus* genomes are available in public databases. The *S. aureus* genome is circular and contains approximately 2.8 million bp that represents approximately 2700 coding sequences (2600 proteins),[27] plus structural and regulatory RNAs. These belong either to, a ***core genome*** containing mostly housekeeping genes, which is quite conserved along various staphylococcal species and accounts for about 80% of the whole DNA, or an ***accessory genome*** that carries mobile genetic elements (MGEs) that contains most *S. aureus* pathogenic and drug-resistance features, which may vary between different species and strains. Genome evolution is driven by random point mutations that lead to single nucleotide polymorphism (SNP), larger variations in core genes (e.g., deletions or duplication of repeat regions) that may differ between lineages, and MGEs that include insertion sequences (IS), transposons (Tn), viruses, and pathogenicity and genomic islands.[2]

2.1 Pathogeneses: Regulation.

At least three families of regulatory elements intertwine to adjust gene expression to specific environmental conditions: (1) two-component regulatory systems (two-CS); (2) DNA-binding proteins or transcriptional regulatory system, largely represented by the Sar family of proteins; and (3) small regulatory RNAs.

2.1.1 Two-Component Regulatory System.

Bacterial ***two-component systems*** (**two-CS**) are signaling pathways, which permit the creation of a communication bridge to the external environment, allowing the cell to translate an external stimulus into an intracellular change in gene expression. The defining components are a membrane-associated sensor histidine kinase and a cytoplasmic response regulator. After activation from an external signal, the histidine

kinase typically dimerizes and trans-autophosphorylates.[28] This leads to phosphorylation of the response regulator, characteristically at a conserved aspartic acid residue, and this phosphorylation induces a conformational change allowing it to bind DNA at a specific consensus sequence in a manner that alters the transcription of the target gene.

S. aureus has at least 16 two-component systems (two-CS).[29] In addition to their role in pathogenesis, at least three of these systems have been shown to modulate resistance to antibacterial agents,[30] thus further emphasizing their important role in pathogenesis of *S. aureus* infection.

2.1.1.1 *The Quorum Sensing and Agr Two Component Regulatory System.*

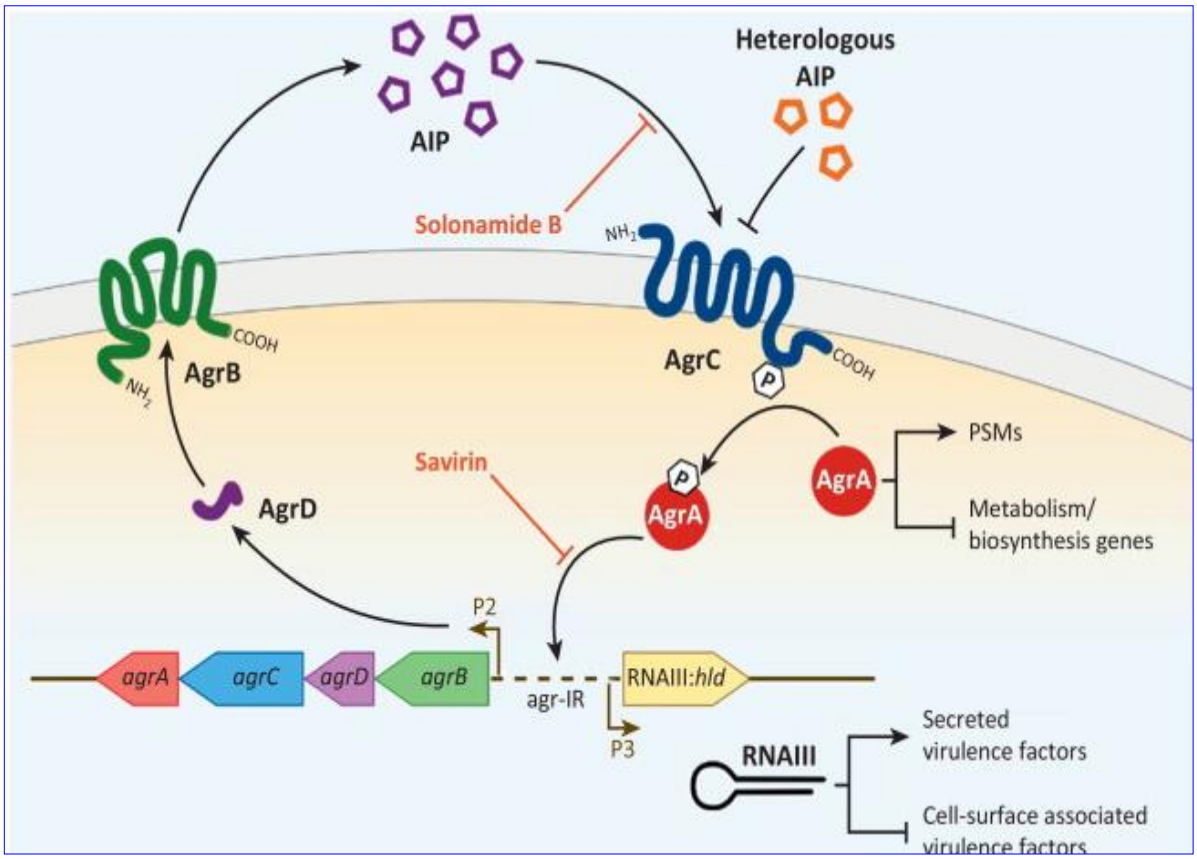
Quorum Sensing (**'QS'**) is a system of stimulus and response, defined as the capacity to detect extracellular, small molecule signals and to alter gene expression in response to bacterial population densities. Bacteria use 'QS' signals to coordinate their behavior via gene expression within their own kind. These sensing signals are used to either inhibit or activate transcriptional programs among competing bacterial strains and other species existing within the same microenvironment.[31,32] These responses to environmental changes include adaptation to availability of nutrients and defence against other bacterias. 'QS' systems control biofilm formation,[33-35] growth potential, sporulation, antibiotic resistance, DNA transfer, autolysis, oxidative stress tolerance, metabolic activity, motility, antibiotic synthesis by antibiotic-producing bacteria, sessile versus planktonic behavior, and most importantly, genetic determinants of virulence.[45]

S. aureus has at least two 'QS' systems together with a large number of other sensing systems and transcriptional and post-transcriptional control mechanisms. In *S. aureus* the dominant 'QS' regulator is the cyclic peptide Agr (accessory gene regulator) system. This major human pathogen, and other staphylococcal species, could in the same way as other bacterias (i.e. *Escherichia coli*), use LuxS signals to regulate virulence and to initiate detachment from biofilms by expression of phenol-soluble modulins (PSMs) peptides.[34]

The ***accessory gene regulator*** (***agr) operon*** constitutes a global regulatory system that controls cell density-dependent virulence factor expression. Several studies have demonstrated that Agr activity is essential for skin and soft tissue infections, and there is considerable interest in developing inhibitors of this system as novel antivirulence drugs for therapeutic use.[36,37]

The *agr* operon consists of two divergent promoters, P2 and P3, where expression from P2 produces the components of a '*quorum sensing*' system (AgrB, D, C, A) (**Figure 2.1.1**), and expression from P3 produces the *agr* effector molecule RNAIII (**Figure 2.1**). The transmembrane P2 product **AgrB** is required for the processing of the propeptide **AgrD** to produce the quorum signal molecule, **autoinducing peptides** (AIP) types I–IV, each one functions as a specific ligand for the corresponding **sensor kinase AgrC**, but as an inhibitor of other AgrC variants. AgrC possesses an extracellular N-terminal domain and seven transmembrane domains (**Figure 2.1.1**).

Figure 2.1.1 The Agr quorum-sensing accessory gene regulator system. Binding of **AIP** (heterologous autoinducing peptide) induces a conformational change within the cytoplasmic helix that links the sensor and kinase domains of specific AgrC, enabling autophosphorylation. However, binding of another AIP that expresses one of the three other different allelic variants of this *agr*, causes the helical linker to twist in the opposite direction, preventing autokinase activity. This specific binding AIP–AgrC is the target for the anti-'QS' molecule solonamide B, which prevents AgrC activation in all allelic variants of *agr*, inhibiting 'QS' and subsequent virulence gene expression via *agr* activation. Activated AgrA dimmers bind to the *agr* intergenic region (***agr*-ir**) between P2 and P3 and up-regulate expression from these promoters. This interaction is the target of a recently identified small molecule, savirin (*S. aureus* virulence inhibitor), which inhibits *agr*-associated virulence gene expression by preventing AgrA binding to the *agr*-ir.



Numerous global regulators enable specific bacterial responses to specific environmental stimuli or collaborations, regulated by *agr*-operon. For example, the global regulator CodY [see below], a stronger repressor, prevents, indirectly, the inappropriate *agr* expression at low cell densities during exponential phase when conditions are nutrient replete.[36,39] Or, the *sarA* family in the early phase of microbial invasion. Thus, when the population density is low, the *agr* expression is lower too, so that most virulence genes are turned off in favor of surface adherence structures and the immunoglobulin inhibitory molecule protein A. Once infection begins, the 'quorum sensing' is activated towards sequential gene activation rapidly with, exotoxin, protease and haemolysin production.[33] Simultaneously, adhesin molecule expression is turned off, thereby facilitating dissemination to other regions in the host. *In vivo*, detection of *agr* expression, indicates this biphasic activation of the system, which occurs with early activation, secondary reduction, and then later reactivation of this 'quorum sensing' system during infection.[40]

The loss of Agr activity creates phenotypes compatible with persistent infection and enhances staphylococcal survival in endothelial cells, as occurs during infective endocarditis.[38] *agr*-defective strains are associated with a high mortality level in bacteremic infections. *agr*-negative strains have a fitness advantage over *agr*-positive strains in the presence of sublethal concentrations of some antibiotics, because the fitness defect of *agr*-positive cells is caused by antibiotic-mediated expression of the *agr* effector molecule RNAIII.[41]

2.1.1.2 *Staphylococcus exoprotein expression, SaeR/S-Two-Component System.*

The ***Staphylococcus exoprotein expression saeRS,*** is transcribed as a 4-gene operon (*saePQRS*), being SaeS and SaeR the sensor and response regulator respectively. The role for SaeP and SaeQ has not yet been determined, although they might be involved in stabilization of SaeS forming a protein complex together with the sensor kinase SaeS and activate the sensor kinase's phosphatase activity.[42] Once phosphorylated, SaeR binds to a specific target sequence to activate transcription of *saePQRS* itself.[43]

Several studies have demonstrated that *sae* also modulates the production of virulence factors, other than toxins, including surface proteins and capsule biosynthesis components.[43] Actually, the loss or deletion of SaeR/S resulted in a decreased amount of more than 17 extracellular proteins and two cell surface-associated proteins, among them α-haemolysin (Hla), β-haemolysin (Hlb), Υ-haemolysin subunit C (HlgC),

leukocidin F (LukF) and leukocidin M (LukM). These proteins are important virulence factors.

"*SaeR/S-mediated transcription is unique to and dependent on specific host stimuli. SaeR/S is strongly involved in a tight temporal control of virulence factor expression. SaeR/S is the major regulator of virulence factors in S. aureus.*"[44]

Agr and SaeR/S each contribute independently to the remarkable virulence of USA300, likely by means of their effects on expression of secreted toxins.[45]

2.1.1.3 *Other Two Component Regulatory Systems.*

The ***staphylococcal respiratory response regulator srrAB,*** responds to oxygen stress by suppressing expression of *agr* and the genes encoding certain exotoxins.[46] The activity of SrrA/B, and the production of multiple types of virulence factors linked to oxygen availability, provide an important example of the link between central metabolic processes and virulence in *S. aureus*. SrrA/B also positively regulates expression of the *icaADBC* operon and production of polysaccharides of cell wall, apparently by repressing transcription of the *icaR*-encoded repressor.[47] One note, the repression of transcription of *spa* ergo the production of protein A, suggests that the impact of *srrAB* is not mediated directly via its regulation of *agr*.

The ***haeme response regulator hssRS,*** is an iron-responsive-system, highly conserved among Gram-positive pathogens suggesting a conserved mechanism of iron acquisition among these organisms. Actually, *S. aureus* can acquire iron in the form of haeme, likely accessed via lysis of erythrocytes, using highly efficient transport systems that can move haeme into the bacterial cytoplasm. In addition, iron is an essential nutrient for many bacterial species during infection,[48] though, a high level of haeme is toxic to the bacterial cell. To avoid this toxicity, *S. aureus* senses haeme by HssS resulting in HssR phosphorylation and binding to the promoter of *hrtAB*. Hrt*AB* encodes an iron efflux pump that maintains intracellular haeme homeostasis,[49] and whose mutant is more virulent than the wild-type,[48] likely due to the stress response induced by increasing intracellular haeme.

The ***arlRS***, is a novel regulator of clumping and pathogenesis, which could be a key regulatory element that defines the "balance" between *agr* and *sarA*. Since, inactivation of *arlRS* results in increased autolysis and an enhanced capacity to form a biofilm.[50] Several studies have related this ArlR/S two-CS, with a production of additional virulence factors including, the exfoliative toxin and capsular polysaccharides, indirect

effect via positive regulation of MgrA,[51] or with the control of agglutination by negatively regulating the expression of the Giant Staphylococcal Surface Protein.[52] Thus, the ArlR/S system is essential for pathogenesis, at least in a rabbit model of sepsis and infective endocarditis.[53] Like *arlRS*, the ***lytSR two-CS*** is a negative regulator of *S. aureus* autolysis and biofilm formation, in fact *arlRS* is an activator of *lytSR* transcription.[54] LytS/R two-CS has led to the identification of the two operons involved in affect stationary-phase survival, murein hydrolase activity, biofilm formation and antibiotic tolerance.[54] On the other hand, recent studies have demonstrated that LytS could act like a staphylococcal "voltmeter sensor". Hence, the LytS/R two-CS plays an important role in an adaptive response, and could be directly involved in the control of programmed cell death and lysis.[55]

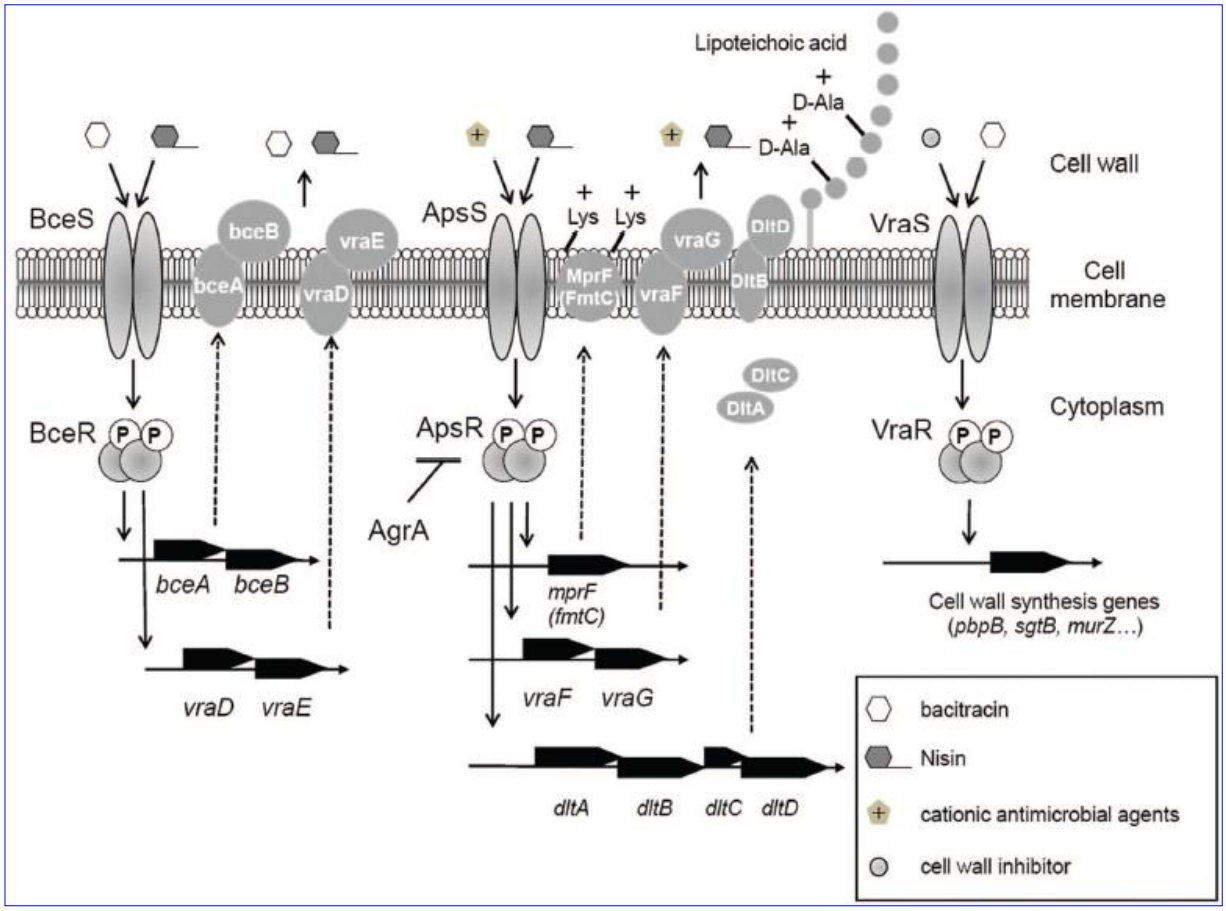
Other less well-characterized sensor protein kinase-systems are, **Kdp*DE system***, which is up-regulated by the Agr/RNAIII system, and linked to the autoinducer peptide (AI)-2/LuxS 'quorum sensing'-system with capsule production,[56] could be an important virulence regulator coordinating the external K^+ sensing and Agr signalling, during pathogenesis.[57] **VraS/R**, which are involved in the stress response to cell-wall inhibitors such as β-lactams and vancomycin.[58] **GraS/R** (ApsS/R), which aid in resistance to oxidative and heat stress, probably due to their role in the modification of wall teichoic acids, and also vancomycin resistance by expression of *vraFG* ABC transporter.[59] **Bce*AB system***, associated with altered susceptibility to bacitracin (Figure 2.1.1.3), and **NsaR/S**, involved in biofilm formation, as well as cell envelope stability in response to cell wall and membrane disruption.[60] Another additional two-CS that stands out from the others, because it is the only one that is essential in *S. aureus,* is **WalK/R** (YycGF). WalK/R has been shown to be involved in peptidoglycan crosslinking and biofilm formation, and plays an important role in virulence and eliciting the host inflammatory response by controlling autolytic activity. Transcriptome analysis of the WalK/R regulon revealed positive regulation of major virulence genes involved in host matrix interactions (*efb, emp, fnbA, and fnbB*), cytolysis (*hlg*ACB, *hla*, and *hlb*), and innate immune defence evasion (*scn, chp*, and *sbi*), via activation of the SaeS/R two-CS.[61]

2.1.2 The DNA-binding proteins or Transcriptional regulatory system.

DNA-binding transcription factors, play an important regulatory role by either repressing or activating genes, in response to environmental and physiological conditions. Generally, they simultaneously bind DNA and RNA polymerase, as well as other agents necessary for the transcription, and can be regulated through reversible

structural alterations such as, phosphorylation or inactivated through such mechanisms as proteolysis.

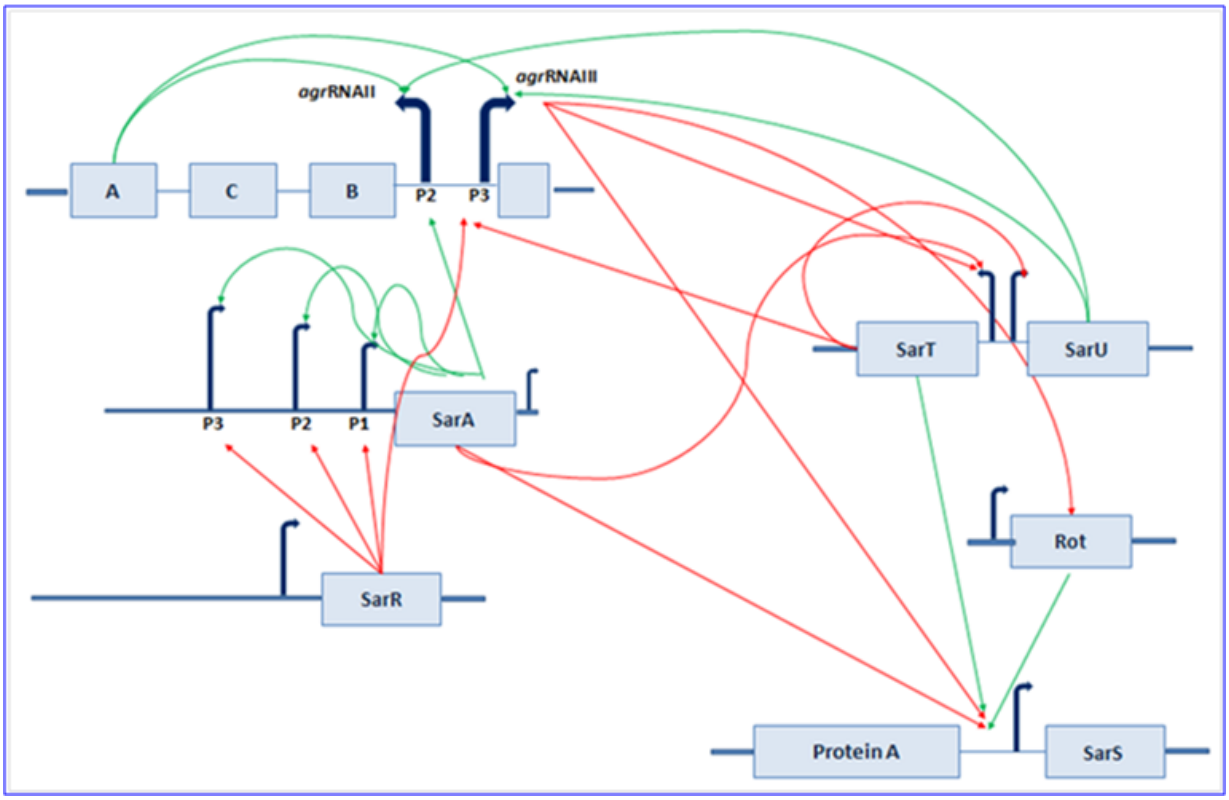
Figure 2.1.1.3. **Resistance mechanism mediated by two-component systems.**



2.1.2.1 *Staphylococcal regulator Sar Family.*

The ***staphylococcal regulator*** specific **Sar** family proteins, are involved in the expression of numerous target genes involving oxidative stresses, biofilm formation, antibiotic resistance, virulence, autolysis and metabolic processes. Ten Sar-family proteins, SarA, SarR, SarS, SarT, SarU, SarV, SarX, SarZ, Rot, and MgrA, have been partially characterized in *S. aureus*, being *sar*A gene well-studied, although the exact mechanism of target gene regulation remains largely unknown. The Sar family proteins are homologous to each other, as well as homologous to the MarR family transcriptional regulators in Gram-negative bacteria. The Sar protein family members are able to regulate numerous target genes, either by binding directly to promoter regions or indirectly via other regulatory systems.[62] **(Figure 2.1.2.1).** The most important is SarA.

Figure 2.1.2.1. Regulatory network of *agr* and *sar* family of DNA-binding proteins. Intertwining of activation *(green arrows)* and repression *(red arrows)* underlines complexity of system. gene expression is further modulated by additional factors (*sigB*, *arlS*, *sae* and *srrAB*), which can act on *agr* promoters or directly on specific genes. Gene promoters are denominated P1, P2, and P3 and represented by *dark blue arrows.* Figure adapted from Mandell [2].



The ***sar*A locus,** is complex and includes three promoters, which drive the production of three transcripts (*sarB*, *sarC* and *sarA*). The *sar*A locus is known to up-regulate the synthesis of fibronectin and fibrinogen binding proteins, haemolysins, enterotoxins, TSST-1 (toxic shock syndrome toxin) and capsule biosynthesis genes, and to down-regulate proteases, protein A and a collagen binding protein. SarA has been also shown to bind to several regulatory and target gene promoter regions (e.g., Agr, SarS, Rot, SarV, SarT, Hla, Fnb, Spa, Cna, Bap, IcaRA) to modulate gene transcription, thus implicating both *agr*-dependent and *agr*-independent pathways, as well as direct and indirect mode for SarA-mediated regulation.[62] Interestingly, the inactivation of *sarA* has been consistently shown to result in a reduced capacity to form a biofilm, the opposite to that of *agr*,[63] suggesting that the role of *sarA* in biofilm formation is independent to its regulation of *agr*. On the other hand, the impact of SarA on exotoxin production has been shown to be heavily influenced by SaeR/S, with an impact on α-haemolysin and PSMs.[64] In addition, in the same way as *agr*, inactivation of *sarA* has been shown to

attenuate virulence in multiple animal models of *S. aureus* infection, including septic arthritis, osteomyelitis, and endocarditis.[65] This, together with the impact of SarA on expression of *agr*, provides direct indications of the interactive role of SarA in *S. aureus* regulatory circuits.

SarR down-regulates the expression of SarA, and its maximum expression is in the exponential phase of growth. Inactivation of *sar*R has positive effects on the transcription of the *agr* locus and also on the maximal transcription of aureolysin and serine protease in *S. au*reus.[62] **SarS**, repressed by Agr and SarA, and activated by SigB (σ^B), is an activator of protein A and a repressor of α-haemolysin.[66] **SarT**, repressed by Agr, also represses *hla* (via *sae*),[67] and induces expression of protein A (indirectly via *sarS*). **SarU**, repressed by SarT, whose inactivation results in a reduction of both RNAII and RNAIII expression, suggests a positive effect of SarU on *agr,* and a key role during biofilm-associated infections by modulating *agr*. **SarV** is involved in regulation of autolysis, which may be part of the common pathway through which SarA and MgrA control autolysis.[68] **SarX** also acts as a repressor of the *agr* locus and can therefore regulate other genes via Agr. Its maximal expression is during the stationary phase of growth,[69] and could be involved in a regulatory cascade that promotes polysaccharide-intercellular-adhesion (PIA)-dependent biofilm formation in *S. aureus*.[70] **SarZ** positively regulates the expression of *agr* and *mgrA* but negatively of *sarA*. Its expression is growth phase dependent, with maximum expression during early exponential phase, and affects surface proteins, toxins and biofilm by modulating the aforementioned global regulators, as well as direct activation on SspA protease.[71]

2.1.2.2 *Others Transcriptional Regulatory Systems.*

Sigma factors (**Sig**, **σ**), are other major mechanism of response to environmental stimuli. There are currently four identified sigma factors (Sig) in *S. aureus*: **SigA**, responsible for transcription of housekeeping genes; **SigB (σ^B)**, responsible for the transcription of stress-response genes; **SigS**, controls expression of genes required for overall fitness and survival; and **SigH**, involved in competence and more recently, prophage integration and excision.[72]

The alternative **SigB σ^B** operon contains: σ^B, anti-σ^B factor RsbW, anti-anti-σ^B factor RsbV, and RsbU, a Mn^{2+}-dependent phosphatase that positively controls σ^B activity by dephosphorylating RsbV (Figure 2.1.3.2). This alternative transcriptional factor SigB σ^B is an essential part of the complex regulatory network controlling the expression of over 200 genes involved in virulence, cell wall metabolism, membrane transport processes, and microbial response to a variety of stress (temperature, energy

depletion,..) and chemical stimuli.[2] It has been also demonstrated to aid in heat tolerance and resistance to cell wall active antibiotics,[73] and contribute to pathogenesis in animal models of infections. More recently, it has been shown that inactivation of SigB has an indirect impact on the *agr* '*quorum sensing*' system by enhancing RNAIII expression,[74] as well as to regulate several extracellular virulence factors and capsule via SpoVG. Hence, it is demonstrating the role in virulence as a response to stress.[75]

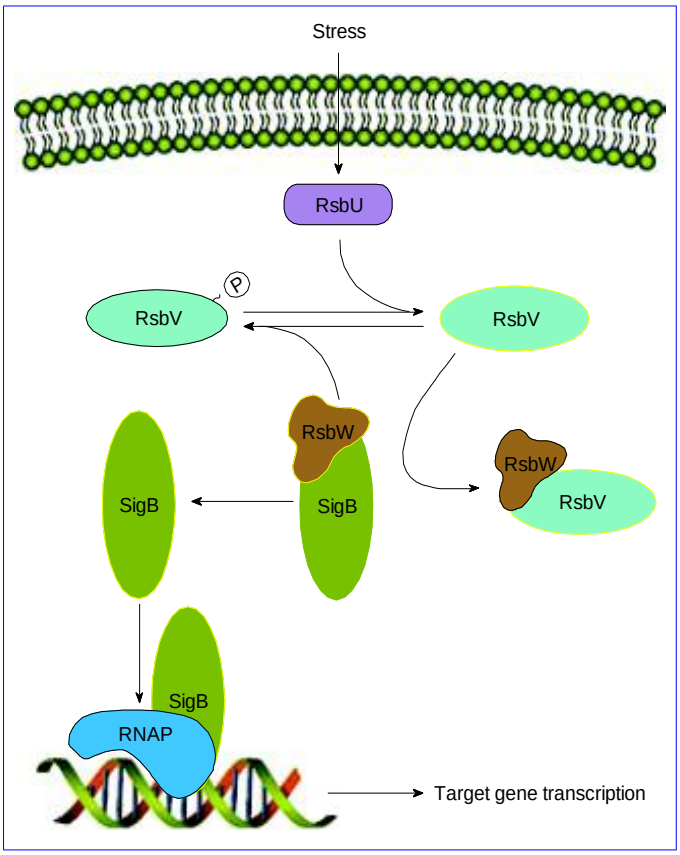


Figure 2.1.3.2 Post-transcriptional regulation of SigB. After stress-induction, RsbU de-phosphorylates RsbV, which can then bind specifically to RsbW thereby removing RsbW from SigB. Phosphorylated RsbV is inactive and therefore cannot bind RsbW. RsbW also promotes phosphorylation of RsbV to maintain its inactivity. RsbW binds to SigB to inhibit transcription by preventing SigB from complexing with the RNA polymerase (**RNAP**). Once SigB is free from inhibition by RsbW, it can complex with RNAP forming the holo-enzyme and activate transcription of target genes. Active proteins are highlighted with yellow.
Figure adapted from [Junecko ME *et al.*, **2012**, *World J Clin Infect Dis*].

MgrA, member of the multiple antibiotic resistance regulator MarR and SarA family proteins, which positively regulates *sarX* gene expression, plays a key role in regulating the expression of major virulence factors in *S. aureus,* including capsule and sortase. Several studies have provided evidence that MgrA regulates *hla* and *spa* expression by *agr*-dependent and independent pathways, and plays an important role in *S. aureus* sepsis, even increasing mortality and accelerating the onset and development of sepsis.[76] In addition, MgrA has been found to repress biofilm formation, in part, by *agr*-dependent pathway and DNA release, probably by affecting LytS/R and the antiholin-like protein Lrg*AB*.[77]

Repressor of toxins **Rot**, is another global regulator that belongs to the Sar family, which mediates modulation of several genes involved in virulence (especially autolysins).[78] Its transcription is growth-phase dependent,[79] however its translation is regulated by the *agr* '*quorum sensing*' system.[80] Rot has also been shown to repress *hla* production by repressing the SaeR/S two-CS, and both, Rot and the Sae-two-CS, had been proposed to work in opposition of one another on their target genes. Otherwise now, it is known that both regulators work in concert to activate promoters.[81]

Other similar transcriptional regulatory system, ***teicoplanin-associated locus regulator*** **TcaR**, belongs to MarR family, appeared to be a weak negative regulator of transcription of the *ica* (intercellular adhesion) locus, necessary for biofilm production, as well as a further activator of *sar*S, and a modulator of *sas*F expression. Obviously, the inactivation of the *tca*RAB operon leads to teicoplanin resistance. And, **AraC/XylS** family, involves in biofilm formation[82] and virulence in *S. aureus.*[83]

Finally, the ***GTP-sensing transcriptional pleiotropic repressor CodY***, well conserved within the low G+C Gram-positive, has been shown to be an important regulator of metabolism and virulence. CodY acts repressing toxin production during times of plentiful nutrition by directly and indirectly *via agr*. It functions by sensing intracellular levels of branched chain aminoacids and GTP during growth, and responds by repressing genes involved in starvation behaviors in nutrient-rich conditions (amino acid transport, sporulation…). When these levels decline (branched chain-aminoacids and GTP), CodY loses its affinity for DNA binding, bringing about de-repression of target genes, and as a result, a physiological transition from growth and division to amino acid metabolism and stress tolerance.[84] Briefly, apart from direct regulation of virulence genes, CodY also affects metabolic regulation in *S. aureus via* carbon flow, nitrogen assimilation, amino acid synthesis and transport systems.[85] Moreover, CodY is also repressed by the intracellular protease/chaperone ClpC, possibly *via* ClpC-induced proteolytic degradation in association with ClpP, eliminating the repressive effect of CodY on virulence genes.[86]

"CodY is activated in nutrient replete environments, repressing virulence factors and metabolic synthesis genes".

2.1.3 Small regulatory RNAs systems (sRNA).

S. aureus also uses, besides these signaling pathways, around 250 regulatory RNAs to coordinate the expression of the numerous virulence genes for growth and survival. Most *S. aureus* sRNAs are located within the core genome, but a few are expressed from

the pathogenicity islands and from plasmids. The sRNAs expressed from the core genome are probably involved in wider biological functions. Most of the few sRNAs, whose physiological roles have been determined, control the expression of genes involved in response to quorum sensing, in central metabolisms, and on virulence by pairing to target mRNAs to modulate their translational activities and stabilities. Several sRNAs encode and express small peptides that may play important roles in virulence or in bacterial growth control. Most of these well-characterized sRNAs act as fine-tuning regulators by repressing the translational level of only one gene, but it is probably that one gene is regulated by different sRNAs.[74]

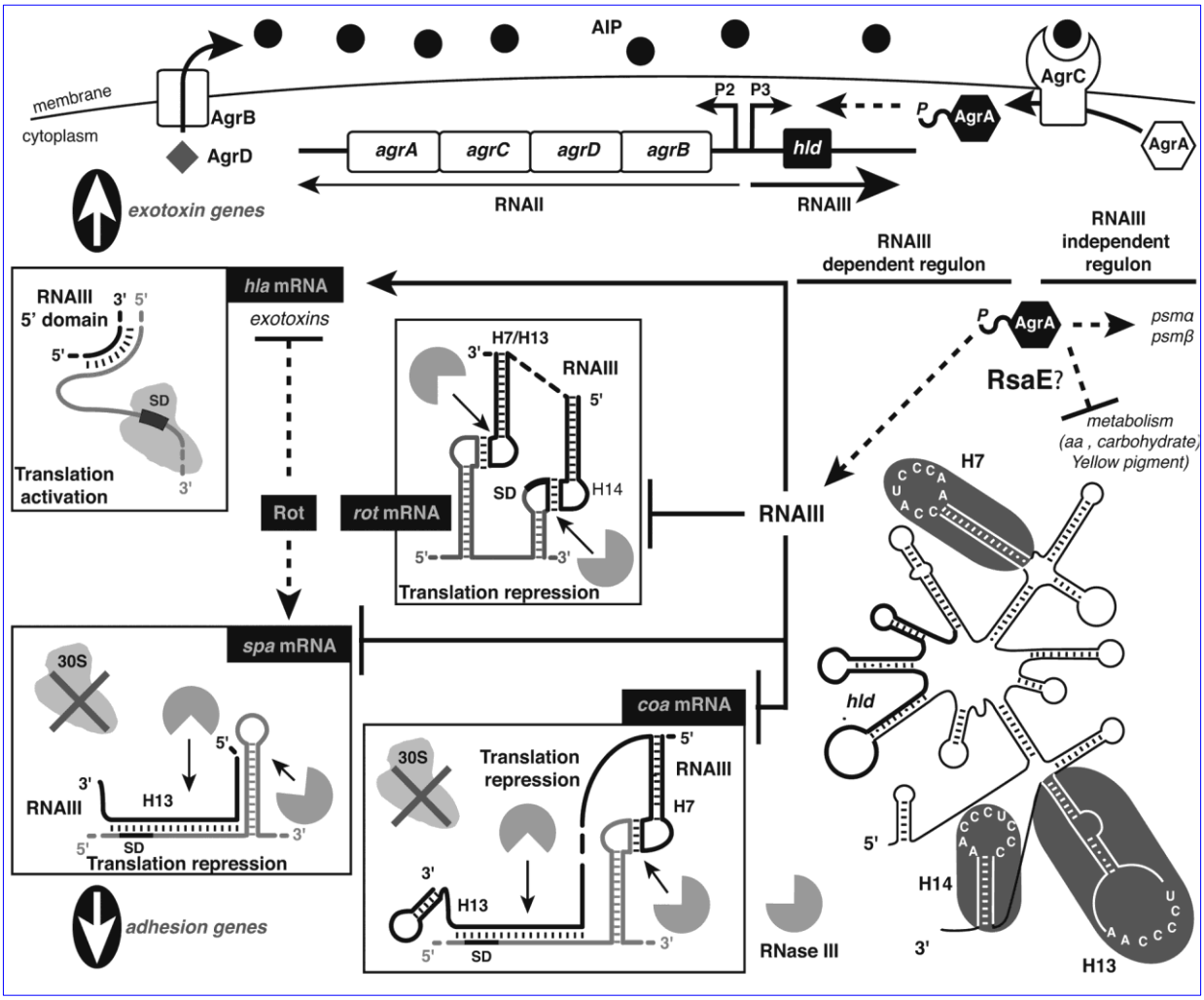
"Multiple sRNAs controlling the expression of a similar component from a regulatory network allows the sharp regulation of virulence genes. It is most probably that S. aureus expresses many other sRNAs that deeply interact with this network to influence bacterial virulence".[74]

SbrA and **SbrB**, highly conserved among Staphylococci, encode putative basic peptides, which are potential virulence factors. Another **SbrC**, encodes for an ABC transporter dedicated in the uptake of manganese, crucial element for defense systems against oxidative stress and contributes to the virulence of *S. aureus*.[74]

RsaA, **RsaD**, and **RsaF,** are differently transcribed in response to environmental stress, heat, cold, osmotic and oxidative stress, as well as acidic pH. Recently, **RsaE**, sRNA conserved in all *S. aureus* strains, has been shown to regulate several metabolic pathways, exactly down-regulates the synthesis of enzymes from the Krebs cycle (tricarboxylic cycle, TCA) and from the folate-dependent one-carbone metabolism. However its expression profile is a subject of controversy,[87,88] because of some *S. aureus* strains. RsaE is expressed at late exponential phase and repressed at stationary phase, it could facilitate the transition of energy metabolisms, the purine biosynthesis, and amino acid transport in response to the nutrients' availability. Moreover, the RsaE expression seems to be dependent on the *agr* '*quorum sensing*' system and SigB activity (Figure 2.1), suggesting that it could modulate the metabolism profile according to stress responses and/or virulence.[43]

Small stable RNAs (**SSRs**) are RNAs specifically produced and/or stabilized in response to various environmental conditions. For example, **SSR42,** expressed during the stationary phase, is involved in host erythrocyte lysis, resistance to human polymorphonuclear leukocyte killing, and pathogenesis in a murine model of bacterial infection.[74,89]

Figure 2.1. The *agr* system and its two main intracellular effectors AgrA and RNAIII. The *agr* system regulates the expression of multiple genes in an RNAIII-independent manner via AgrA and in an RNAIII-dependent manner. **RNAIII-independent regulation**: AgrA activates the synthesis of several peptides by binding to their promoter regions and represses metabolic enzymes by an unknown mechanism. RsaE regulates enzymes of the central metabolism. **RNAIII-dependent regulation**: RNAIII encodes ***hld*** (δ-haemolysin) and the secondary structure of RNAIII and three of the hairpin domains containing a redundant UCCC motif (gray color). The 3' non-coding region of *hld,* which contains the three redundant hairpin loops, binds to the ribosome binding sites of *coa* mRNA encoding coagulase, *rot* mRNA encoding repressor of toxins *rot,* and *spa* mRNA encoding protein A. The 5' non-coding region of *hld* binds to *hla* mRNA to facilitate ribosome binding, and to activate translation. Dashed bars and dashed arrows are for transcriptional regulation while black bars and arrows are for post-transcriptional regulation. Bars are for repression and arrows for activation.[90]



Small pathogenicity island rNA D9 (**SprD**), is expressed from the genome of a converting phage, a horizontally-acquired pathogenicity island, being the repository of superantigens, toxins, adherence, invasion factors, and secretion systems, playing important roles during host infection. SprD down-regulates, the expression of the immunoglobulin binding (Sbi) immune evasion molecule, located on the core genome.[74]

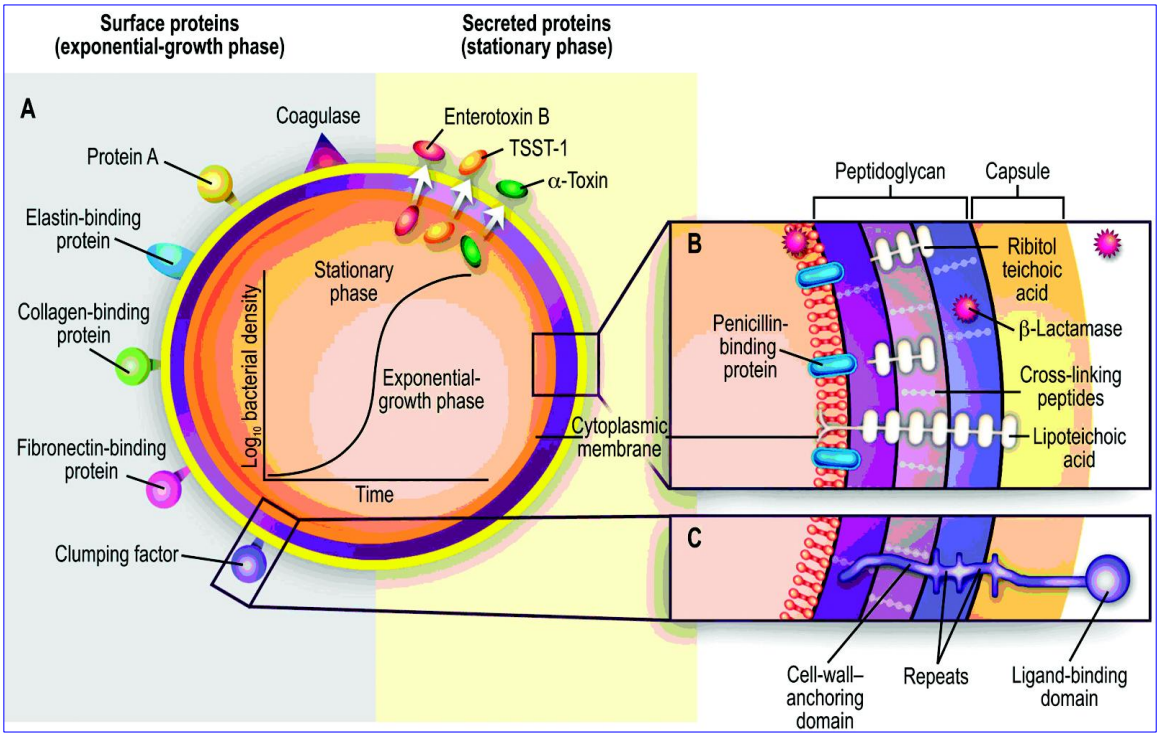
Finally, **RNAII** encoding a 'quorum sensing'-cassette and an *agr*-locus-two-CS [see above], and **RNAIII** encoding δ-haemolysin (Hld). (**Figure 2.1**) RNAIII is one fascinated regulator and structured mRNAs that regulates multiple targets involved in virulence and peptidoglycan metabolism.[74,90] RNAIII is responsible for post-transcriptional regulation of multiple virulence factors, by mediating a switch from expression of cell-surface associated proteins, such as staphylococcal protein A and fibronectin-binding proteins A and B (FnbA and FnbB), to secreted toxins, such as α-haemolysin and δ-haemolysin, PSMs, and leukocidins such as Panton-Valentine leukocidin (PVL).[36,37,91]

Recently, a new sRNA, named **ArtR** (AgrA-repressed, toxin-regulating sRNA), has been reported to activate α-haemolysin (Hla) expression by binding to the *sar*T mRNA.[92] Although both, RNAIII and ArtR, similarly regulate *hla* expression, in contrast to RNAIII, ArtR transcription is repressed by *agr*A, suggesting that up-regulation of *hla* mediated by ArtR could be enhanced in *agr*-deficient strains.[74]

2.2 Pathogenesis: Virulence determinants.

S. aureus is known due to its virulence, which is multifactorial and dependent on a series of toxins, adhesion proteins, another virulence determinants and immune system evasion; the same is true for MRSA strains. The molecular basis of infection by *S. aureus*, has been an active field of research for several decades. However, now such research has further intensified to become decisive, with the recent emergence of highly pathogenic CA-MRSA strains that combined antibiotics resistance, rapid ability for spreading and exceptional virulence.[93] The last decade has been decisive for the identification of yet unrecognized *S. aureus* virulence factors such as the PSMs,[94] as well as the characterization of the pathogenic role of long-known toxins such as the PVL.[95,96] This virulence can be gained via acquisition of new toxin genes by horizontal transfer, such as the phage-borne *pvl* genes, or can be gained via overexpression of core genome-encoded toxins, such as PSMs or α-haemolysin.[94,97,98] Moreover, to this already intricate scheme of complexity, it has also been added, the observation that the acquisition of new genes, can be harbored by MGEs.[99] (**Figure 2.2**)

Figure 2.2 **Pathogenic factors of *Staphylococcus aureus*,** with structural and secreted products, both playing roles as virulence factors. **(a)**, surface and secreted proteins.**(b)** & **(c)**, cross-sections of the cell envelope. Reprint from [100,101] with permission from Lowy, FD (author) and Oxford University Press. Copyright © 2008 Copyright Clearance Center, Inc. All rights reserved.



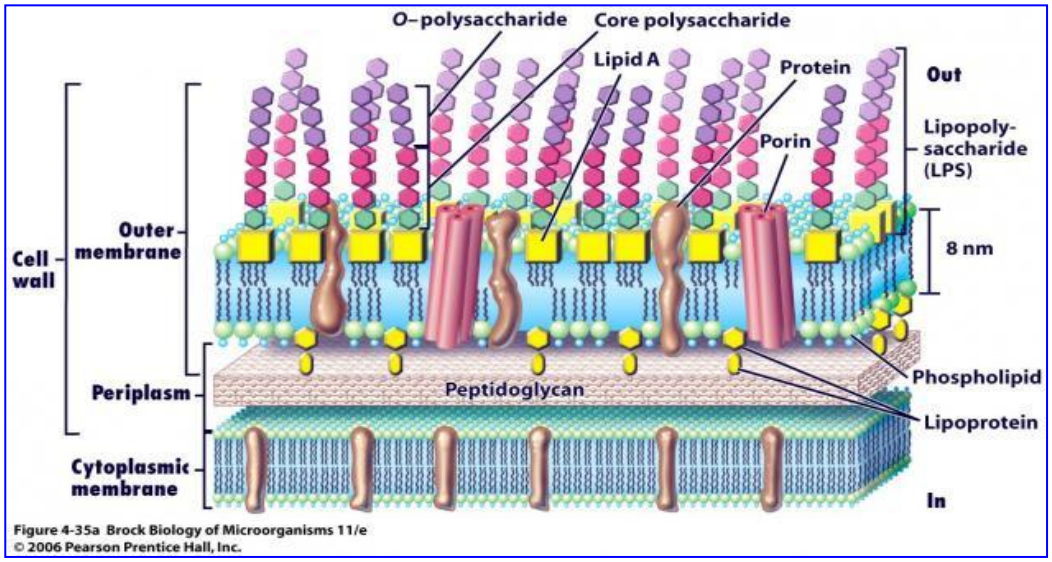
2.2.1 Cell Surface Determinants Involved in Pathogenesis.

Cell surface proteins are proteins embedded in cell membrane with a multiple important roles in *S. aureus* pathogenesis, such as key functions in bacterial cell wall metabolism. They bind to host tissue, facilitate internalization and immune evasion, and are involved in bacterial aggregation and biofilm formation. Most surface proteins are encoded on the core genome.[102]

2.2.1.1 *Teichoic, Lipoteichoic Acids and peptidoglycan.*

Teichoic acids are bacterial polysaccharides constituted of polyribitol-phosphate polymers cross-linked to N-acetylmuramic acid residues of the peptidoglycan, and decorated with D-alanine and N-acetylglucosamine residues. They represent up to 50% of the dry weight of purified staphylococcal walls, and play an important physiologic role in cell wall metabolism, probably to be a site of attachment of cell wall for active enzymes and other proteins. Teichoic acids have also been involved in adherence to nasal epithelia, nevertheless, their role in invasive infection and host inflammatory response is unclear.[2,102] (**Figure 2.2.1.1**)

Figure 2.2.1.1. Cell wall organization



Lipoteichoic acids are the major constituent of cell wall in gram-positive, with a plasma membrane-bound counterparts of teichoic acids. They have a similar general structure to teichoic acids, except that they contain polyglycerol-phosphates and are linked to a diacylglycerol-moiety, which serves as a plasma membrane anchor. Lipoteichoic acids can act like receptors, and have been implicated in inflammation via triggering the release of cytokines by macrophages and other players of the innate immune system. In particular, the stereochemistry of the D-alanine and the presence of the diacylglycerol lipid anchor, were shown to be determinants for host recognition and subsequent inflammation. Certainly, lipoteichoic acids may facilitate bacterial recognition by host innate immunity, but at the same time, they protect bacteria from killing by cationic antimicrobial peptides, which are produced by professional phagocytes. Native lipoteichoic acid is polyanionic (negative charge) and therefore, attracts cationic antimicrobial peptides. To circumvent the problem, lipoteichoic acids become decorated with D-alanyl residues (positive charge) by the *dlt*ABC gene products, which render the structure more positively charged, and thus repulse cationic antimicrobial peptides. Indeed, mutants impaired in *dlt*ABC are also less adherent to endothelial cells and less able than wild type to produce experimental endocarditis in rabbits. Hence, the microbial cover is not an amorphous scaffold that only ensures bacterial shape, it is also a sophisticated structure indispensable to mediate adherence, sensing, and growth in complex environments.[2] (Figure 2.2.1.1).

Peptidoglycan, also known as murein, is a critical cell structure highly conserved constituent of both the gram-positive and gram-negative, however in gram-positive

bacteria is a thick structure while in gram-negative is thin. It is constituted of glycan chains made of N-acetylglucosamine and N-acetylmuramic acid disaccharide subunits, in which the N-acetylmuramate moiety is linked to highly conserved pentapeptide or tetrapeptide stems (L-alanine-D-isoglutamine-L-lysine-D-alanine-[D-alanine]). In *S. aureus*, the peptidoglycan is cross-linked via a characteristic pentaglycine interpeptide bridge, piece that comprises 1 to 5 glycine residues. The addition of glycines to the wall precursors is driven by *fem*ABC and *fmh*B genes. These determinants are implicated in the plasticity of the wall and are indirectly implicated in staphylococcal resistance to methicillin and vancomycin (see subsequent Antibiotic Resistance section).[2]

Peptidoglycan is the major scaffold for anchoring most MSCRAMMs (microbial surface component recognizing adhesive matrix molecule), thus it plays a key role in pathogenesis. However, peptidoglycan is recognized by the innate immune system and triggers cytokine release and inflammation, therefore it is made important for the microorganisms to be able to hide these structures (peptidoglycan and lipoteichoic acids) from host recognition. The objective is achieved by producing antiphagocytic components such as a capsule, or protein A. [2]

2.2.1.2 *Capsule and biofilms.*

More than 90% of clinical isolates of *S. aureus* elaborate a ***polysaccharide capsule.*** Heretofore, 11 serotypes have been reported, of which capsule type 1 and 2 produce large quantities of polysaccharides and appear mucoid on culture plates, though they are rarely found in human clinical samples. Nevertheless, capsule type 5 and 8 are responsible for up to 75% of clinical infections, indeed antibodies against these capsular types are protective in animal models of sepsis, and naturally occurring antibodies are detected in normal human serum. This could be because of, both capsule type (type 5 and 8) are antiphagocytic and can increase virulence in several animal models. Capsule type 5 and type 8 are made of various sugars, including mannose and fucose. Thus, the capsule is an antiphagocytic constituent that might be a promising target for vaccination. Actually, in patients for haemodialysis, a conjugate vaccine addressed against type 5 and 8 capsules has been shown temporally effective, however, no definitive human studies are available on this issue.[2]

Biofilms are surface-attached bacterial agglomerations embedded in extracellular matrix. The production of a series of surface molecules that promote extracellular matrix formation, allow Staphylococci to be known as a very good biofilm formers.[102] **Biofilm-formation** evolves in two steps, starting with nonspecific adherence of

individual cells to the materials, followed by growth and biofilm formation. Biofilm-formation is associated with the production of polysaccharide intercellular adhesion (PIA), which is synthesized by an operon called *ica* (intercellular adhesion) composed of a regulator (*ica*R) and biosynthetic (*ica*ADBC) genes.[2] Throughout this chapter, we will continue to explain the factors involved in biofilm formation, Figure 2.2.1.3 and Figure 2.2.2. The strategy of colonizing or biofilm-formation of *S. aureus*, to remain in or on human epithelia in relative 'stealth', is opposed to an aggressive status of active toxin production during acute *S. aureus* disease. Moreover, biofilms enable the bacteria to remain attached to surfaces, and provide considerable protection from antibiotics and host defenses. Therefore, biofilms may contribute to prolonged colonization and infection, and the spread of MRSA in hospital and community settings. It is noteworthy in that regarding many colonizing *S. aureus* isolates were shown to be defective in the global virulence regulator *agr*.[102]

2.2.1.3 *Surface Adhesines.*

Surface adhesines-anchored are binding-proteins that are covalently attached to peptidoglycan,[2,102,103] which are known as cell wall-anchored (**CWA**) proteins. These surface proteins are crucial to the success of the organism as a commensal bacterium and as a pathogen (Figure 2.2.1.3). The repertoire of CWA proteins on the surface varies among strains, and *S. aureus* can express up to 24 different CWA proteins. Moreover, the expression of CWA proteins can be altered by growth conditions; for example, some proteins are expressed only under iron-limited conditions, whereas others are found predominantly on cells in the exponential or stationary phases of growth. The most prevalent group is the ***microbial surface component recognizing adhesive matrix molecule*** (**MSCRAMM**) family, which is defined by tandemly linked IgG-like folded domains and likely play a role in nasal colonization, especially when the mucin layer is breached and matrix proteins are exposed.[100] All these MSCRAMMs obey a relatively similar type of structure, a *N-terminal signal sequence*, which is followed by variable functional domains that carry the binding activity and are themselves followed by a series of repeated sequences, a *LPXTG* (Leu-Pro-any aminoacid-Thr-Gly) wall-anchoring domain, and a *membrane-spanning domain*, which is cleaved off during wall anchoring by an enzyme called sortase.[2] Secretory signal sequences are located at the amino-terminal, which direct the translated proteins to the secretory (*sec*) apparatus in the membrane, to cleave during secretion. Carboxyl-terminal has a characteristic sorting signal, which facilitates to each protein their covalent anchorage to peptidoglycan. Some examples of CWA is, the protein A, a multifunctional CWA protein

ubiquitous in *S. aureus*, which is often used in strain typing on the basis of *spa*-typing. Surface protein G (SasG), closely related to the accumulation-associated protein (Aap), which is needed for biofilm formation in *S. epidermidis*. And near iron transporter (NEAT) motif proteins, involved in haem capture from haemoglobin, which help bacteria to survive in the host, where iron is restricted. Haem is transported via several CWA proteins, called iron-regulated surface (Isd) proteins, to a membrane transporter and then to the cytoplasm, where haemoxygenases release free iron.[103] Relevant CWA for pathogenesis are summarized in Table 2.2.1.3.[103]

Figure 2.2.1.3 Functions of cell wall anchore (CWA) proteins of *S. aureus*. The CWA protein iron-regulated surface determinant (*isd*) binds haemoglobin and extracts, and transports haem across the cell wall and membrane into the cytoplasm, where iron is released. Phagocytosis by neutrophils is inhibited by the binding of CWA proteins to IGg and other plasma proteins, by reducing the level of, or access by, neutrophil receptors to the complement opsonin C3b. CWA proteins promote adhesion to the extracellular matrix, to the surface of host cells and to biomaterial surfaces. Interactions between CWA proteins on adjacent cells contribute to the accumulation phase of biofilm formation. CWA proteins directly or indirectly interact with integrins and promote the invasion of non-phagocytic host cells. Intracellular bacteria can cause host cell apoptosis or necrosis, or they can enter a non-disruptive semi-dormant state known as small colony variants. By binding to and activating tumour necrosis factor receptor 1 (TNRF1) on host epithelial cells, protein a triggers the synthesis of cytokines and causes disruptive inflammation, which contributes to pathogenesis.

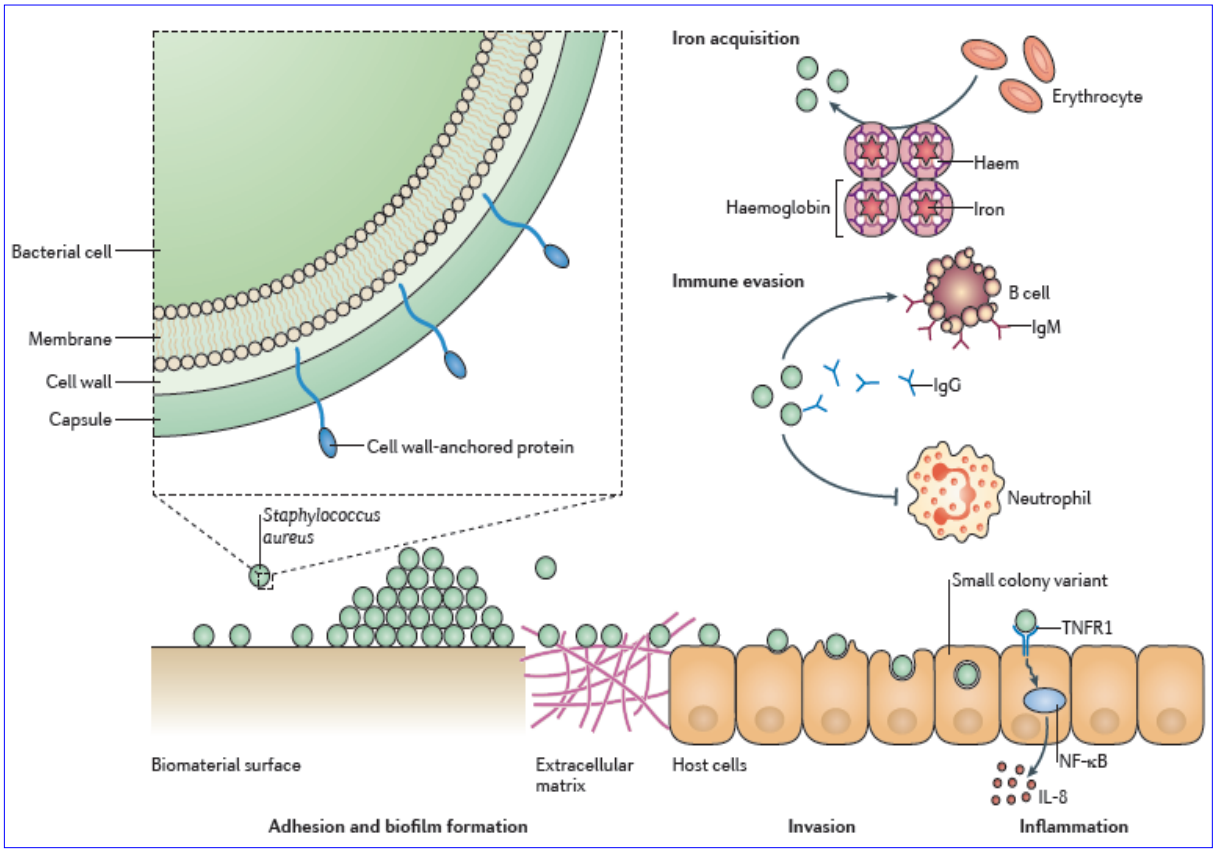


Table 2.2.1.3 The main groups of cell wall-anchored (CWA) proteins as virulence factors of *Staphylococcus aureus.* Role in the colonization.

#ECM: extracellular adhesion; LPXTG: Leu-Pro-any aminoacid-Thr-Gly; Ct: carboxyl-terminal; Nt: amino-terminal; gp: glucoprotein; TNFR1: tumour necrosis factor receptor 1; VH3: variable heavy chain 3; SAg: superantigen.[103]

	PROTEIN GROUP	LIGAND AND BINDING MECHANISM	FUNCTION	ROLE
MSCRAMMs (*microbial surface component recognizing adhesive matrix molecule)*				
ClfA	Clumping factor A	Fibrinogen γ-chain carboxyl-t	Adhesion to immobilized fibrinogen; (thrombus) immune evasion by binding soluble fibrinogen	Endocarditis Kidney abscess following survival in blood Septic arthritis Septic death; survival in blood
		Complement factor I	Immune evasion; degradation of C3b. Reduce opsonophagocytosis	
ClfB	Clumping factor B	Fibrinogen a-chain repeat 5, keratin 10 and loricrin	Adhesion to desquamated epithelial cells; nasal colonization. Adhesion to thrombus	Nasal or skin colonization Endocarditis Kidney abscess following survival in blood
SdrC	Serine-aspartate repeat protein C	β-neurexin	Unknown	
		Desquamated epithelial cells	Nasal colonization?	
SdrD	SdrD			
SdrE	SdrE	Complement factor H	Immune evasion; degradation of C3b	
	Bone sialoprotein-binding protein (isoform of SdrE)	Fibrinogen α-chain	Adhesion to ECM	
FnbA, FnbB	Fibronectin-binding proteins A and B	FnbA A domains binds the Ct of fibrinogen γ-chain and elastin FnbB A domain also binds fibronectin but not only		Mastitis Foreign body infection Endocarditis
		Fibronectin (FnbA and FnbB Ct repeats b-zipper)	Adhesion to ECM; invasion. Adhesion to thrombus; adhesion to intra-aortic patch.	
Cna	Collagen adhesion	Collagen triple helix; collagen hug	Adhesion to collagen-rich tissue. Adhesion to cartilage	Ocular keratitis Septic arthritis
		Complement protein C1q	Prevention of classical pathway of complement activation. Enhanced colonization and evasion	
Near Iron Transporter, NEAT mofit family				
IsdA	Iron-regulated surface protein A	Haem, fibrinogen, fibronectin, cytokeratin 10, locicrin (Nt NEAT-mofit region)	Haem up-take and iron acquisition; adhesion to desquamated epithelial cells; resistance to lactoferrin	Nasal colonization Kidney abscess following

		Unknown ligand (Ct domain NEAT. Mofit region)	Resistance to bacterial lipids and antimicrobial peptides; survival in neutrophils	survival in blood
IsdB	IsdB	Haemoglobin, haem (Nt NEAT-mofit region)	Haem up-take and iron acquisition	Kidney abscess following survival in blood
		β3-integrins (NEAT mofit regions)	Invasion of non-phagocytic cells	
IsdH	IsdH	Haemoglobin, haem (Nt and/or Ct NEAT-mofit region)	Haem up-take and iron acquisition	Septic death; survival in blood
		Unknown ligand (Nt domain NEAT. Mofit region)	Accelerated degradation of C3b	
Structurally uncharacterized proteins				
AdsA	Adenosine synthase A	Function not mediated by binding	Promotion of survival in neutrophils by inhibiting the oxidative burst	
SasX	*S. aureus* surface protein X	Unknown ligand	Biofilm formation; cell aggregation; and squamous cell adhesion	Nasal or skin colonization Septic death; survival in blood
SraP	Serine-rich adhesion for platelets	Salivary agglutinin gp340 and an unidentified ligand on platelets	Endocarditis; and endovascular infection	Endocarditis
SasC	SasC	Unknown ligand	Promotes primary attachment and accumulation phases of biofilm formation	
	SasB, SasD, SasF, SasJ, SasK and SasL	Unknown ligand	Putative LPXTG proteins identified from genome sequences. No knows structure or function	
Bap	Biofilm –associated protein	gp96	Promotes biofilm formation; prevents invasion of mammary gland epithelial cells; and promotes aggregation on epithelial cell surfaces. Only found in bovine strains	
G5-E repeat family				
SasG	*S. aureus* surface protein G (SasG) and plasmin-sensitive surface protein (PIs) (a SasG homologue in MRSA)	Unknown ligand (A domain)	Adhesion to desquamated epithelial cells	
		Unknown ligand (G5-E repeat)	Biofilm formation	
Three-helical bundle				
Prot A	Protein A	IgG, IgM Fab VH3 subclass, TNFR1	Inhibition of opsonophagocytosis; B cell SAg; inflammation	Pneumonia Septic death; survival in blood Septic arthritis
		vonWillebrand factor	Endovascular infection; endocarditis	
		Unknown ligand (region Xr)	Biofilm formation	

2.2.2 Secreted Enzymes and Haemolysins.

S. aureus is known for producing a plethora of toxins, with a wide variety and common repertoire, because many toxins together with other virulence determinants are encoded on MGEs, whose presence varies considerably between strains. Such MGE-encoded toxins include, superantigens such as TSST, some leukotoxins such as PVL, and exfoliative toxins. Whereas, α-haemolysin (Hla), Υ-haemolysin (Hlg), other leukotoxins and PSMs are produced by most strains. Notwithstanding, differential expression of every core-genome encoded toxin genes, could cause considerable differences in the pathogenic potential between *S. aureus* strains, *i.e.*, mutants *agr*-defective, which controls the expression of many *S. aureus* toxin genes.[102] (Table 2.2.2)

Table 2.2.2 Main toxins of *S. aureus.*

NAME		FUNCTION
β-haemolysin/toxin	**Hlb**	Sphingomyelinase with cytolytic activity
α-haemolysin/toxin	**Hla**	Cytolytic pore-forming toxin
Leukocidins D, E and M	**LukD/E/M**	Kill leukocytes; bicomponent pore-forming leukotoxins
PSM peptides	**PSMs**	Pore-foring toxins or detergent activity
Exfoliative toxins A, B and D	**ETA/B/D**	Exotoxins with superantigen activity: glutamate-specific serine protease that digest desmoglein1
Enterotoxins	**SEs**	Gastroenteric toxicity; immunomodulation via superantigen activity
SE-like proteins	**SEIs**	Unknown. Non gastroenteric toxicity; immunomodulation via superantigen activity
Toxic shock syndrome toxin-1	**TSST-1**	Endothelial toxicity; superantigen activity
Formylpeptides	**fMLPs**	Ligands for formyl peptide receptor

2.2.2.1 *Haemolysins.*

S. aureus has a minimum of four *haemolysins* referred: α- (Hla), β- (Hlb), γ- (Hlg) and δ- (Hld, see in PSMs) toxins, which are present in most *S. aureus* isolates, encoded on the chromosome and subject to *agr* regulation. Also, they are able to lyse erythrocytes and other eukaryotic cells.[2]

***α-toxin/α-haemolysin* Hla,** the main and most characterized virulence factor, is a cytolysin produced by most *S. aureus* strains.[104,105] The *hla* gene is not mobile, and its expression is regulated by at least three global regulatory systems including Agr. It is a water-soluble monomer of 33 kDa with pore-forming and pro-inflammatory properties.[65] Upon binding to a membrane receptor, Hla forms heptameric pores, thereby destroying a variety of host cells, including epithelial cells, erythrocytes, fibroblasts, lymphocytes, monocytes and macrophages, but not neutrophils.[104] This cytolytic activity of Hla is dependent on the interaction with the ADAM10 (disintegrin

and metalloproteinase domain containing) receptor; in fact, ADAM10-deficient mice are protected from lethal pneumonia and severe skin infection, by *S. aureus*. Moreover, α-toxin leads to neutrophil chemotaxis and has pro-inflammatory effects, including induction of the inflammation and generation of highly pro-inflammatory cytokines, IL-1 and IL-18.[53] (Figure 2.4) In addition, α-toxin has been shown to contribute to the penetration of the epithelial barrier during skin infection by USA300,[106] and has also been demonstrated to impact on virulence in many infection models, such as pneumonia, where it has a significant effect on morbidity and mortality.[107] This strong impact on the development of USA300 skin infection,[37] can be prevented by passive or active immunization,[108] with anti-α-toxin antibodies or derivative of α-haemolysin, respectively.

β-toxin*/*β-haemolysin **Hlb** is peculiar, because it is a sphingomyelinase of type C that damages membranes by enzymatic alteration of their lipid leading to cell lysis. And, in many virulent *S. aureus* strains, a pathogenicity island disrupts the gene encoding *hlb*.[2,109] β-toxin does not form pores in the plasma cell membrane, in fact the lysis of erythrocytes is only observed after the cells are switched to low temperature, thus suggesting that the lytic activity of β-toxin is not as efficient as that of other haemolysins, at least towards erythrocytes. Although β-toxin is selectively cytotoxic to monocytes, and is inactive against fibroblast, lymphocytes and granulocytes, the mechanism leading to cytotoxicity is still poorly understood. Currently, it is unknown if this result is due to a specific targeting of β-haemolysin to monocytes (possibly mediated by a specific receptor) or to a higher sensitivity of these cells to the toxin.[110]

2.2.2.2 *Leukocidins.*

Leukocidins or leukotoxins, bicomponent (hetero-oligomeric) pore-forming, can lyse cells of the myeloid lineage, namely monocytes, macrophages and neutrophils, which is considered important for *S. aureus* immune evasion. These leukotoxins consist of one class S and one class F subunit proteins of 32-35 kDa, whose genes are either core genome-or phage-encoded. The class S and F subunit proteins are non-toxic on their own, but upon oligomerization, they form a β-barreled pore-structure. Five class F subunits (HlgB, LukF-PV, LukD, LukF`-PV and LukG) and six class S subunits (HlgA, HlgC, Luks-PV, LukE, LukM and LukH) have been described (see Table 2.2.2.2)[104]

Luk*DE* and **Luk*GH***, are expressed by the majority of CA-MRSA strains, and both contribute to the virulence of *S. aureus* in murine sepsis and renal abscess models.

Luk*GH* exhibits potent cytolytic activity towards neutrophils acting in synergy with the PVL *in vitro*.[104]

***γ-haemolysin* Hlg ,** also named ***leukocidin*,** can lyse white blood cells as well as other cells. It is encoded by two distinct operons, one that encodes a unique HlgA (S protein) and another encodes for HlgC (S protein) and HlgB (Table 2.2.2.2). Both proteins (S and F) have to be assembled to form membrane-perforating complexes. Active Hlg is encoded in the core genome, and exists in two bioactive forms, haemolysin-c (**Hlg**) and haemolysin-c2 (**Hlg2**). Hlg is present in 99% of *S. aureus* strains and appears to contribute to septic arthritis with weight loss in mice, and to endophthalmitis in rabbits.[104]

Table 2.2.2.2 **Leukotoxins of *S. aureus*.**[104] # PMN: polimorphonucleares.

Leukotoxins	Components		Localization	Prevalence	Cell specificity
	Class F subunit	**Class S subunit**			
Haemolysin-c (Hlg)	HlgB (Hlg1,LukF)	HlgA (Hlg2)	*hlg* gene cluster; genome	~99%	Erythrocytes from humans and other mammalian species
Haemolysing-c2 (Hlg2)/Leukocidin (Luk)	HlgB (Hlg1, LukF)	HlgC (LukS)			Human and rabbit PMN and rabbit erythrocytes
Panton-Valentine Leukocidin (PVL)	LukF-PV	LukS-PV	*pvl* locus; phage	0-5%	Human and rabbit PMN
LukDE	LukD	LukE	pathogenicity island	30-87%	Murine and rabbit PMN
LukFM	LukF`-PV	LukM	phage	0%	Bovine PMN
LukGH (LukAB)	LukG (LukB)	LukH (LukA)	n.d.	n.d.	Human PMN

***Panton-Valentine leukocidin* PVL,** two-component leukocidin, belongs to a β-barrel forming family of cytolytic toxins, comprising also other leukocidins, γ-haemolysin (Hlg) and α-haemolysin (Hla). PVL is encoded by the prophage-encoded adjacent *lukS* and *lukF* genes, which produce the two toxin parts. LukS and LukF, both are needed for the cytolytic activity of the toxin. Since 1932, when Panton and Valentine noted an association between PVL production and abscess formation,[105] the interest in PVL has been increased enormously due to an epidemiological association between the presence of the *lukSF* genes and CA-MRSA.[111] Most CA-MRSA strains have both *lukSF* genes, while their frequency in MSSA is much lower, and they are absent from predominant *hospital acquire* (HA)-MRSA clones.

PVL is lytic toxin to human neutrophils at concentrations between 0.3–2 μg/ml.[105] Concentrations of PVL reaching or exceeding that range were demonstrated in human skin abscesses and in some clinical specimens from different infection types.[105] In laboratory experiments, the contribution of PVL to the lytic activity towards human neutrophils when assayed with CA-MRSA culture filtrates proved strongly dependent on the type of growth media used.[112] For these reasons, such studies are barely conclusive when judging the role of PVL in CA-MRSA virulence.[105] (Figure 2.2.2.2) On the other hand, animal infection models have yielded conflicting results, which has been attributed to differences between models, inoculum sizes, and, very importantly, the host species.[104,105] Actually, the leukotoxic activity of PVL differs dramatically between species: human and rabbit neutrophils are lysed by very low toxin concentrations, whereas 1000-fold higher amounts are required for the lysis of mouse or java monkey neutrophils.[113] Consequently, and the same way as with many other toxins, it is not clear whether pro-inflammatory effects are of benefit to the bacteria, as they may cause excessive phagocyte infiltration and tissue damage, or whether they serve the human innate immune system to recognize infiltration bacteria and launch a defensive response. Most probably, both mechanisms contribute to infection out-come and it depends on the specific scenario, which has a stronger impact. Finally, it needs to be noted that an increasing number of CA-MRSA clones have been found that do not contain *lukSF* genes.[98] For example, in Korea[114] and the United Kingdom,[115] and Li *et al.*,[97] have showed in rabbit skin infection studies that these clones are o average not less virulent than *lukSF* containing CA-MRSA clones.

γ-haemolysin (HlgC/B) and PVL probably target the same cell types. Both toxins are similarly potent at lysing granulocytes and human macrophages. Whereas, PVL and γ-haemolysin are extremely potent, leukocidins LukAB/GH and LukED are only active at concentrations 100-fold higher. The relative secretion of the different toxins in different conditions remains unknown.[110]

2.2.2.3 *Phenol-soluble modulins.*

Phenol-soluble modulins **PSMs** are a family of amphipathic α-helical peptides produced by staphylococci.[94] Many members of the PSMs family have pronounced cytolytic activity towards a variety of human cells, including neutrophils and erythrocytes.[94] In addition, PSMs trigger inflammatory responses by interaction with the formyl peptide receptor 2 (FPR2).[116] The α-type PSMs, ~20–25 amino acids in length, may have strong cytolytic activity, and β-type PSMs are barely cytolytic and with a ~40–45 amino acid long.[94] In particular, the PSMα peptides of *S. aureus*, PSMα1-PSMα4, are encoded in the *psm*α

operon that contains the potent cytolysin PSMα3. This PSMα3 or δ-haemolysin (Hld), is a moderately potent yet often strongly expressed. Three different mechanisms have been proposed to explain the haemolytic activity of δ-haemolysin, (i) bind to the cell surface and aggregate to form transmembrane pores; (ii) bind to the cell surface and affect the membrane curvature, thereby destabilizing the plasma membrane; or (iii) at high concentration, act as a detergent to solubilize the membrane.[110]

PSMs also contribute to staphylococcal biofilm formation by forming fibril-like structures, structure biofilms, and cause biofilm detachment, resulting in the dissemination of biofilm-associated infection.[105] Actually, in *S. epidermidis* have been demonstrated to participate in the maturation of the biofilm structure, specifically intra-biofilm channels, and at high concentrations, in mediating bacterial detachment from the biofilm. Hence and by extension, probably performing the same biofilm regulatory functions in *S. aureus.*[110] (Figure 2.2.2)

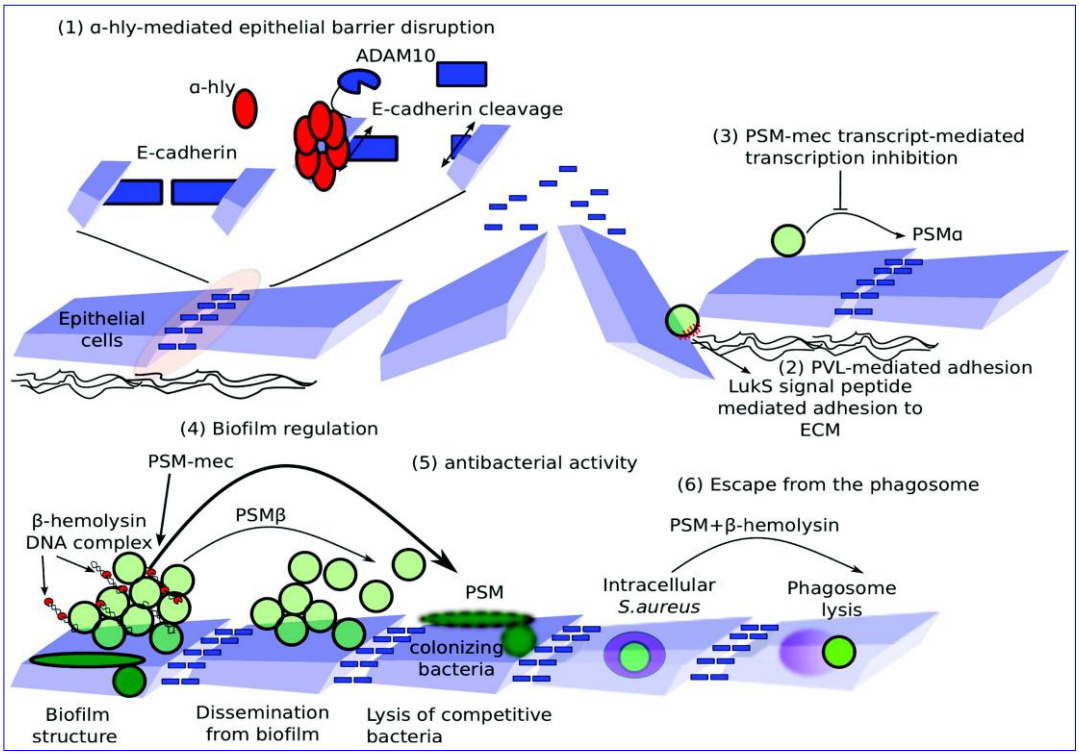
CA-MRSA strains produce high amounts of PSMs, whereas production is on average lower in typical HA-MRSA strains (USA100 and USA200 strains) where the *psm-mec* gene is located on a chromosomal cassette.[117] [94] This might, at least in part, be due to the facts that (i) Agr virulence regulator exerts an exceptionally strict control over PSMs expression,[91] and (ii) HA-MRSA strains often show low, while CA-MRSA strains commonly have high, Agr activity.[117] Briefly, the PSMα peptides have a significant impact on CA-MRSA virulence, in experimental skin infection using mice or rabbits and bacteremia in mice.[37,94] Notably, PSMα peptides, especially PSMα3, are responsible for the increased neutrophil killing capacity that distinguishes CA- from HA- MRSA strains.[118] Recent findings suggest that PSMs are expressed after neutrophil ingestion of the bacteria, in the neutrophil phagosome as a result of *agr* induction, identifying PSMs as the main mediator of quorum- (or diffusion-) sensing-induced neutrophil killing, after *S. aureus* ingestion.[119] Moreover, the last research are setting towards the possibility that production of α-haemolysin could be modulated by PSMs expression, suggesting one coordinated action among Hla and PSMs in host tissue during early pathogenesis, confirming a major role for α-haemolysin in epithelial injury during *S. aureus* infection.[120]

2.2.3 Other toxins and enzymes.

Others secreted *S. aureus* proteins, exactly proteases, are enzymes that degrade host molecules in a broad fashion, or interfere with host metabolic or signaling cascades, leading to tissue destruction, and may also have a more specific impact. The protease

aureolysin, cleaves many proteins including insulin B, with a preference of cleaving after hydrophobic residues, or even can have a major impact on the pathogenesis of osteomyelitis because of inactivating PSMs. Up-regulated by Agr at the end of exponential phase, aureolysin also leads to maturation of another non-specific *S. aureus* exoprotease, such as the glutamyl endopeptidase ***SspA* (o V8 protease)**, which cleaves after glutamate residues. Aureolysin, glutamyl endopeptidase, and the cysteine proteases staphopain B ***SspB***, all interfere with complement factors, leading to evasion of complement-mediated bacterial killing. The biological function of further *S. aureus* proteases, a series of ***serine proteases***, is not well understood, except for the exfoliative toxin serine proteases [see below]. Finally, *S. aureus* may produce a protease that degrades collagen, called ***collagenase***.[109]

Figure 2.2.2 Non-lytic functions of haemolysins, bicomponent toxins, and PSMs. (**1**) α-hly binds ADAM10 and relocalizes it to E-cadherin-containing micro-domains. ADAM10 cleaves E-cadherin, leading to loss in epithelial barrier function. (**2**) Exposure of the underlying glycosaminoglycans-rich extracellular matrix to LukS-PV signal peptide favors adhesion. (**3**) *psm-mec* RNA controls the transcription of virulence factors. (**4**) β-haemolysin binds DNA, forming nucleoproteins nucleating *S. aureus* biofilm. PSMβ acts as surfactant, promoting the formation of intra-biofilm tunnels and bacterial dissemination from biofilms. *psm-mec* enhances biofilm formation (**5**) in addition, PSMs have antibacterial activity. (**6**) Finally, PSMs in synergy with β-haemolysin participate in phagosome lysis and escape into the host cytosol [see below Figure 2.4].
α-hly: α-haemolysin; ADAM10: disintegrin and metalloproteinase domain containing.



Some *S. aureus* secreted host-damaging factors, such as the fibrinogen-binding protein **Efb** and staphylococcal complement inhibitor **SCIN** *(scn)*, which are potent inhibitors of the function of convertase C3, a crucial enzyme in the complement pathway.[109]

Staphylokinase (**Sak**) is enzyme that activates plasminogen to plasmin, which degrades fibrin clots, whose biological significance is to diminish the function of the fibrin meshwork in keeping a staphylococcal infection localized. It also cleaves the complement factor C3b, adding to the broad attack of other staphylococcal proteases and further molecules, such as Efb (fibrinogen-binding protein) and SCIN (staphylococcal complement inhibitor), on complement function. Aside staphylokinase facilitates bacterial penetration through the skin barrier, while it decreases the severity of skin infections by leading to drainage.[109]

S. aureus further produces two coagulases, ***staphylocoagulase*** and ***von Willebrand factor***, which contribute to the formation of fibrin clots after binding to prothrombin (forming a complex called staphylothrombin) and several other plasma proteins, thereby triggering the conversion of fibrinogen to fibrin. This leads to fibrin clots on the surface of *S. aureus* cells, inhibiting phagocytosis, causing abscess formation and adhesion of *S. aureus* to catheters during biofilm-associated infection.[109]

Moreover, *S. aureus* produces other proteins such as lipases and nucleases, whose functions in pathogenesis are poorly understood. Possibly, nucleases may decrease the antibacterial activity of neutrophil extracellular traps, which consist of DNA released from lysed neutrophils.[109]

On the other hand, *S. aureus* also harbors up to 15 ***enterotoxins*** (**SEs**, A, B, C_n, D, E, G, H, I, J, K, L, M, N, O), which are defined as superantigens, and able to produce gastrointestinal symptoms that include vomiting and diarrhea. For example, SEA, SEB, and SEC are the most frequent enterotoxins associated with food poisoning. Although many of these toxins have potential superantigen activity, others do not have a clear role in human disease, and the mode of action or mechanism at the surface of the intestinal mucosa is unclear. SEB can traverse the mucosa via transcytosis[121,122] nevertheless, SEA, which is one of the first causes of food intoxication, apparently cannot. Likewise, SEB and SEC are associated with non-menstrual TSS.

Finally***, Staphylococcal scalded skin syndrome*** (**SSSS**) is a superficial skin disorder by toxigenic *S. aureus* that produces either exfoliative toxin A (ETA) or B (ETB), encoded by the *eta* and *etb* genes, respectively. These toxin genes are located either on a

phage *(eta)*[123] or on a plasmid *(etb)*. Two additional isoforms of SSSS toxins (exfoliative toxins C [ETC] and D [ETD]) were isolated through pathologic observations in animals and with genome screen.[124] A recent study indicates that the proportion of *S. aureus* carrying *eta* or *etb* in overall staphylococcal nasal carriers or clinical isolates is low (0 to 2% of isolates),[125] which may explain the rarity of the disease and its clustering in favorable milieus.

2.2.4 Superantigens.

Aforementioned staphylococcal enterotoxins (**SEs**), and TSST-1 are the paradigm of a large family of pyrogenic exotoxins, called ***superantigens*** (**SAgs**) and denominated SET1 to SET15.[126–128] Superantigens are proteins that do not activate the immune system via normal contact between antigen-presenting cells and T-lymphocytes. All of them share a common architecture, though quite some variation exists in the primary structure of many superantigens. They consist of A and B globular domains, β-sheet barrels and α-helices, rejoined by a discrete linking piece. A genealogy study of superantigens built on the base of their sequence homologies, has segregated them into five groups. Group I represented only by TSST-1, and group III contains only staphylococcal superantigens (SEs H, I, K, L, and P). Groups II and V contain both staphylococcal and streptococcal superantigens (staphylococcal SEs B, C, and G and SEs I, K, L, and P, respectively), and group IV only streptococcal superantigens. This underlines the likelihood of horizontal gene transfer between these two genera, a fact that is becoming increasingly apparent with genome comparisons.[129,127]

Recall that toxin genes are dispensable elements, not necessary for growth in rich media and in the absence of competition, but obviously provide a way for the bacterium to escape host immunity. Thereby, superantigens can result in harm of the host immune system, however they are not ultimate bacterial weapons, as they only affect to restricted subgroup of anergic patients.[123,126,129] This survival advantage of provoking allergic diseases, including rhinitis, asthma[130] and Kawasaki syndrome,[131] is true but less intuitive. For example, TSST-1 and few SEs (A,B and C), have been involved in the etiology of psoriasis and atopic dermatitis,[132] where toxin-induced skin modification could promote bacterial survival. TSST-1, associated with the toxic shock syndrome, is secreted locally by toxigenic strains, and can cross the mucosal membrane, and then disseminate throughout the body. An experimental study suggests that TSST-1 could activate directly epithelial cells and the innate immune system to promote its translocation.[121] Actually, the clinical relevance of this multiplicity of toxins is not entirely understood.

In short, CA-MRSA and CA-MSSA USA300 do not produce the superantigens SEB and SEC, but instead produce SEl-Q (and others superantigens including occasionally SEA and often SEl-K). Also it produces an apparently a N-terminal one-half deletion variant of TSST-1, whose activity is incompletely characterized, and has recently been associated with a newly described illness, extreme pyrexia syndrome, in which patients rapidly developed fevers in excess and quickly succumbed and died.[133] USA300 also secretes the recently described superantigen SEl-X, which has been associated with necrotizing pneumonia caused by USA300 strains.[134]

2.3 Pathogenesis: Genomic and mobile elements.

Mobile genetic elements (**MGEs**) can be defined as DNA fragments, which are able to encode one or more virulence and/or resistance determinants, including enzymes that mediate for their own transfer and integration into another DNA. Thus, they play a central role in the adaptation process, and are a means to transfer genetic information (DNA) between and within bacterial species. MGEs are called a ''mobilome'' because they are able to display a mobility in the same cell and between cells.[135] MGEs can insert through vertical gene transfer phenomena various size of DNA sequences, such as phages, transposons, pathogenicity islands, plasmids and chromosome cassettes.

"*This extra-chromosomal DNA elements play a crucial role in the plasticity of the genome, allowing bacteria to adjust readily to new environments*".[136]

2.3.1 Plasmids, transposons and bacteriophages.

The definition of ***Plasmids*** could be, any small-DNA-molecule able to auto-replicate in the own genome within a suitable host, and with the ability to transfer high frequency both resistance and virulence determinants, from one bacterium to another (even of another species) via horizontal gene transfer. For *S. aureus* have been classified into three different classes, (1) *class I*, composes of small (1.3-4.6 kbp), multicopy (10-55 copies per cell) plasmids with either cryptic or caring a single (rarely two) resistance determinants (pT181, pC194, pE194); (2) *class II*, are larger (15-46 kbp) and exist in lower copy numbers (4-6 per cell), however, this group includes most of the penicillinase and aminoglycoside/trimethoprim resistance plasmids (e.g. pSK1); and (3) *class III* consists of most large (30-60 kbp) plasmids caring a determinant of transfer (*tra*) by conjugation and in most of them, a combination of resistance markers; this group includes glycoside-resistance plasmids (pGO1 or pCRG1600) and usually possess one or two transposons and many copies of insertion sequences.[135] In addition, staphylococcal plasmids encode resistance to a variety of organic and inorganic ions,

such as cadmium, mercury, arsenate, etc., which are highly toxic for living cells, as well as molecules involved in metabolism.[136]

Insertion sequences (**IS**) are transposable elements, responsible for the recombination and stabilization of some resistance genes, which carry only that genetic information required for transposition, although it does not code for any resistance. Their presence is really important in the evolution of bacterial genome by inducing changes in the chromosomal genes expression,[135] and can even inactivate several genes either by direct insertion or by polar effect on nearby genes transcription. For example, IS256 and IS257 are mediated by Tn4001 and Tn4003, and form a hybrid pair promoting resistance to aminoglycoside (*aac*A-*aph*D) and trimethoprim (*dfr*A), respectively.[135,136]

Staphylococcal ***transposons*** (**Tn**) are probably the smallest elements that encode resistance genes predominantly. The smaller transposons are usually presented in multiple copies in the genome, either inserted into the chromosome or into MGEs, such as SCC or plasmids. The larger transposons are present in single copies and encode resistance to antibiotics, such as tetracycline, trimethoprim, aminoglycosides, or vancomycin.[136]

Transposon Tn552 carries *bla* for penicillinase. Tn554 carries resistance to erythromycin, spectinomycin and macrolide-lincosamide-streptogramin B, presented in multiple copies, which can be found integrated into SCC (staphylococciccal cassette chromosome), plasmids or on the chromosome.[137] Tn5801, found as unique conjugative transposon in Mu50, carries *tet*M encoding the resistance to both tetracycline and minocycline.[138] Transposon Tn4291 carries resistance gene to methicillin, on the penicillinase plasmid (pI524).[135] Tn1546 encodes the *van*A operon, (*van*A, *van*H, *van*X, *van*S, *van*R, *van*Y and *van*Z) to vancomycin resistance, within a conjugative plasmid, whose gene expression occurs only in the presence of vancomycin, a process mediated by a two-CS-signal transduction encoded by *van*S and *van*R.[136] *Van*Y and *van*Z encode an accessory protein that could play a role in teicoplanin resistance.[139] Transposon Tn1546, was likely transferred to MRSA from vancomycin-resistant *Enterococcus* (VRE) during co-infection.[136]

Bacteriophages (phages) or bacterial viruses, perhaps, have the greatest impact on staphylococcal diversity and evolution. All phages are classified into one of three distinctive groups: lytic, chronic and temperate. **Lytic** phages (*Myoviridae* family) have been used in phage therapy, as they lyse completely to bacteria during release of progeny phages. Bacteria infected with **chronic** phages, release progeny into the extracellular environment without killing the host, which allows bacteria to grow and

divide. **Temperate** phages (*Siphoviridae* family), the most numerous group, have the ability to lyse bacteria after infection, but they typically form a long-term relationship with the host cell, whereby the phage DNA integrates into the staphylococcal genome as a prophage. Anyhow, phages can impact expression of virulence determinants by lysogenic conversion, positive or negative. Following positive lysogenic conversion, bacteria express prophage-encoded virulence determinants. Negative lysogenic conversion occurs when there is insertional inactivation of genes (e.g., *hlb* of *S. aureus*) by integration of the phage DNA into the bacterial chromosome. Although there is a loss of β-haemolysin during lysogeny, these prophages contain genes encoding immune-modulator proteins, such as staphylokinase, staphylococcal inhibitor of complement (Scn), and chemotaxis inhibitory protein (Chp). Other *S. aureus* prophages encode virulence molecules such as enterotoxins and PVL.

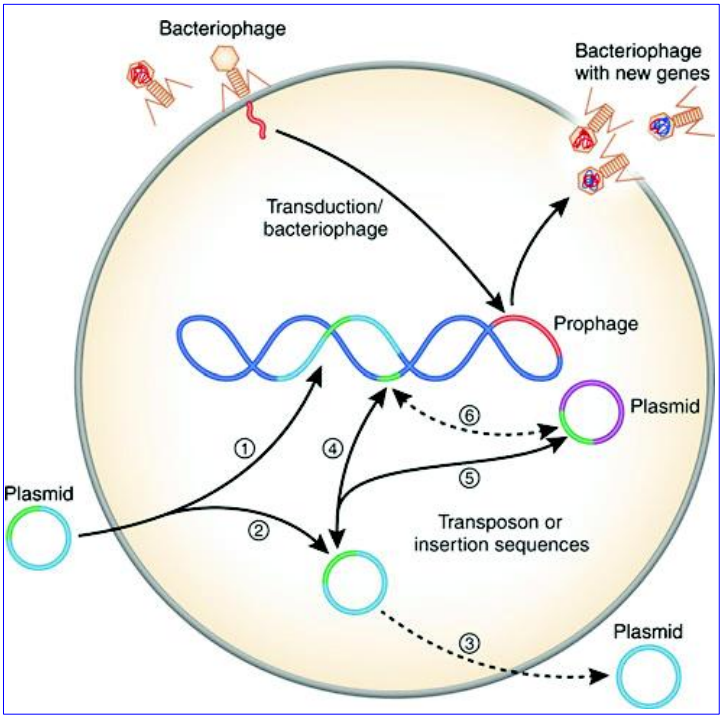


Figure 2.3.1 **Acquisition of MGEs by *S. aureus*.** (**1**) Incorporation of plasmids or plasmid elements into genomic DNA. (**2**) Plasmids can be maintained as free circular DNA. (**3**) Suicide plasmid. (**4**) Transfer of a transposon or an insertion sequence between plasmid and genomic DNA. (**5**) Transfer of a transposon or an insertion sequence between plasmids within the cell. (**6**) Transfer of a transposon or an insertion sequence from genomic DNA to another plasmid.
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Prophages and ***prophage-encoded molecules*** also work together with other MGEs within staphylococci. They, for example, can create mobility for some staphylococcal pathogenicity islands, i.e., helper phage 80a mediates excision and transfer of pathogenicity island SaPI1 to other staphylococci. And others, have also the ability to transfer antibiotic resistance by transduction of plasmids or plasmid elements previously incorporated into chromosomal DNA, i.e., plasmid pS194 with a chloramphenicol resistance determinant and pI258.[135,136]

2.3.2 Pathogenicity and genomic Islands.

Pathogenicity and genomic islands are continuous structures with variety in size (approx. 15 kb to 70 kb) that can harbour many virulence or resistance genes. They mostly contain heterologous DNA pointing out exogenous acquisition. A common feature of these elements is that they are bracketed by direct or inverted repeats and carry recombinase genes. These repeats serve as attachment site *(att)* for integration into homologous regions of the bacterial chromosome. The recombinase, which is often an integrase, catalyzes integration into the chromosome.[2]

Staphylococcal pathogenicity islands (**SaPIs**) are MGEs of 14–17 kb of which, at least 23 have been sequenced, and SaPI1 is considered as the prototype.[136,135] SaPIs family core genes are highly conserved,[136] and include two open reading frame (ORF) encoding transcriptional regulatory proteins, and a region encoding integrase (*int*), replication initiator with helicase activity (*rep*), phage interference function (*pif*) and phage terminase small subunit homologue (*ter*S). SaPIs are integrated in one of six different specific sites on the chromosome (*att*s) and each is always in the same orientation. In addition to core genes, almost all SaPIs encode integrase, resistance and virulence genes, and other superantigen-related diseases, such as food poisoning or host adaptation. SaPIs can be mobilized following infection by certain staphylococcal bacteriophages or by induction of endogenous prophages,[135,136] such as induced excision of SaPI1 by phage 80a. Six kinds of superantigens genes (*tst*-1, *seb*, *sec*, *sel*k, *sel* and *sel*q) are harbored by several kinds of SaPI.[136] Agr-regulated enterotoxin genes (*seb* and *sec)* are carried on SaPI3.[135]
On the other hand, several hypotheses to explain the origin and evolution of SaPIs have been proposed, between them, Yarwood *et al*. propose the existence of a common ancestral genetic element, probably, a prophage for all SaPIs that then generated diversity of islands through modular recombination events.[140]

Staphylococcal genomic islands are larger segment of DNA (10–200 kb) commonly acquired by horizontal gene transfer.[136] The families of genomic islands among the *S.aureus* strains whose genomes have been sequenced, are named νSAα, νSAβ and νSAY, and are flanked by a broken transposase gene (up-) and partial restriction-modification system type I (down-). Both flanking DNA segments contribute to the stability of genomic islands within the *S. aureus* chromosome.[135,136] Upstream, the direct-repeat genes are commonly associated with phages and plasmids and encode for both integrases and transposases.[136]

νSaα carries a lipoprotein gene cluster (*lpl*) and staphylococcal superantigen-like genes (*ssl*). **νSAβ** (also known as SaPIn3/m3) encodes bacteriocin, enterotoxins, hyaluronate lyase, and a serine protease gene cluster. The third, **νSAγ**, contains genes encoding β-type PSMs and a cluster of *ssl* genes similar to that present within mSAa.[135,136]

2.3.3 The resistance Island SCC*mec*.

Staphylococcal cassette chromosomes (**SCC**) are considered to be a relatively large DNA fragments always insert, on the *S. aureus* chromosome, into the *orfX* gene that may vary between 15-60 kb. The SCC is other of the MGEs able to carry virulence determinants and/or antibiotic resistance, this latter for the expression of broad spectrum beta-lactam resistance encoded by the mecA gene.[2,135,136] Thus, SCC are usually classified into two groups: **SCC*mec*** and non-SCC*mec*.[135,136] The SCC*mec* critical genes are the recombinases *ccrA* and *ccrB,* which can mediate mobilization of the whole element, and are responsible for site-specific integration and excision from the chromosome at *attBscc*.[141] And the *mecA* gene, which mediates β-lactam resistance. The rest of SCC*mec* contains various additional determinants and is referred to as *J* for junkyard.[2]

2.3.3.1 *Staphylococcal cassette chromosome (SCC) mec.*

All MRSA strains contain SCC*mec*, which encodes the *mec*A gene. Resistance of the S. aureus strains to β-lactam antibiotics is expressed by the failure of the methicillin and the other β-lactam antibiotics to link a specific low-affinity penicillin-binding protein (PBP2a) produced by MRSA, [135,136,] thus conferring resistance to methicillin and all β-lactam antibiotics.[142] This SCC*mec* could have been acquired by *S. aureus* from *S. sciuri*,[136] being necessary for its integration and excision on the *S. aureus* chromosome, a specific attachment site (*att*Bscc) at the 30 end of *orfX* by recombinases (*ccr*). [135,136,]

Based on the organization of *mec* (A, B, C1, C2, D and E),[135] of which A-C are the most common in *S. aureus*,[136] and the 6 different *ccr* allotypes, the SCC*mec* elements have been classified into 11 types (I-XI) (Table 2.3.3.1).[135] Furthermore, SCC*mec* types can be differentiated into subtypes depending on variations in the J regions.[136] These elements also differ in what other antimicrobial resistance genes are carried on them. Summarizing, types I, IV, V, VI, and VII generally do not carry other resistance genes; types II, III, and VIII may contain one or more other resistance genes, such as *ermA* (erythromycin), *aadD* (tobramycin), and *tetK* (tetracycline);[143] and the last SCC*mec* type identified, type XI has been associated to *mec*C,[144] gene a new variant of *mec*A.[145]

Interestingly, these types are also used to help distinguish CA-MRSA and HA-MRSA strains. CA-MRSA strains typically carry SCC*mec*IV, with some carrying types V and VII

elements, whereas HA-MRSA typically contain the larger SCC*mec*I, II, III, VI, or VIII elements[143] that may encode resistance determinants in addition to *mec*A. These additional resistance determinants are often encoded by plasmids, transposons, or insertion sequences incorporated into the J regions of SCC*mec*. For example, the J1 region of SCC*mec*VIII encodes a putative copper-transport ATPase (*cop*A), and the J2 region has a Tn554 transposon encoding erythromycin (*erm*A) and streptomycin/spectinomycin resistance (*aad*9) genes.[136]

Table 2.3.3.1 Currently identified SCC*mec* types in *S. aureus* strains.

SCC *mec* types	*ccr* gene complexes	*mec* genes complexes	strains
I	**1** (A1B1)	**B** (IS431-*mec*A-D*mec*R1-IS1272)	NCTC10442, COL
II	**2** (A2B2)	**A** (IS431-*mec*A-*mec*R1-*mec*I)	N315, Mu50, Mu3, MRSA252, JH1, JH9
III	**3** (A3B3)	**A** (IS431-*mec*A-*mec*R1-*mec*I)	85/2082
IV	**2**	**B** (IS431-*mec*A-D*mec*R1-IS1272)	CA05, MW2, 8/6-3P, 81/108, 2314, cm11, JCSC4469, M03-68, E-MRSA-15, JCSC6668, JCSC6670
V	**5**	**C2** (IS431-*mec*A-D*mec*R1-IS431)	WIS(WBG8318), TSGH17, PM1,
VI	**4** (A4B4)	**B** (IS431-*mec*A-D*mec*R1-IS1272)	HDE288
VII	**5 (C1)**	**C1** (IS431-*mec*A-D*mec*R1-IS431)	JCSC6082
VIII	**4**	**A** (IS431-*mec*A-*mec*R1-*mec*I)	C10682, BK20781
IX	**1**	**C2** (IS431-*mec*A-D*mec*R1-IS431)	JCSC6943
X	**7** (A1B6)	**C1** (IS431-*mec*A-D*mec*R1-IS431)	JCSC6945
XI	**8** (A1B3)	E (*bla*Z-*mec*ALGA251-*mec*R1LGA251-mecILGA251)	LGA251

(http://www.sccmec.org/Pages/SCC_TypesEN.html)

2.3.3.2 *Non-mec staphylococcal cassette chromosome (SCC).*

Non-*mec* SCC and ψSCC (without or no functional recombinase) contain virulence or fitness/survival determinants, but not encode methicillin resistance.[135,136] Thus, in this case include SCC-like elements, an ACME, a SCC, and a SCC*mec* insertion site genomic sequence, for what has been suggested that these elements should be described as pseudo-SCC elements (ψSCC). Obviously, these SCC-like regions are similar to classic SCC*mec*, so that commonly share the three following characteristics, (i) carriage of *ccr*AB and/or *ccr*C in a *ccr* gene complex, (ii) integration at ISS in the staphylococcal

chromosome, and (iii) the presence of flanking direct repeat sequences containing an ISS. For the nomenclature of such elements, it was recommended to add the suffix describing the genes' names or their functions after SCC.[135] As an example, we can have SCCHg carries mercury resistance operon. It was probably obtained from CoNS by integration of a plasmid that carried the resistance determinant, or by direct transfer of the SCCmercury element. If there is no gene carrying inferable functions in the SCC, it is recommended to add the name of the strain to the described SCC elements. For example, the strains MSSA476 (methicillin susceptible *S. aureus* strain 476), contains a *mec*-like element (SCC476) that encodes fusidic acid resistance. Moreover, some *S. aureus* strains have been describe to produce capsular polysaccharide 1, confering resistance to phagocytosis. The genes encoding synthesis of capsular polysaccharide 1 are located on a special SCC element named SCCcap1, immobile because of it lacking an active *ccr*A homologue and the *ccr*B homologue contains a no-sense mutation.[135,136]

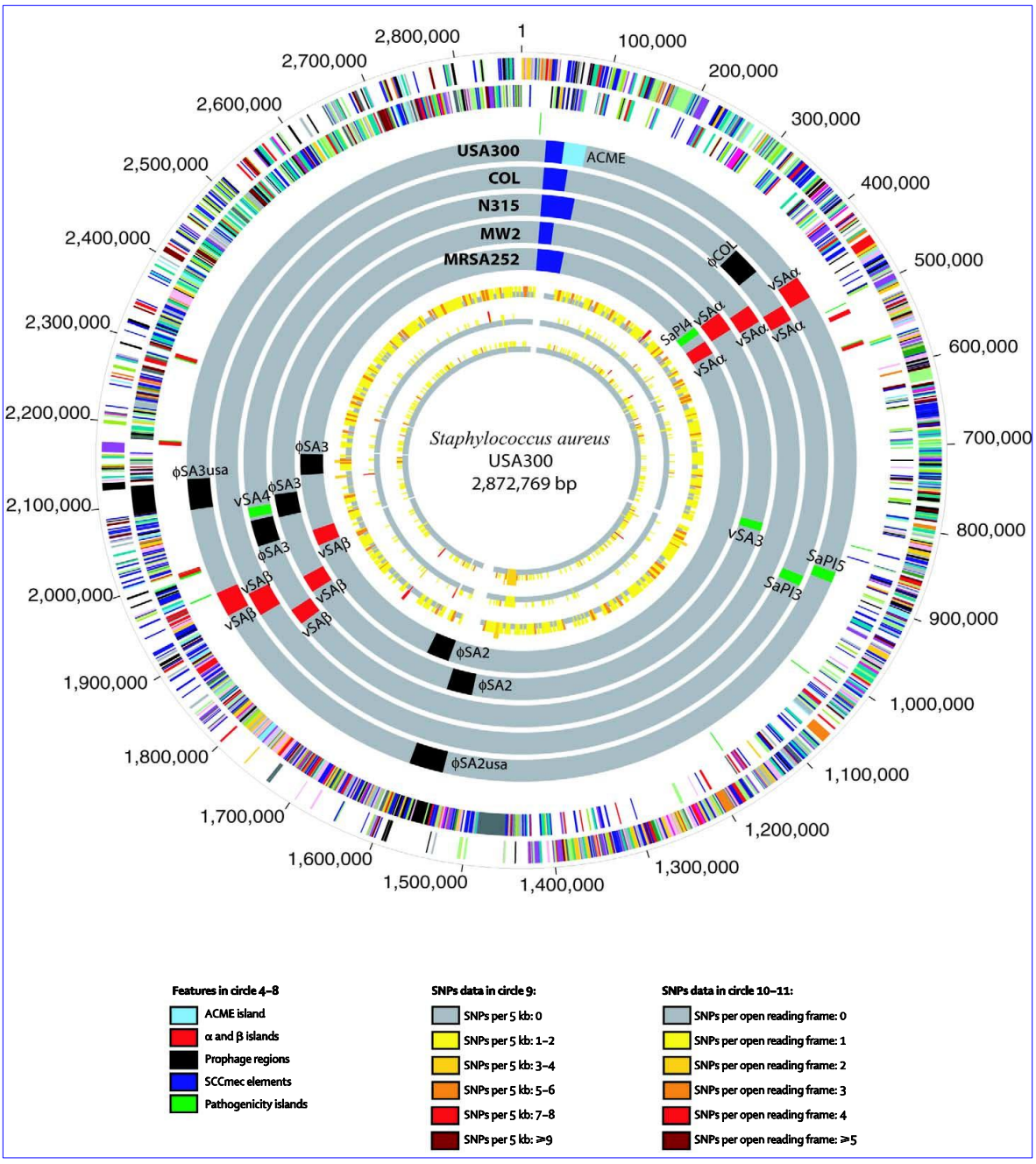
2.3.4 Arginine catabolic mobile element.

The ***arginine catabolic mobile element*** (**ACME**), discovered by sequencing the complete genome of USA300,[146] encodes a complete arginine deiminase pathway that converts L-arginine → CO_2 + ATP + NH_3. Three ACME types have been identified, **ACME type I** (*arc* and the *opp* gene cluster), **ACME type II** (*arc* gene cluster) and **ACME type III** (*opp* gen cluster). ACME type I and a truncated form of ACME type II have been identified in *S. aureus*, and exactly in USA300 was ACME type I. ACME is adjacent to SCC*mec* and integrated at the same *att*B site within *orfX*. Therefore, it is probably that the recombinases that mediate excision of SCC*mec* also mobilize ACME.[146] Actually, five novel types of ACME-SCC*mec*-CI (Composite Island) have been reported from MRSA strains with distinct genotypes.[147]

Nowadays, it seems that the role played by ACME in the success of USA300 starts to be known. Although ACME could not be associated with enhanced virulence of USA300, Diep *et al.*,[146] suggest that enhances fitness of *S. aureus*, likely facilitating colonization and/or haematogenous dissemination to target organs.[146] On the other hand, Montgomery *et al.*,[148] found no significant difference between ACME-positive and ACME-negative USA300 strains, in a rat model of necrotizing pneumonia and a mouse model of skin infection. Anyhow, the presence of ACME enhances colonization of the skin by neutralizing the acidic pH of human sweat,[136] and improves *S. aureus* survival and growth on human skin, due to ACME-encoded arginine deiminase system. This arginine deiminase system achieves via production of ammonia from L-arginine catabolism, and by depletion of L-arginine, only substrate for production of NO via

nitric oxide synthase isoform (iNOS)-producing macrophages. Additionally, USA300-ACME carried *speG* gene which helps to enhance its fitness. Spermidin N-acetyltransferase encoded by the ACME-*speG* gene, counters polyamine toxicity. *S. aureus* exhibits a unique sensitivity among bacteria to this polyamines, USA300 is able to survive.[147] Indeed, the study of Joshi et *al.*, demonstrated this decisive function in fitness by *spe*G gene.[149]

Figure 2.3. Atlas of the chromosome of *S. aureus* USA300 compared to others MRSA.



2.4 Pathogenesis: Evasion from innate immune defence.

The first line of defense against invading pathogens in humans is innate immune system. This complex system consists mainly of three major effector mechanisms: antimicrobial peptides and enzymes, complement system, and phagocytes. The importance and efficiency of these effector mechanisms is different depending on the site of infection and on bacteria characteristics, as well as the own immune system. In the past, leukocytes were considered the main source of molecules involved in inflammatory and immunological responses, but eventually it has been shown that other cells could be an important source of these mediators. Indeed, lung epithelial cells could modulate the inflammatory response in the airways and modulate cell recruitment by producing cytokines, chemokines, receptors and adhesion molecules. Similarly, mammary gland epithelial cells could produce both cytokines and immunomodulating molecules.[150]

The invasion of host tissues and organs by *S. aureus* triggers a response from innate immunity system, which is characterized by a quick response against the pathogen. The timing of the immune response is strictly dependent on the first indispensable step: **the recognition of pathogen associated molecular patterns**.[150] Additionally, *S. aureus* should adhere to host cell to multiply and, sometimes, even invade host cells,[151] then the host must reply by the immediate release of antimicrobial components and of chemoattractants, while it recruits immune cells for a more efficient response. Understandably, bacterial pathogens use very efficient strategies to evade host defenses in order to colonize and invade human and animal tissues.[151] *S. aureus* virulence factors repertoire, has plenty of mechanisms to evade host innate immunity, including modifications of structural component and secretion of a large array of specific immune-modulating proteins that act in concert to counteract innate immune defenses, and create a microenvironment to enable even better survival. *S. aureus* is also able to avoid Toll-like receptors (TLR) recognition, and furthermore, their adhesins are quite efficient in reducing the effectiveness of complement system and phagocytes, and they have the capacity to develop resistance mechanism against host defense peptides, similarly to the one developed against conventional antimicrobials.[150] Regrettably, *S. aureus* can also survive within an intracellular compartment,[152] which may be relevant during persistent infections, indeed can be found intracellularly in non-professional phagocytes, either in a membrane bound compartment or in the host cytosol.[110] Table 2.4 reports a list of the known factors involved in *S. aureus* immune evasion classified by target: complement system, neutrophils and phagocytosis, host defense peptides, immunoglobulins and opsonins.[150]

Table 2.4. Virulence factors to evade innate immune defenses related to complement, leukocyte migration phagocytic activity, immunoglobulins and opsonization. # C: complement; SAg: superantigen; PMN: polimorphonucleares; ROS: reactive oxygen species; TNFR: tumoral necrosis factor receptor.

EFFECTOR MECHANISM	EVASION FACTOR		FUNCTION
Virulence factors to evade innate immune defenses related to complement			
Complement inactivation	Capsular polysaccharides	**CPSs**	Alter C3 (CPS5 and 8) or C3b (CPS1) deposition
	Staphylokinase	**Sak**	Plasminogen activator
	Staphylococcal complement inhibitor	**SCIN**	Inhibits convertase
	Extracellular complement-binding protein	**Ecb**	
	Clamping factor A	**ClfA**	Platelets adhesion; binds complement regulator factor I
	Surgface protein E	**SdrE**	Binds C regulator factor H
	Extracellular fibrinogen-binding protein	**Efb**	Binds fibrinogen; inhibits C3 and C5 convertase; binds C3
Virulence factors to evade innate immune defenses related to leukocyte migration and phagocytic activity			
Neutrophil migration	Staphylococcal SAg-like 5	**SSL5**	Specific binding to P-selectin glycoprotein ligand-1 blocking PMNS rolling
	Staphylococcal SAg-like 11	**SSL11**	
	Staphylococcal SAg-like 1	**SSl10**	Binds chemokine receptors
	Chemotaxis inhibitory protein	**Chp**	Blocks C5a receptor and formyl peptide receptors
	Staphylococcal SAg-like 7	**SSl7**	Binds to Fc region of IgA and block recognition by neutrophils
Neutrophil lysis	Ɣ -toxin	**Hlg**	Bicomponent leukocidin; haemolysin
	δ-toxin	**Hld**	Cytolytic toxin; binds neutrophils and monocytes
	Panton Valentine leukocidin	**PVL**	Bicomponent pore-forming; kills leukocytes
	Leukocidins A and B (alternative names H and G)	**LukAB/HG**	Bicomponent pore-forming leukotoxin that kills PMNs
Resistance to oxidative stress	Staphyloxanthin		Carotenoid (protects against ROS)
	Catalase and alkylhydroxide reductase	**CatA, AhpC**	Inactive hydrogen peroxide; pivotal for nasal colonization
	Thioredoxin and thioredoxin reductase		Inactive ROS
Virulence factors to evade innate immune defenses related to immunoglobulins and opsonization			
Degradation of Ig	Protein A	**Spa**	Binds Fc domain of Ig, vonWillebrand factor and TNFR-1; binds C3 and promoters c3-c3b conversion
	Staphylokinase	**Sak**	Plasminogen activator
Cloaking of opsonins	Clamping factor A	**ClfA**	Platelets adhesion; binds C regulator factor 1
	Clamping factor B	**ClfB**	Platelets activation; binds cytokeratin 10

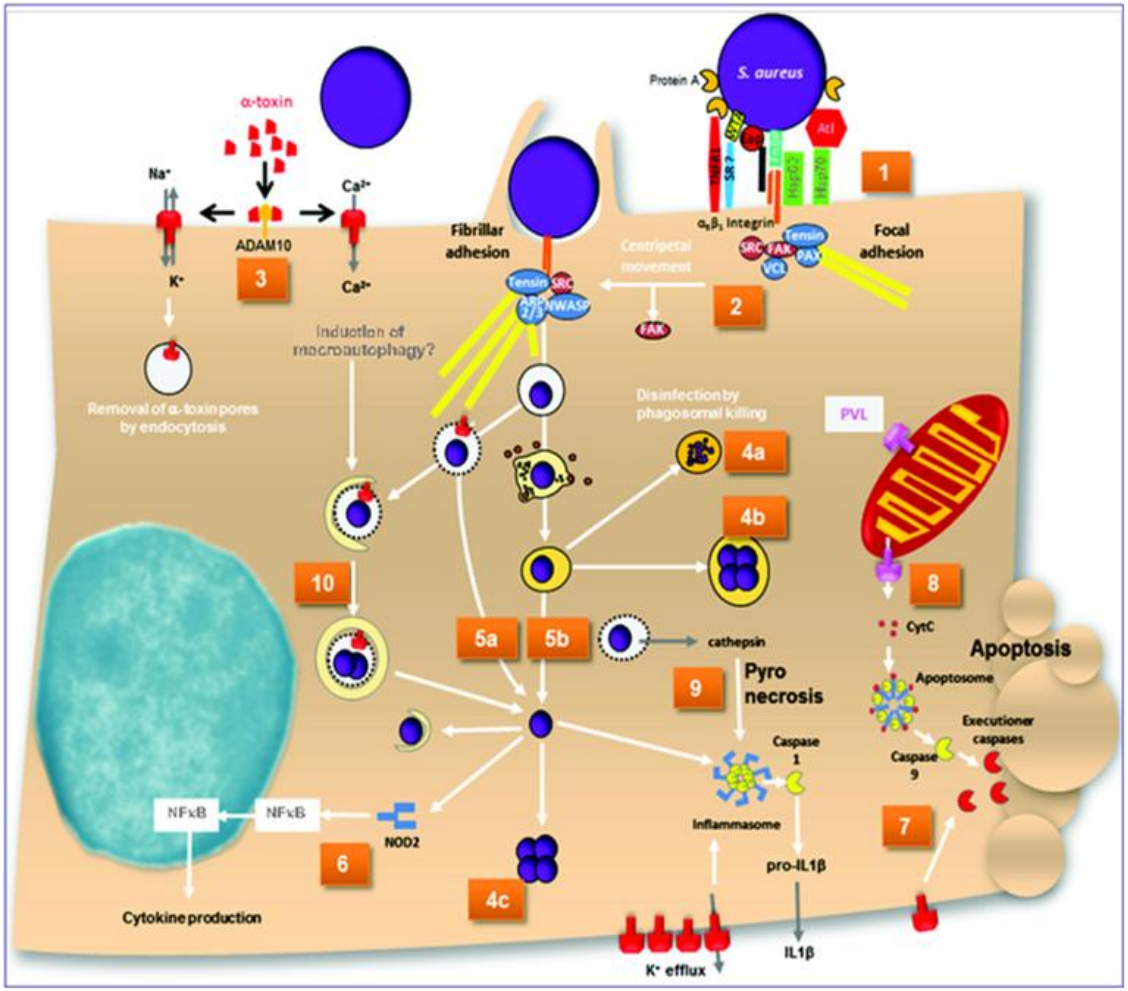
To ***avoid complement system components,*** gram-positive pathogens employ several proteolytic strategies through specific proteases capable of cleaving C5a or degradation of complement system proteins. Notable between these, is the serine protease V8[153] and the metalloprotease aureolysin,[154] which degrade key complement system components, including the opsonin C3b and the chemoattractant C5a. (**Figure 2.4**) Alternatively to the degradation of the complement-derived chemoattractant C5a, the neutrophil recruitment can be blocked by antagonizing the chemoattractant receptors. This is possible by antagonizing of formyl-methionyl-leucylphenylalanine (fMLP) peptides, one powerful neutrophil chemoattractants that arise from bacterial protein degradation, and can activate high-affinity fMLP peptide receptors on neutrophils. How? Chemotaxis inhibitory protein (Chp) is secreted by the pathogen and binds specifically to the fMLP peptide (and C5a) receptors to impair their sensing function. Likewise, *S. aureus* releases FLIPr, another virulence factor capable of antagonizing the fMLP peptide receptor to impair leukocyte responses to the bacterial-derived chemoattractant agonists.[155] (**Figure 2.4**)

The next steps to avoid the phagocytic activity, gram-positive bacteria are not sensed effectively by extracellular Toll-like receptors (TLRs), but intracellular detection is important. TLR family recognition *S. aureus* invasion triggers a complex mechanism through the activation of specific receptors for pathogen associated molecular patterns, eventually starting innate immunity response. Recently, a novel mechanism for staphylococci to escape the host immune system via interference with recognition by immune cells has been identified. Actually, the "phagocytized *S. aureus",* have been shown to evade detection by limiting phagosome acidification or phagosome fusion with granules or lysosome, or by escaping the phagosome altogether, as mediated by toxins such as leukocidins.[155]

In all of this mechanism, *Staphylococcal* superantigens-like proteins play an important role. These proteins family of molecules are able to interfere with multiple components of host immunity, including humoral immunity, opsonization, and trafficking of leukocytes.[150] Other mechanisms of evading intracellular killing include using *protein mimicry* to immune signaling. On the other hand, *S. aureus* can be observed intracellularly in non-professional phagocytes, membrane bound in a compartment or in the cytosol. Respect to this last point, the escape into the host cytosol would involve a synergism between δ-haemolysin or PSM and β-haemolysin.[152] Curiously, in Cystic Fibrosis patients, α-haemolysin is involved in phagosomal membrane lysis in CFTR-deficient cells.[156] (**Figure 2.4**)

Howsoever, *S. aureus* is able to reply the bacteria to phagocytosis, by inducing the oxidative burst response, a key host microbicidal process in which oxygen is consumed, and toxic ROS are generated. This pathway is catalyzed by two major reactions in the cell. To survive the oxidative burst response, gram-positive pathogens have mechanisms to either prevent the production of or dispose of harmful oxidants. Invasive gram-positive species like *S. aureus*, encode a bacterial superoxide dismutase (SOD)[157] that accelerates conversion of $O_2 \rightarrow H_2O_2$, and catalase (Kat) is a common mechanism to oxidize $2H_2O_2 \rightarrow O_2 + 2H_2O$. Whereas, reducing pathways such as thioredoxin systems provide electrons to small molecules that can react with H_2O_2, the tripeptide glutathione can reduce H_2O_2 directly.[155]

Shortly, other relevant concepts related such as the **autophagy**, which serves for degradation of organelles or self-digestion during nutrient limiting conditions (starvation), presumably to constitute a cellular survival mechanism. During bacterial infections, eventually, the bacteria escape from their intracellular confinement into the host cell cytoplasm in an *agr*-dependent manner (**Figure 2.4**), and consequently the host **cell death** is induced. Indeed, *agr*-deficient *S. aureus* fails to induce autophagy, which results in maturation of bacteria containing phagosomes followed by lysosomal degradation of the pathogens. As stated, *S. aureus*-induced autophagy resulted in a vacuolization of the host cell cytoplasm ("Swiss cheese phenotype"). And even more, α-haemolysis is able to permeabilize membranes for Ca2+, an inducer of autophagy.[156] The phagocytised bacterial pathogens, also can evade lysosomal killing, *e.g.*, by disintegration of the organelle membrane in order to translocate into the host cell cytoplasm. This translocation into the cytoplasm of host cell, and the growing without an immediately ensuing cell death, illustrates that phagosomal escape is not identical with cytotoxicity. The virulence factors required for **apoptosis** by *S. aureus* induced in endothelial cells, also depend on *agr* and the alternative stress-response SigB (σB), independently of SarA. Aside from apoptosis *S. aureus* is also able to induce **pyronecrosis** (**Figure 2.4**).



ADAM: Metalloprotease and Disintegrin; Arp2/3: Actin-Related Protein 2 And 3; Atl: Autolysin; Cytc, Cytochrome *C*; Eap: Extracellular Adherence Protein; Fak: Focal Adhesion Kinase; Fnbp: Fibronectin-Binding Protein; Hla: α-haemolysin; Hsp: Heat Shock Protein; Il: Interleukin; Nfκb: Nuclear Factor Kb; Nwasp: Neural Wiskott-Aldrich Syndrome Protein; Pax: Paxillin; Sr: Scavenger Receptor; Vcl: Vinculin; WTA: Wall Teichoic Acid.

Figure 2.4. **A map of intracellular fates of *S. aureus*.** (**1**) α5β1 integrins are sequestered by Fnbp-dependent fibronectin cross-linking at focal adhesions. (**2**) Bacteria are eventually endocytosed. (**3**) Assembly of Hla pores on the plasma membrane of host cells leads is dependent on ADAM10. Hla-pores are permeable for cations. [Ca^{2+} has been reported to induce macroautophagy]. (**4a**) Bacteria are disinfected by phagolysosomes or (**4b**) survive and grow within endosomes or (**4c**) in the cytoplasm after phagosomal escape. (**5a**) Phagosomal escape can be mediated by Hla, in Cystic Fibrosis cells, and (**5b**) also by a combination of PSMs and phospholipases. (**6**) Cytoplasmic *S.aureus* peptidoglycan is recognized by NOD2. (**7**) The mode of cell death induced by *S.aureus* is not completely understood. While caspase-independent cell death exists, Hla is capable of inducing extrinsic apoptosis. Up on Hla induced K^+ efflux caspase2 has recently been shown to lead to mitochondrial outer membrane permeabilization. (**8**) PVL has been reported to permeabilize mitochondrial outer membrane thereby releasing cytochrome *c* and thus inducing the apoptosome in a Bax-independent pathway of intrinsic apoptosis. Caspase 9 subsequently activates executioner caspases. (**9**) Cathepsin release from permeabilized phagosomes activates the inflammasome. Activated caspase1 leads to Il1β maturation and inflammatory pyro-necrotic cell death. (**10**) Toxin-permeabilized endocytic vesicles are targeted by autophagy. During autophagy isolation membrane engulfs leaky endosomes or cytoplasmically located bacteria. Within these autophagosomes bacterial replicate and eventually escape the organelle ultimately lead into host cell death. Reprint from [156] with permission from Frontiers Group.

"The spread of highly virulent CA-MRSA is a major concern. Increased prevalence of leukotoxins, such as PVL, and increased expression of membrane-damaging factors, such as α-haemolysin and PSMs, have been observed in these strains".[110]

Over decades, haemolysis and leukotoxicity have been highlighted as the major role of these virulence factors, and increasingly it is clear that the membrane-damaging toxins and peptides could have other functions besides killing host cells. Certainly, *in vitro* studies, suggest that these virulence factors are secreted in response to different environmental signals or at different stages during colonization or infection, and that this differential diffusion of the toxins and peptides within tissues could lead to different functions.[110] Study of the interaction between these virulence factors and host cells in more physiological models should provide us novel important findings.

3 Antibiotic Resistance.

S. aureus is a magnificent example of evolving pathogen, this organism has been shown an effective adaptation to the changing conditions of the surrounding medium, and therefore, from the point of view of the pathogenicity and the evolution to antimicrobial resistance, is regarded as the evolutionary paradigm progression. *S. aureus* has been able to develop resistance to virtually all antibiotic classes available for clinical use. These encompass cell wall inhibitors such as β-lactams and glycopeptides; ribosomal inhibitors that include macrolide-lincosamide-streptogramin B (MLS_B resistance), aminoglycosides, tetracyclines, and the new oxazolidinones; the RNA polymerase inhibitor rifampicin; the DNA gyrase blocking quinolones; the antimetabolite trimethoprim-sulfamethoxazole; and the newer lipopeptides and lipoglycopeptides.[2] The main resistance mechanisms are summarized in Table 3. Some are discussed subsequently.

Table 3. Antimicrobial resistance genes and mechanism in *S.aureus*.

ANTIBIOTIC	RESISTANCE GENE(S)	GENE PRODUCT(S)	MECHANISM(S) OF RESISTANCE	RESISTANCE TO	LOCALIZATION
Inhibitor(s) of cell wall synthesis					
β-lactams	*bla*Z	β-lactamase	Enzymatic hydrolysis of β-lactam ring. Inactivation.	Penicillins	Plasmid: Transposon
	*mec*A, *mec*C[144]	PBP2a	PBP2 modified with reduce affinity. Modified Target.	Penicillins, cephalosporins, monobactams, carbapenems	Chromosome: SCC*mec*
Glycopeptides	Mutations in *vra*RS, *rpo*B?	Altered peptidoglycan	Trapping of vancomycin in the cell wall. Cell wall thickness: VISA.	Vancomycin	Chromosome
	*van*A, *van*H?	D-Alanine-D-Lactate	Reduce affinity. Modified target: VRSA	Vancomycin	Plasmid: Transposon
Lipoglycopeptides	vanA, vanZ?	D-Alanine-D-Lactate	Unknown	Oritavancin	Plasmid: Transposon
Lipopeptides	Mutation in *mpf*R, *dlt*A.	Multifactorial	Change in the cell membrane charge. Decrease drug binding.	Daptomicin	Chromosome
Bacitracina[158]	*bra*DE, *vra*DE	ABC transporters	BraS/R-two-CS (Bacitracin resistance associated) responds to low bacitracin concentrations.	Bacitracin	Chromosome
Fosfomycin[159]	*fos*A, *fos*B, *fos*X	Glyoxilases	Ring opening of the epoxide motif. Inactivation	Fosfomycin	Chromosome and Plasmid
Inhibitor(s) Proteins Synthesis					
Aminoglycosides	*ant*(4´)-Ia	O-Adenyltransferases (ANT)	Catalyzes ATP-dependent adenylation of hydroxyl group	Tobramycin, amikacyn	Plasmid. Plasmid: Transposon
	aph(3´)-IIIa	O-Phosphotransferases (APH)	Catalyzes ATP-dependent phosphorylation of a hydroxyl group	Kanamycin, neomycin, amikacyn, gentamicin,	

	aac(6´)-Ie+*aph*(2´´)	N-Acetyltransferases (AAC)	Catalyzes acetyl CoA-dependent acetylation of an amino group	Gentamycin, tobramycin, amikacyn	
Macrolide-lincosamide-streptogramin	*msr*A, *msr*B	MsrABC pump	Active efflux	Macrolides, streptogramin B (MSb)	Plasmid or chromosome
	lnu	Nucleotidyltransferase	Inactivation	Lincosamines	
	vgb	Phosphorilases	Inactivation.	Macrolides, streptogramin B (MSb)	
	*erm*A, *erm*B, *erm*C, (*erm*T or *erm*Y).	Erythromycin methylase	Methylation of ribosome. Reduce binding to the 23s ribosomal.	Macrolides, lincosamines, streptogramins (MLSb)	Plasmid or chromosome
Quinoprustin /Dalfopristin	*vat*	Acetyl transferases	Enzymatic modification of dalfopristin	Streptogramin A	Plasmid
	vga	ATP-binding cassette (ABC) transporters	Active efflux.		
Oxazolinones	*rrn*	23sRNA	Mutations in domain V of 23S rRNA component of the 50S ribosome. Interferes with ribosomal binding.	Linezolid	Chromosome
	cfr	23S rRNA methylase	Methylation of ribosome. Modified target.	Linezolid, chloramphenicol, streptogramins, lincosamides	Plasmid
Tetracyclines	*tet*K, *tet*L	Efflux pump	Active efflux.	Tetracyclines	Plasmid or chromosome
	*tet*M, *tet*O	Ribosome protecting protein	Protection of drug target	Tetracyclines, minocyclines	
Fusidic Acid[160]	Mutation in *fus*A, *fus*E	Elongation factor EF-G (*fus*A) and RNApol RplF (fusE)	Reduce affinity. Modified target.	Fusidic ac.	Chromosome
	*fus*B, *fus*C, *fus*D		Protection of drug target		Plasmid

Chloramphenicol	*cat*	Chloramphenicol-acetyl transferase	Inactivation	Chloramphenicol	Plasmid or chromosome
	*fex*A	Efflux pump	Active efflux of phenicoles.	Florphenicol, chloramphenicol	Chromosome
Mupirocin[161]	Mutation in *ileS*	Isoleucyl tRNA synthetase	Low resistance (MuL) Reduce affinity. Modified target.	Mupirocin	Chromosome
	mupA, mupB[162]		High resistance (MuH)		Plasmid
Inhibitor(s) of metabolites					
Sulfonamides	Mutations in *dps*A,	Dihidro-pholate synthetase (DHPS)	DHPS with reduce affinity. Modified target. Overproduction of PABA (p-aminobenzoic ac)	Sulfonamides (sulfamethoxazole, etc)	Chromosome. Plasmid?
Trimethoprim/ sulfamethoxazole	Mutations in *dfr*A	Dihidro-pholate reductase (DHFR)	DHFR with reduce affinity. Modified target.	Trimethoprim	Chromosome, Plasmid
Inhibitor(s) of nucleic acids function or synthesis					
Quinolones[163]	Mutations in *grl*A (*par*C), *grl*B (*par*E)	*par*C and *par*E, components of topoisomerase IV	Mutations in the quinolones-resistance-determining-region QRDR, reducing affinity of enzyme-DNA complex for quinolones. Modified target. Stepwise resistance.	1st generation of quinolones (nalidixic ac, etc) 2nd generation, fluorquinolones (norfloxacin, ciprofloxacin, etc)	Chromosome
	Mutations in *gyr*A, *gyr*B	*gyr*A, *gyr*B component of gyrase			
	*nor*A, *nor*B, *nor*C	Efflux pump	Active efflux of hydrophilic quinolones.	Quinolones, fluorquinolones	Chromosome
Rifampicin	Mutations in *rpo*B	β-subunit of RNA polymerase	RNA polymerase with reduced affinity	Rifampicin, vancomycin?	Chromosome

3.1 β-Lactams.

β-lactams are inhibitors of bacterial growth. They act by irreversible inhibiting of penicillin-binding proteins (PBPs) via preventing the final cross-linking (transpeptidation) step in the synthesis of the peptidoglycan, and thus, they disrupt the cell-wall synthesis. Penicillin and other β-lactams are steric analogs of the cell wall precursors D-alanine- D-alanine terminal. They compete with it for binding to the active site of the membrane-bound transpeptidase and act as mechanism-based inhibitors of PBP coined for these enzymes.[2]

In *S. aureus*, three different mechanisms of resistance has been identified, (i) enzymatic inactivation by β-lactamases or penicillinases, (ii) alteration on PBPs, and (iii) tolerance phenomena which affect to all β-lactams too.[1] Tolerant microorganisms have reduced autolytic activity, and in the case of staphylococci, the experiments indicate that this is due to an excess of an autolysin inhibitor. Hence, tolerance is manifested as an increased resistance to the lethal, rather than the inhibitory, action of β-lactams.[164]

3.1.1 Resistance to Penicillin.

The most common resistance mechanism of *S. aureus* to β-lactams is penicillinase production, encoded by the *blaZ* gene, usually carried on a plasmid. The gene is inducible and proceeded by the *blaR1* and *blaI* regulatory determinants. Penicillinase is a secreted enzyme that hydrolyses penicillin and other penicillinase-susceptible compounds into inactive penicilloic acid (**Figure 3.1.a**). Penicillinase-producing *S. aureus* emerged rapidly after penicillin was introduced as a therapeutic agent in the mid-1940s. It is prevalent both, in the hospital and in the community, where it represents >95% of the isolates.[2,165]

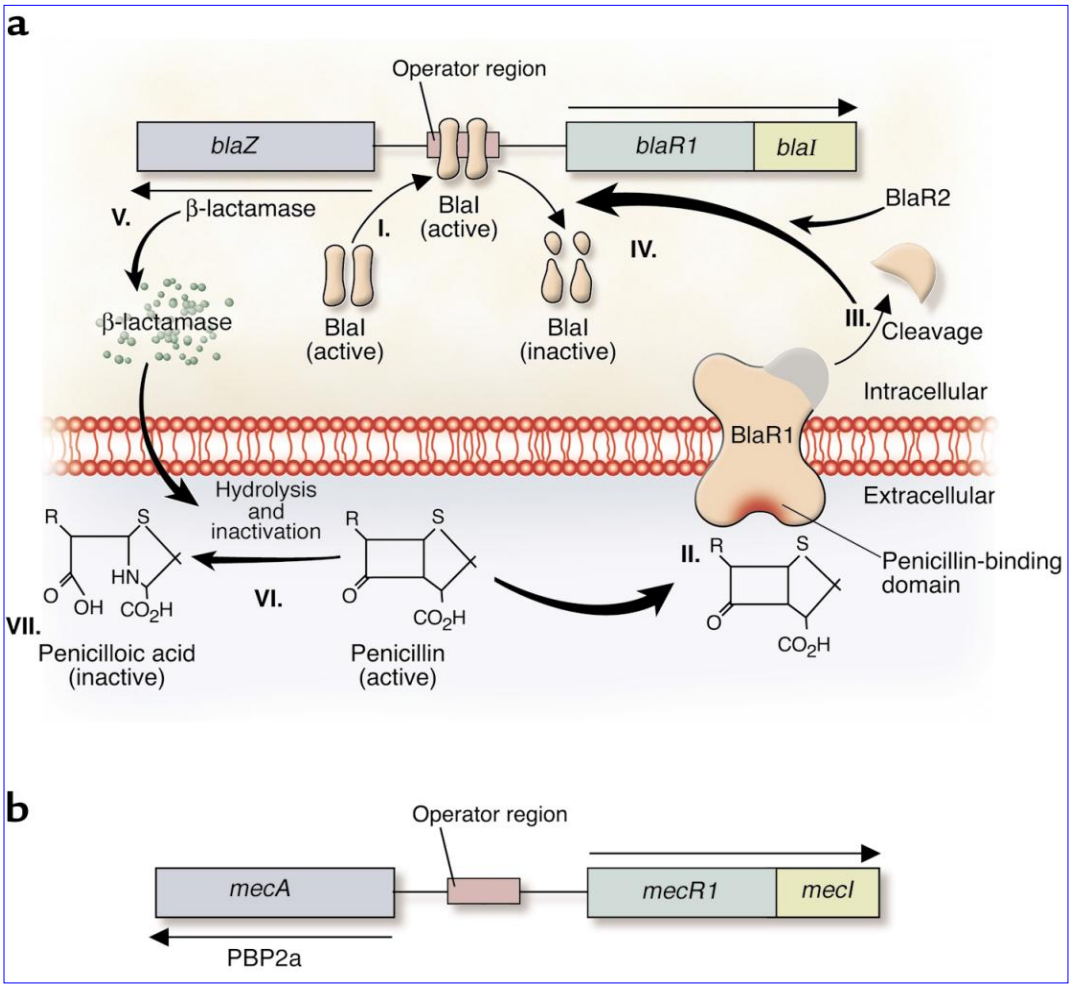
3.1.2 Mechanism of Methicillin Resistance.

The first penicillinase-stable β-lactams such as semisynthetic methicillin, nafcillin or cephalosporins became available in the late 1950s. Ironically, the first MRSA was described at about the same time. The prevalence of MRSA progressively increased thereafter, and nowadays MRSA constitutes 25-50% of clinical isolates in the North America, Europe and Asia.[165]

Higher-level β-lactam resistance (MRSA) results from the acquisition of the *mecA* gene, which encodes the penicillin-binding protein 2a (PBP2a) whose genes is controlled by the MecR-MecI-MecA regulatory systems,[165] which responds to β-lactam antibiotics in a fashion similar to that of the regulation of *blaZ*. PBPs are membrane-bound enzymes

that catalyze the transpeptidation reaction that is necessary for cross-linkage of peptidoglycan chains. Their activity is similar to that of serine proteases, from which they appear to have evolved. PBP2a substitutes for the other PBPs and, because of its low affinity for all β-lactam antibiotics, enable staphylococci to survive exposure to high concentrations of these agents. Thus, resistance to methicillin confers resistance to all β-lactam agents, including cephalosporins.[141] (Figure 3.1.b)

Figure 3.1 (**a**) **Induction of staphylococcal β-lactamase synthesis in the presence of the β-lactam antibiotic penicillin**. (**i**) The BlaI binds to the operator region, repressing RNA transcription from both *blaZ* and *blaR1-blaI*. In the absence of penicillin, β-lactamase is expressed at low levels. (**ii**) Binding of penicillin to the transmembrane sensor-transducer BlaR1 stimulates BlaR1 autocatalytic activation. (**iii**)-(**iv**). Active BlaR1 either directly or indirectly (via a second protein, BlaR2) cleaves BlaI into inactive fragments, allowing transcription of both *blaZ* and *blaR1-blaI*. (**v**)-(**vii**) β-lactamase extracellular (**v**), hydrolyzes the β-lactam ring of penicillin (**vi**), thereby rendering it inactive (**vii**). (**b**) **Mechanism of *S. aureus* resistance to methicillin**. Synthesis of PBP2a proceeds in a fashion similar to that described for β-lactamase. β-lactam antibiotic induces MecR1 synthesis. MecR1 inactivates MecI, allowing synthesis of PBP2a. MecI and BlaI have co-regulatory effects on the expression of PBP2a and β-lactamase.



Provision of this adequate substrate to PBP2a requires the functionality of numerous accessory genes implicated in the normal wall building,[165] some of which, *femABCD* essential factors for the expression and *fmhB,* are responsible for adding the glycine residues critical for the PBP2A function.[1] Any alteration in these elements decreases the expression of methicillin resistance, even though PBP2a is present. Another fragility of PBP2a is that it carries only a transpeptidase domain and misses a transglycosidase activity. Thus, for successful assembly of the peptidoglycan, PBP2a needs to hijack the transglycosidase domain of normal staphylococcal PBP, namely PBP2.[166] This is a salient example of heterologous protein cooperation in antibiotic resistance, but also represents the 'Achilles' heel of the system. According to that, successful treatment of experimental endocarditis from MRSA achieved with an array of older and newer β-lactams with good PBP2a affinity could be explained. This approach is a driving force for the development of new anti-MRSA compounds, or the re-using of older β-lacatms.[2]

As noted, the *mecA* gene is invariably part of a larger unique MGE, SCC*mec*, which may also contain additional genes for antimicrobial resistance and insertion sequences, as well as genes of uncertain function.[141] These elements include, transposons such as Tn*554*, which carries resistance genes for spectinomycin and erythromycin, or integrated plasmids such as pUB110, which encodes tobramycin and kanamycin resistance among others.[167]

The different situation about the full-resistance to β-lactams, classified different types of strains: (i) higher-producing to β-lactamases, (ii) borderline oxacillin resistant *mecA*(+) strains, BORSA showing heterogeneous resistance, (iii) intermediate level of resistance to methicillin due to production of modified (PBP1, PBP2) or normal (PBP4) PBPs, achieving a reduced affinity for beta-lactams, and *mec*A (-), called MODSA,[168] (iv) cefoxitin/oxacillin susceptibles *mec*A (+) OS-MRSA,[169] and (v) the own MRSA.

3.2 Glycopeptides.

In susceptible strains, ***glycopeptides*** inhibit cell wall through hydrogen bond interactions with the terminal D-alanyl-D-alanine moieties of the N-acetyl-muramic acid/N-acetyl-glucosamine (NAM/NAG)-peptides, and block both transpeptidation and transglycosylation. As a general rule, current glycopeptides are less effectives than β-lactams against MSSA. Thus, they should not be used as first-line treatment against β-lactam-susceptible organisms. However, vancomycin is still a "gold standard" against severe MRSA infections, until proof is found of newer anti-MRSA drugs, such as daptomycin, linezolid and anti-MRSA β-lactams.

The first strains of *S. aureus* with reduced susceptibility to vancomycin were reported at 1997 in Japan.[170] Strains considered to have reduced susceptibility include (i) vancomycin-resistant *S. aureus* (VRSA), characterized by minimum inhibitory concentration (MIC) >16 µg/ml, (ii) vancomycin intermediate *S. aureus* (VISA), characterized by MIC = 4-8 µg/ml, and (iii) heterogeneous vancomycin-intermediate *S. aureus* (hVISA), defined as the presence of isolated populations of VISA at the rate of 1 organism per 10^5-10^6 organisms susceptible to vancomycin.[14] Both phenotypes have been already reported in clinical isolates and might show different clinical and epidemiologic relevance.

3.2.1 Intermediate Resistance to Glycopeptides: GISA / hGISA.

This first isolate detected to have reduced susceptibility to vancomycin (MIC = 8µg/ml, detected by standard broth dilution methods) was called Mu50.[170] At that time, the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee on Clinical Laboratory Standards [NCCLS]) defined for staphylococci: "MIC of vancomycin $\leq$ 4µg/ml as *susceptible*, MIC = 8-16 µg/ml as *intermediate*, and MIC $\geq$ 32 µg/ml as *resistant"*. Therefore, the Mu50 isolate was defined as a vancomycin (or *glycopeptide)* intermediate *S. aureus* (GISA). The same author reported a second *S. aureus*, called Mu3, where the vancomycin MIC for this isolate was 4 µg/ml, formally considered as susceptible. However, Mu3 contained VISA subpopulations ($\leq 10^{-6}$ colony-forming unit [CFU]) that grew in the presence of 5 to 9 µg/ml of vancomycin and were not detected with standard drug-susceptibility testing. The term *heteroresistant* (*hGISA*) was coined to define the Mu3 phenotype. Since then, a number of cases of VISA (*GISA*) and hVISA (*hGISA*) were described worldwide, and were associated with vancomycin treatment failures both in animal experiments and in human cases.[171]

The VISA (*GISA*) may cause glycopeptide treatment failure, because of its low level of resistance, and sometimes its heterogeneous phenotype makes it hard to detect in the laboratory. The brain-heart-infusion (BHI) screen agar with 4 µg/ml vancomycin and casein (BHI-V4), together a 0.5 McFarland inoculum keep on being the best sensitivity and specificity combination (90% sensitive and 95% specific with a 0.5 McFarland inoculum and 100% sensitive and 68% specific with a 2.0 McFarland inoculum), easy to perform, which should be useful for clinical detection of hVISA. BHI-V3 (3 µg/ml of vancomycin) and BHI-V4 agars provide more precise identification of hVISA and VISA, respectively, and they may be reasonable alternatives to gold standard PAP/AUC (Population Analysis Profile / Area Curve).[172]

All these GISA showed a thickened cell-wall that contained an increased number of free uncrosslinked D-Alanine-D-Alanine terminals, which was thought to act as a shill-lure and trap glycopeptide molecules before they reach their target (Figure 3.2.a).[141] Recent genomic analyses indicate that mutations in a two-CS, *vra*SR, *gra*SR and *tca*RAB, could be involved.[173] However, other studies over clinical VISA isolates implicated to the *walk*R, *vra*SR, and *rpo*B genes. Watanabe *et al.*, [174] showed that most of the VISA strains carried mutations in the RNA polymerase gene *rpo*B. Regardless, in most, but not all cases, the relevant mutated genes appear to be directly or indirectly involved with the biosynthesis/metabolism of the staphylococcal cell wall, specifically two-CS that control the transcription of genes in cell wall synthesis.

3.2.2 Full Resistance to Glycopeptides: GRSA.

In 2002, the first vancomycin-resistant *S. aureus* (VRSA) strain (with a vancomycin MIC ≥ 32 μg/ml) was reported in the United States. Clearly using vancomycin against such isolates would be impossible.[175] The strains carried plasmid-borne copies of the transposon Tn1546, which was acquired from vancomycin-resistant *Enterococcus faecalis* (VREF). The mechanism of resistance identified in Tn1546 was shown to involve alteration of this dipeptide residue from D-Alanine-D-Alanine to D-Alanine-D-Lactate, a dipeptide with substantially lower affinity for the antibiotic,[174] which in the presence of vancomycin, allowing to continue the peptidoglycan assembly (Figure 3.2.b). Hence, resistance in VRSA occurs via specific modifications of the peptidoglycan cell wall target and is mediated by the VanA operon. This VanA operon is carried on the mobile genetic element Tn1546 in *Enterococcus* and can be transferred to a recipient *S. aureus* strain by transconjugation. Notably, all characterized USA VRSA strains belong to the *S. aureus* CC5 phylogenetic lineage.[176] Most strains appeared in a unique epidemiological scenario in diabetic wounds of patients that were infected by both vancomycin-resistant *enterococci* and VRSA.

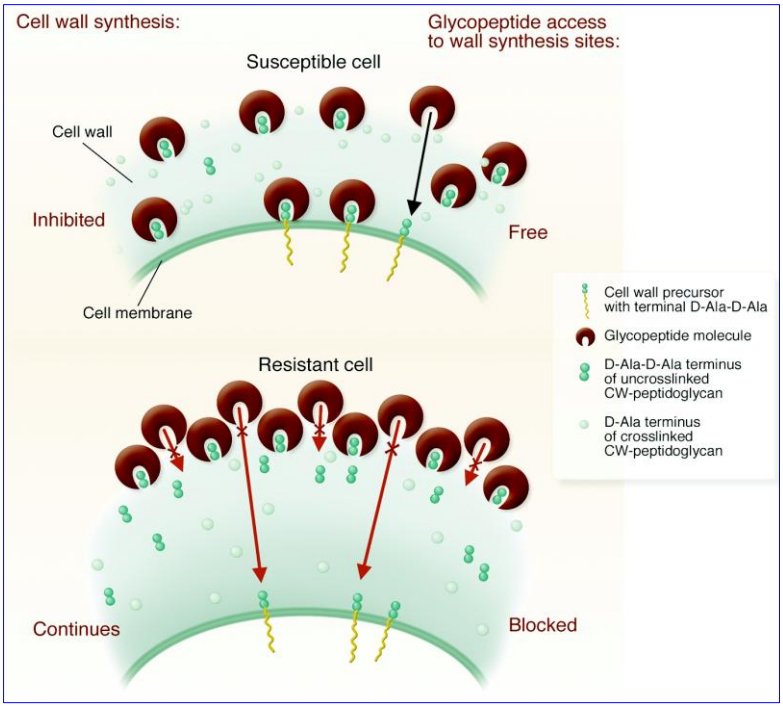
3.2.3 High Vancomycin MIC susceptibility.

Besides of hVISA and VISA, one high number of changing patterns of vancomycin MICs have been significantly reported regarding the *S. aureus* population. This has been partly driven by studies demonstrating poorer outcomes of vancomycin treatment for MRSA infections, even when these MICs are within the susceptible range. This changing pattern has been referred to as 'MIC creep'. The data suggest that the phenomenon of elevated vancomycin MICs, now appears to be common to all geographical regions. Consequently, vancomycin 'MIC creep' will likely further cause concerns as time progresses, and the absence of high-level resistance to vancomycin is not noteworthy.

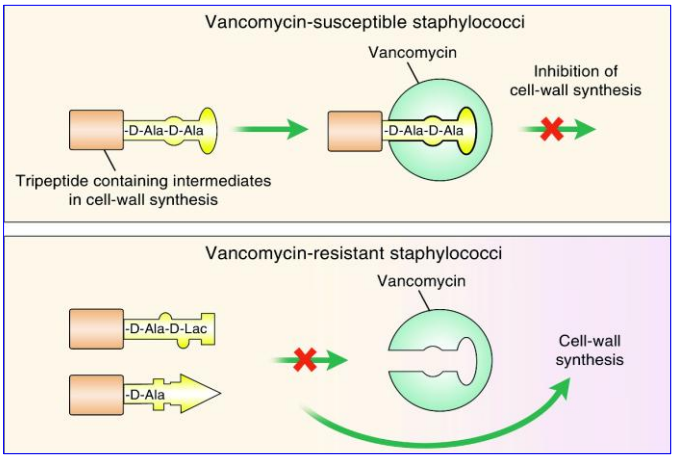
Regardless, 'MIC creep' may be still overcome, with appropriate and adequate drug dosing regimens, wherever possible.[177]

Figure 3.2 Mechanisms of *S. aureus* resistance to vancomycin. (**a**) These VISA strains synthesize additional quantities of peptidoglycan with an increased number of D-Ala-D-Ala residues that bind vancomycin, preventing the molecule from getting to its bacterial target. (**b**) VRSA strains are resistant to vancomycin because of the acquisition of the *van*A operon from *Enterococcus*, which synthesizes one new dipeptide with a dramatically reduced affinity for vancomycin.
Reprint from [141] with permission from American Society for Clinical Investigation.

(**a**)



(**b**)



3.3 Linezolid and new oxazolidinones.

The oxazolidinone ***linezolid*** is another essentially bacteriostatic antibiotic, inhibitor of the elongation of protein synthesis and only active against gram-positive bacteria. It was approved in the USA for complicated skin and soft tissues infection and nosocomial pneumonia from susceptible organisms, including MRSA. Linezolid inhibits, as clindamycin, the secretion of TSST-1 and other toxins, thus should be considered against toxin-associated infections including CA-MRSA hemorrhagic pneumonia.[178] The published guidelines for the treatment of MRSA consider linezolid an alternative first-line agent for MRSA pneumonia.

Linezolid resistance has been reported episodically in clinical settings. It is primarily the result of mutations in the 23S rRNA gene.[179] *Staphylococci* harbors 6-7 copies of rRNA genes, so that mutations in only one of them do not yield high-level resistance at once. MICs increments are progressive, and MICs of such mutants are usually 4 to 8 µg/ml compared with a baseline of 2 µg/ml. However, plasmid-mediated high-level resistance was recently detected in one clinical isolate of *S. aureus* and one of *S. epidermidis* (MIC, 8 and >257 µg/ml, respectively).[180] The resistance gene involved, *cfr,* encodes a 23S rRNA methylase that confers cross resistance to other drugs that bind at the same site, including chloramphenicol, lincosamides (i.e., clindamycin), and streptogramin A. Though as yet anecdotal, this plasmid-mediated resistance to linezolid is potentially transmissible to other organisms, and might become a clinical problem.[181]

Tedizolid phosphate, a prodrug of the active agent tedizolid or torezolid, has been recently approved by the FDA (Food and drug administration) for indication in the treatment of acute bacterial skin and skin structure infections. Tedizolid, a next generation oxazolidinone, is one of a very few prospective agents with a spectrum of activity including both, MRSA and vancomycin resistant *Enterococcus* (VRE). Similar in many ways to linezolid, tedizolid possesses several potential advantages over linezolid, by the moment, in the case of linezolid resistance mediated by *cfr*, tedizolid potency remains nearly unaffected.[182]

3.4 New Tetracyclines: Tigecycline.

Tigecycline is a modified minocycline, from the tetracycline family, also a bacteriostatic and protein synthesis inhibitor antibiotic, which acts by binding to the 30S ribosomal subunit of bacteria and thereby blocking entry of aminoacyl-tRNA into the A site of the ribosome during translation. It is almost universally active against gram-negative and gram-positive pathogens, with the notorious exception of *Pseudomonas aeruginosa* and

a few other gram-negative organisms that can extrude the drug via efflux pumps.[183] It was approved in the United States and Europe for the treatment of complicated skin and soft tissues infection. However, the recent MRSA guidelines do not include tigecycline, because the FDA's september 2010 Safety Statement described one increased overall mortality among patients with serious infections treated with tigecycline (4%) *vs* comparator therapy (3%), although it is recommended as a second- or third-line agent for MRSA infections when alternative agents cannot be used. Besides, because of its low plasma drug concentrations, its bacteriostatic activity and higher mortality rates, tigecycline should not be used in patients with MRSA bacteremia, therefore it has not been included in the recent IDSA-MRSA treatment guidelines.[181]

The resistance has only been described in *Klebsiella pneumoniae* by mutations in *acr*R. efflux pump AcrAB is regulated by its local transcriptional repressor AcrR, and a global transcriptional activator RamA. High-level expression of *acr*AB can result from mutation in *acr*R and up-regulation on *ram*A.[184] Moreover, tigecycline overcomes current *S. aureus* tetracycline-resistance mechanisms, including ribosome protection and active efflux, and thus is effective against all tetracycline-resistant isolates.

3.5 Other Antibiotic Resistance.

3.5.1 Macrolide-Lincosamine-Streptogramin B Antibiotics.

The ***macrolide-lincosamide-streptogramin B*** (**MLS$_B$**) family, bind to the bacterial ribosome and block protein synthesis. Resistance proceeds by any of the three classic mechanisms: (i) modification of the bacterial drug target by methylation or mutation (as detail below), (ii) modification-inactivation of the drug itself by phosphorilases (*vat* and *vgb* genes),[1] and (iii) decreasing intracellular accumulation of the drug controlled by the Msr(A) pump, which is coded by the *msr*(A) or *msr*(B) gene,[185] conferring resistance to macrolides and type B streptogramins (MSb phenotype). Ribosome modification and drug efflux are the most frequent resistance mechanisms in *S. aureus*.[2]

Ribosome modification, is mediated by the *erm* gene (erythromycin methylase), being the most common *erm*A and *erm*C for *S. aureus,* and conferring MLSb phenotype. The *erm* genes are preferentially located on MGEs such as transposons (e.g., Tn*554* and *ermA*) or plasmids (e.g., pE194 and *ermC*). The expression of *erm* is inducible,[186] that means, the *erm* product is synthesized only in the presence of inductive drugs (macrolides). Mutations that result in constitutive *erm* expression (consititutive-cMLSb phenotype), confer global resistance MLS$_B$.[187]

Quinupristin-dalfopristin (**Q/D**), a combination of streptogramin B and A respectively, active against MLSB-resistant staphylococci, has showed an excellent *in vitro* activity against *S. aureus*, including MRSA, VISA and VRSA, being an alternative to vancomycin for the treatment of MRSA infections. Although Q/D resistance among *S. aureus* clinical isolates is rare, one case was reported in China, caused by a combination of *ermA-ermB-ermC-msrA-msrB-vatC-vgaA* genes.[188]

3.5.2 Quinolones.

Quinolones, are an important class in the anti-infective armamentarium that originated in the 1960s as a byproduct from the synthesis of antimalarial quinines. Known quinolone-resistance mechanisms result from chromosomal mutations. Plasmid-mediated resistance has been described in gram-negative pathogens and is associated with the *qnr* gene, which protects the quinolone targets. A *qnr*-like gene has been described in *Enterococcus faecalis,* and could confer resistance to *S. aureus,*[189] however such a mechanism has not been described in clinical isolates yet.

Quinolone resistance proceeds by two types of mechanisms, including overexpression of the efflux pump NorA,[190] and structural mutations in the quinolone targets topoisomerase IV *(grlA* and *grlB)* and gyrase *(gyrA* and *gyrB)* genes.[191] Resistance is acquired stepwise. A first *grlA* mutation, (10^{-7} – 10^{-8} frequency) , produces a moderate increase in MIC (e.g., from 0.5 to 2 µg/ml of ciprofloxacin) and is still considered susceptible. However, this first mutation paves the way to a second mutation in the *gyrA* gene, which combined with the *grlA* mutation results in high-level resistance. Therefore, prevention of first-level quinolone resistance by ensuring appropriate drug levels in the tissues is important.[2] Recently, a new mutation of GyrB has been involved in high-level resistance to third-generation quinolones.[192]

The prevalence rate of quinolone resistance in *health care-acquired* -MRSA has been around 90% for a long time and is close to 40% in CA-MRSA, which makes older and newer quinolones mostly inappropriate against MRSA.[2]

3.5.3 Lipoglycopeptides and Lipopeptides.

Lipoglycopeptides: ***dalbavancin***, ***oritavancin*** and ***telavancin,*** are semisynthetic derivatives of glycopeptides. Like vancomycin, they bind to the D-Alanine-D-Alanine inhibiting both transpeptidation and transglycosylation.[2] Oritavancin also inhibits RNA synthesis. Their lipophilic nature allows the molecules to interact with the plasma membrane, which leads to dispersion of membrane potential and rapid bacterial killing. Isolates producing peptidoglycan precursors terminating in D-Lactate may exhibit

resistance to oritavancin if all precursors terminating in D-Alanine are eliminated, and the expression of the *van*Z gene of the *van*A gene cluster also results in resistance to oritavancin (MIC $\geq$ 8 µg/ml) via an unknown mechanism. Anyhow, the selection of dalbavancin resistance among staphylococci is slower than that with vancomycin. Dalbavancin and telavancin are active against VISA and oritavancin is active against both VISA and VRSA.[193]

Daptomycin is a lipopeptide and an amphophilic molecule that requires calcium to solubilize as octamer-micelles in liquid phases,[194] necessary to interact with the plasma membrane and destabilize its electric potential.[195] It is proposed as a replacement for vancomycin against MRSA, especially in right-sided infective endocarditis.[2] The mechanisms of resistance to daptomycin might involve more than one mechanism, including (i) increased cell membrane fluidity, (ii) significantly reduced susceptibility to cationic host defense peptides of platelet and leukocyte, and (i) altered expression of two key determinants of net positive surface charge, either during exponential or stationary growth phases. *DltA gain-in-function*, reflected by a significant increase in teichoic acid D-alanylated content, and *MprF gain-in-function*, reflected by a heightened elaboration of lysinylated phosphatidylglycerol. Taken together, *S. aureus* appears to involve multi-factorial and strain-specific adaptive mechanisms for resistance to daptomycin. This issue is especially relevant to MRSA strains in the context of invasive endovascular infections.[196]

4 Characteristics of CA-MRSA.

The emergence of CA-MRSA as an important pathogen has occurred over the past 15-20 years.[197,198] The first cases described, 1997-1999, were associated with necrotizing pneumonia or pulmonary abscesses and sepsis[100] with a resolution rapidly fatal. The strain responsible was USA400 (also known as the MW2 strain).[199] Subsequently, clonal outbreaks of skin and soft-tissue infections caused by CA-MRSA were also reported.[100] This time, the strain responsible was USA300.[200] These CA-MRSA, both ST8, appear to have enhanced virulence, enhance capacity to colonize multiple body sites and to survive on environmental surfaces,[98,146] as well as one important characteristic, their transmissibility due to their small SCC*mec*IV. Although cases of pyomyositis, purpura fulminans with toxic shock syndrome, and Waterhouse-Friderichsen syndrome,[100] were described, their fatality is due to necrotizing fasciitis and necrotizing pneumonia, associated especially to USA300.

Pointing out, CA-MRSA is different from *health care-acquired* (HCA)-MRSA, from both epidemiologic and molecular points of view, as well as for clinical syndromes.[201] *Health care-acquired* -MRSA is associated with risk factors that included recent hospitalization or surgery, living in a nursing-home, or carrying an indwelling catheter or device, producing mostly hospital-related pneumonia and bacteremia. Otherwise, CA-MRSA is not associated with any risk factors, and produces primarily skin and soft tissue infections, recently necrotizing fasciitis and bone and joint infections and sometimes rapidly fatal necrotizing pneumonia.[202] In addition, *health care-acquired*-MRSA is multiresistant and highly clonal, whereas CA-MRSA is pauciresistant and seemingly more polyclonal,[111] except for USA300 clone.[203] Thus, *health care-acquired*-MRSA and CA-MRSA are not alike. Practically, MRSA in patients at risk is likely to be of the multiresistant hospital type, whereas MRSA in patients without risks is likely to be more susceptible but more invasive.

4.1 Genetics of methicillin resistance in *S. aureus* and CA-MRSA clones.

Aforementioned in a previous chapter, the main characteristic of CA-MRSA is the acquisition of a MGE SCC*mec* of smallest size,[204] usually SCC*mec*IV (sometimes SCC*mec*V), and especially the strains that cause virulent infections. The capacity to acquire novel elements have been and is essential to the success of these clones, so much as to its transmissibility, as well as to its adaptation or evolution, which has also permitted them to hold over time (**Figure 5.1**).

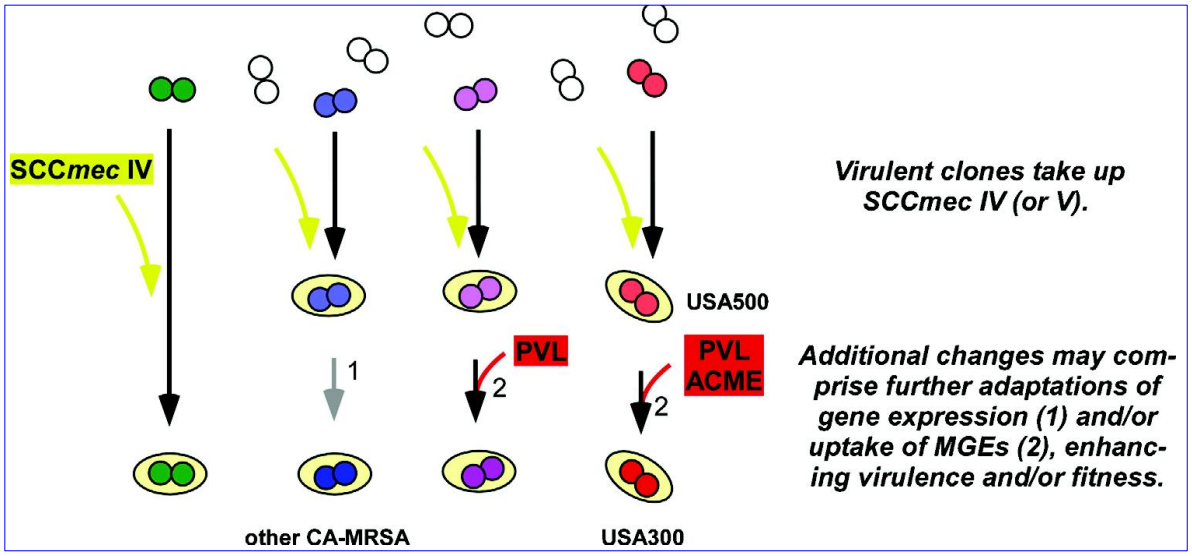
"*The evolution of virulence in S. aureus clones and lineages is frequently only analyzed in terms of acquisition or loss of virulence-associated genes. However, significant changes in virulence may arise from minute changes in the genome and all of these changes contribute to the evolution of highly virulent CA-MRSA. The peculiar in all CA-MRSA, is the higher expression of PSMs and* α-haemolysin *(Hla) compared to HA-MRSA strains,* [117] *likely or at least in part due to the high activity in CA-MRSA of Agr, which controls expression of most S. aureus toxins and has a strong impact on CA-MRSA virulence*".[205]

4.2 CA-MRSA transmissibility and fitness.

CA-MRSA isolates carry SCC*mec* elements of type IV or V, which may be associated with lower fitness costs[105] due to smaller size, so that they may also show increased transmissibility and colonization characteristics. Although, the "in vitro[206] *vs* in vivo"[202] studies have been dissimilar in this aspect about the fitness. Actually, the fact that all

CA-MRSA strains detected so far harbour one type of the novel, short SCC*mec* type IV or V, indicates that they played a crucial role in the evolution of CA-MRSA.

Figure. 4.1. Evolution of CA-MRSA. The acquisition of SCC*mec* Type IV (or V) by virulent strains appears to have been a common first step in the evolution of CA-MRSA strains. In some strains, USA300, additional steps were required. These additional steps may have involved adaptations of gene expression. Uptake of SCC*mec* type IV by a virulent ST8 strain resulted in a virulent MRSA strain (USA500), whose fitness was further improved by uptake of ACME. Acquisition of PVL-encoding genes appears to have increased virulence at least in some infection types.



On the other hand, although there is only limited data, this potentially increased colonization capacity present in CA-MRSA is related to the presence of the ACME, exactly ACME-*speG* gene, which encodes a spermidin acetyltransferase gene that transfers resistance to spermidin and other polyamines molecules.[147] Thus, the synthesis of this *speG*, could serve to explain augmented colonization capacity in USA300, since the exceptional sensitivity towards polyamines is only abolished in ACME-containing strains. Briefly, SpeG detoxifies polyamines and may, thus enhance survival of USA300 on the human skin.[105]

4.3 CA-MRSA virulence.

Many studies about the virulence of CA-MRSA, where MSSA infections *vs* MRSA infections are compared, have been published. To date, there is no compelling evidence that MRSA, in general, is more virulent than MSSA. Although the issue remains unresolved, invasive MRSA infection is associated with greater costs, limited treatment options[100] and worse

long-term outcomes after MRSA bacteremia *vs* those after MSSA bacteremia.[207] The major virulence of CA-MRSA is a fact. These strains exhibit high capacity to circumvent the first line of defence of the human body against staphylococcal infections, even the killing by human neutrophils. Hence, it is exactly the accountable for the ability of CA-MRSA strains to infect healthy people.[102]

In general *S. aureus* is not commonly known for producing a plethora of toxins, some of which are found virtually in all *S. aureus*, while others are linked to MGEs and restricted to a subset of strains. Among these toxins, some are involved in evasion of neutrophil killing, which was at least one of the predominant factors assumed to be associated with that enhanced virulence. In short, staphylococcal leukocidins, a specific toxin repertoire or an enhanced production of toxins appeared as a likely basis for the enhanced virulence characteristics of CA-MRSA.[105]

α-haemolysin (Hla) is a cytolysin that has demonstrated a big impact on virulence in many infection models. Hla contributes to the penetration of the epithelial barrier during skin infection by USA300,[106] and it could contribute to the observed systemic severity by CA-MRSA. Thus, α-haemolysin is a critical virulence factor provable in numerous animal infection models, for example, brain abscesses, skin and soft tissue infections, and has a significant effect on morbidity and mortality in CA-pneumonia.[107]

The **PSMs** peptides have a significant impact on CA-MRSA virulence, due to being responsible for the increased neutrophil killing capacity that distinguishes CA-MRSA from HA-MRSA strains.[94]

And the leukocidin **PVL,** a virulence factor whose role is more controversial. Because, depending on specific infection types or scenarios (animal model) and even of strains, could significantly contribute to the severity of the disease caused by CA-MRSA.[105] Actually, its effect keeps on being studied in different experimental models,[96,97, 114] because an increasing number of CA-MRSA clones, not containing *luk*SF genes are found equally virulent.[98] On the other hand, the contribution of lytic activity in this PVL toxin towards human neutrophils is studied in laboratory experiments.[112] Therefore, perhaps initially overestimated their virulence.

CA-MRSA, especially **USA300**, has been shown to overexpress a number of core-genome-encoded virulence factors, such as α-haemolysin (Hla) and PSMs. PVL and Hla seem to be required for early lung involvement via haematogenous spread. α-haemolysin, but not

PVL, significantly impact severe sepsis-related mortality. PVL is the predominant factor determining late-stage bone abscesses.[208]

Figure 4. Hypothetical virulence factors in USA300 and other CA-MRSA strains.

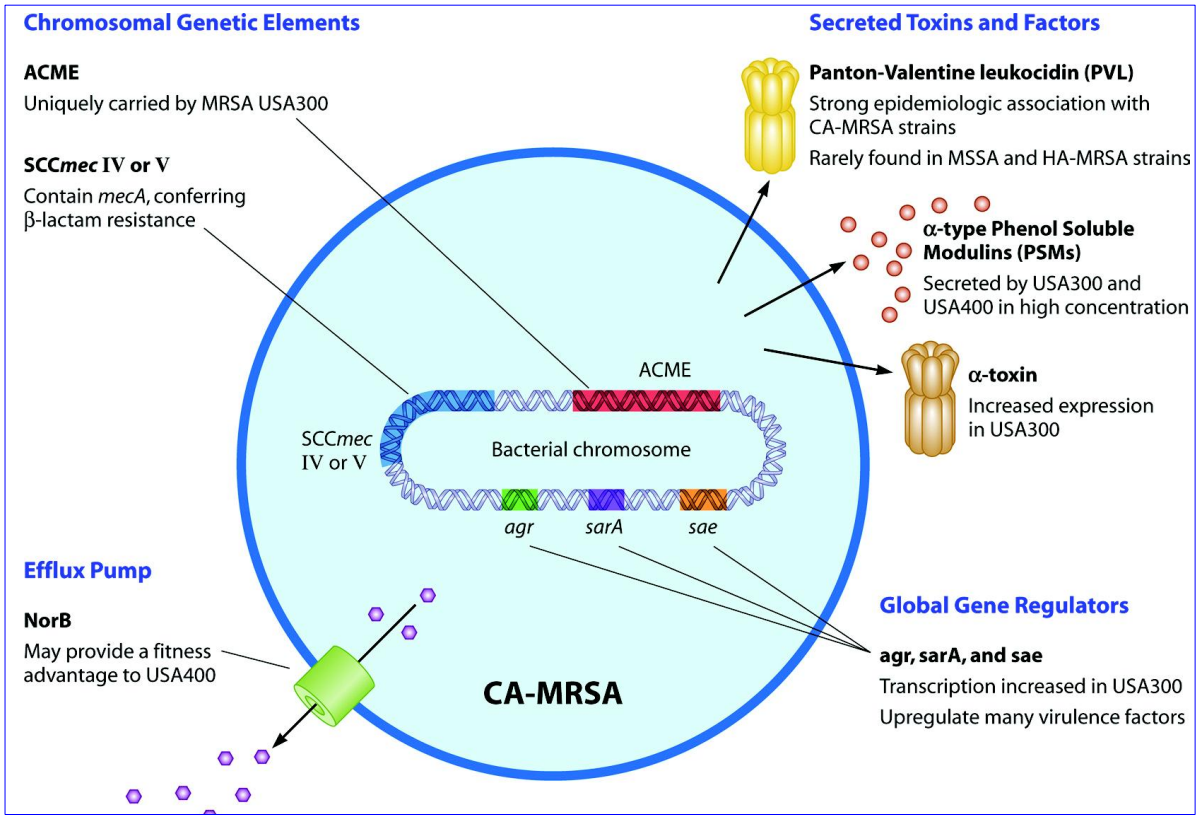


Table 4. Plasmid and characteristics MGEs of *S. aureus* USA300.

	Size (bp)	Characteristics genes	Function
Mobile genetic elements			
SCCmec type IV	-	*mec*A	Methicillin resistance
ACME type I	-	*arc* cluster + *opp*3 cluster	Arginine desaminase + oligopeptide permease system
SaPIs	-	*seq*2, *sek*2	Pyrogenic toxin superantigens
ϕSA2USA	-	*luk*S-PV, *luk*F-PV	PVL
ϕSA3USA	-	*sak*, *chp*	Fibrin-specific-blood-dot dissolving enzyme, chemotaxis inhibitory factor
Plasmids			
pUSA01	3125	-	Cryptic
pUSA02	4439	*tet*K	Resistance to tetracycline
pUSA03	37136	*erm*C, *ile*S	Resistance to macrolides, lincosamides, streptogramins B an mupirocin

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Since 1900s we have known about mass spectrometry and, since a lack of suitable ionization techniques for high mass biomolecules, proteins remained inaccessible to MS analysis for decades. Due to the introduction of soft ionization techniques, such as Matrix Assisted Laser Desorption Ionization (MALDI) and Electrospray Ionization (ESI), mass spectrometry at the end of the 1980s[209,210] and protein analysis by mass spectrometry underwent a rapid phase of development. Nowadays, has become a widely established technique for analyzing chemical structures in low quantities to trace levels. In parallel, an increasing number of full genome sequences for a variety of organisms are now available and numerous protein databases were constructed from this information. Well-annotated, high-quality protein databases built the ground on which high-output of protein identification with mass spectrometry can be performed. Another major approach has been the combination, or the modular arrangement of different types of mass analyzers (MALDI- or ESI), which has resulted in a wide variety of different mass spectrometric instrumentation (e.g., MALDI-TOF, ESI-Q-TOF, ESI-ion trap, MALDI-TOF/TOF, etc.). Although they always required additional sample preparation techniques, all of these mass spectrometry techniques allowed the determination of the primary structure of a protein. Now, modern mass spectrometers are combined with high sensitivity, to improve mass accuracy, mass resolution, and rapid analysis as well as getting sophisticated data handling in a system-dependent manner. In addition to these technical aspects in mass spectrometry, greatly improved sample separation and preparation techniques have also lead to enhanced sensitivity. Currently, the multienzyme digestion (MED) filter-assisted sample preparation strategy (FASP) approach appears to be especially useful for the analysis of samples available only in minute amounts, significantly increasing the number of identified proteins and their sequence coverage. The MED-FASP offers efficient exploration of previously unused sample material, increasing depth of proteomic analyses and sequence coverage.[211]

Quantitative proteomics yields comprehensive data on protein expression levels enabling the detailed characterization of biological processes, by comparison of various physiological states or different biological entities (such as different bacterial strains or samples from healthy and diseased organisms), on a proteome-wide scale. Mass spectrometry-based label-free quantification (LFQ) can be achieved by spectral counting[212] or by determination of peptide signal intensities.[213] However, the reliable quantification via these approaches requires a highly reproducible chromatographic sample separation, which has been resolved with the pre-treatment of the samples by nano-LC (Liquid Chromatography). Although it is known that LFQ techniques suffer from the fact that peptide signal intensities are not strictly proportional to the peptide

concentration owing to ion suppression effects,[214] LFQ is a great alternative a label-techniques such as ITRAQ for these proteogenomic studies. The applicability of label-free approaches was shown in different proteomic studies[215] and even in the challenging field of biomarker discovery.[216]

Furthermore, bioinformatics software's such as, MaxQuant software suite, developed for data acquired by high-resolution instrumentation, which permits the identification of proteins and analysis later, linked to the large "online" bioinformatics tools. Thus, nowadays, an exhaustive-wide and in depth proteogenomic analysis, is possible.

The proteogenomic studies give us information of each protein within its own functionality and its adaptation in different environments or contexts.

Background

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In the 1940s, *S. aureus* developed fast resistance to the first known antibiotic, penicillin, by acquiring the gene for penicillinase. After penicillinase-resistance, methicillin was introduced into use, and resistant strains were found about one year later.[98] These first MRSA strains spread sustainably on a global level in the next decades, leading to several epidemic outbreaks.[217] Since the detection of the first MRSA strain in 1959, different MRSA clones have emerged.[218] At that time, almost all MRSA clones detected worldwide belong to only five clonal complexes (CCs): 5, 8, 22, 30 and 45 among them, the archaic MRSA clone [strain COL, sequence type (ST) 250] harbored the SCCmec element of type 1 and belonged to CC8. Since then, important MRSA clones emerged and thus one worldwide expansion of common clones. For example, in the 1980s, the MRSA pandemic belonging to the same CC8, but had a new SCCmec types (II and III) including the Iberian (EMRSA-5, ST247) clone, a descendant of COL, or the Brazilian/Hungarian (EMRSA-1, ST239) clone. A further major MRSA clones was the New York/Japan clone (ST5, USA100) relevant in the last decade, nowadays shifted by the 'pediatric' clone (ST5), both of which belong to CC5,[119] being SCCmec-II the first and SCCmec-IV the second.

In September 2000, in the USA two MRSA strains belonged to different pulsed-field types were isolated, USA300 and USA400 (represented by the isolate MW2), and they were the predominant cause of community acquired infections. Since then, USA300 has been implicated in epidemiologically unassociated outbreaks of skin and soft tissue infections in healthy individuals in USA, Canada, and Europe.[199,200,219-221]

In Spain, MRSA strains comprise approximately 30% of the *S. aureus* isolated during 00s,[222] one resistance rates maintained over the years. Until 1995, the Iberian clone predominated in hospital outbreaks and, in recent decades, the strains of the Iberian clone have been replaced by others, such that, nowadays, the ST125-MRSA-IV clone represents approximately 50% of those MRSA strains isolated in Spain.[223,224] In recent years, the epidemiology of MRSA infections has undergone a series of changes towards one "homogeneity" of clones, in the same way as in other countries, and concurrently the resistance. In the first decade, Spain, had high rates of HA-MRSA and the incidence of CA-MRSA seem to be low, and the few cases of PVL-positive CA-MRSA isolates have frequently been associated with immigrants from South America, mainly from Ecuador.[225-229] However, considering also the small number of studies published from a few institutions, mostly Hospitals, and descriptions of sporadic cases and outbreaks, could have been the reason the divergence in the first spanish studies, limited to a lower population and zone.

The study carried out by Cercenado *et al*.,[230] analyzing the present situation of CA-MRSA in Spain, and its evolution over the last 9 years, shows one more exact map about the real situation in our country. This study identified the main clonal lineages of CA-MRSA, and its population structure by analyzing all MRSA isolates received at the Spanish National Reference Centre for Staphylococci (SNRCS) from 2004 to 2012, confirming a recent increase in the rates of CA-MRSA in Spain. This situation found by Cercenado *et al*, was also found in our studies, and though it was in contrast to that described in the USA, where a single USA300 epidemic clone is the cause of the majority of CA-MRSA infections, it was very similar to what happens in Europe. On the other hand, although the prevalence of the USA300 clone in these spanish studies was low, this was according to real situation, since this clone did not appear until 2008 and was uniformly distributed approximately by 2010.

Obviously, the urgency of USA300 clone in Spain is a cause of concern since it shows multiresistance and is highly virulent, is well adapted to the community, and has a high capacity for dissemination, which is helped by international travellers.

AIMS of the studies

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This study has enormous potential from a clinical point of view for improving clinical practices. The main objectives of this thesis have been split, original manuscript and scientific letters published (annexed).

The rapid development of resistance to most antibiotics marketed, despite the development of new antibiotics, makes the global knowledge about the whole mechanism of action of these antibiotics in bacteria, a need.

Thus, in this **original manuscript,** the following objectives will be developed:

1. **To know a global "picture" of the response of CA-MRSA USA300 to antibiotic pressure of subinhibitory concentrations of main antibiotics to clinical practice, and**
2. **To understand the adaptation of CA-MRSA USA300 clone when it is exposed to these subinhibitory concentrations.**
3. **To investigate the specific cellular response of CA-MRSA USA300 clone under subinhibitory concentrations of four antibiotics in clinical use: linezolid, vancomycin, oxacillin and tigecycline, and**
4. **To evaluate the effect of these subinhibitory concentrations**.

AIMS of letters:

1. To know the clonal distribution in our community.
2. To compare the displacement of the clones over a short time 2005-2009.
3. To compare the susceptibility to antibiotics in that time.

The importance of maintaining constant epidemiologic control, and the need for the implementation of new initiatives to prevent their dissemination, are made essential.

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Quantitative Proteomic Analysis of Community-Acquired Methicillin-Resistant *Staphylococcus aureus* USA300 in Response to Subinhibitory Concentrations of Antibiotics

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Keys words: USA300, MRSA, pathogenesis, LFQ proteomics, virulence factors

The version of this manuscript may undergo minor modifications for publication.

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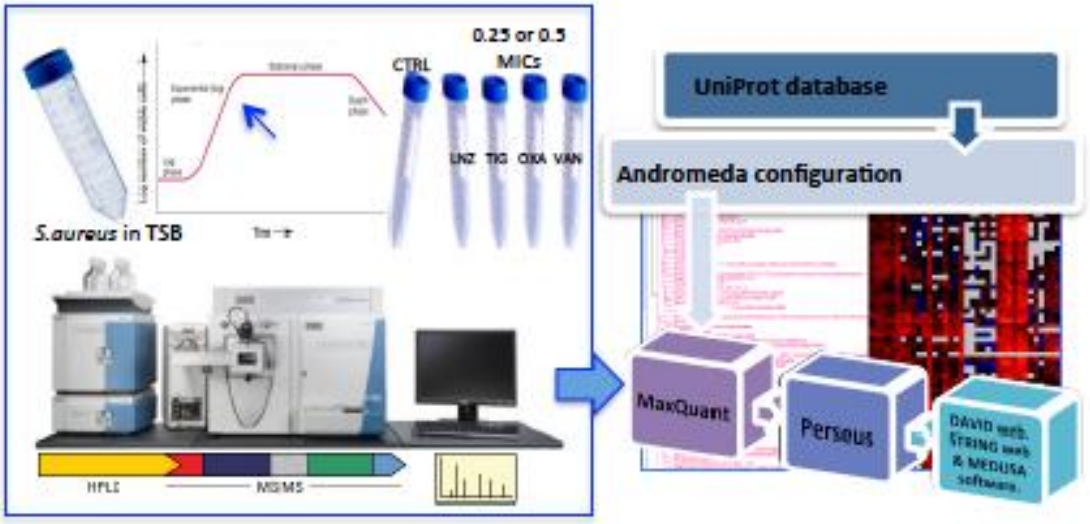
Synopsis



This study gives an overview of the expressed proteome of a hypervirulent, community acquired and resistant *Staphylococcus aureus* isolate, *S. aureus* USA300 strain, and its response to antibiotic pressure. Unlike previous genome-wide transcriptome studies, which monitored changes on mRNA level, we show qualitative analysis of USA300 cell at the protein level that directly reflects physiologically relevant adaptations. We focused on several groups of main proteins regulatory system, response to stress (include mechanism of resistance) and virulence. Here we provide evidence of the *S. aureus* USA300 cells adaptation to different antibiotics, and the possible therapeutic effect on the *S. aureus* USA300 strain of subinhibitory concentrations of several clinical used antibiotics. This study shows how the bacteria adapts to overcome low-level antibiotic stress and what proteins are involved.

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Summary

Antimicrobial resistance of the human pathogen *Staphylococcus aureus* is an ongoing problem in the healthcare sector. Community-acquired methicillin-resistant *S. aureus*, USA300 strain, has the ability to infect healthy individuals because of its high virulence. By using tandem mass spectrometry, we investigated the expressed proteome of USA300 in response to two subinhibitory concentrations of linezolid, tigecycline, oxacillin and vancomycin, and compared it to a control condition without antibiotic. Analysis of the respective USA300 cell extracts identified over 40% of USA300 predicted proteome in total.

Analysis of expression patterns of virulence and pathogenesis proteins showed that protein synthesis inhibitors had the most effect during the conditions used and cell-wall synthesis inhibitors had less effect. The treatment with protein synthesis inhibitors, linezolid and tigecycline, increased the expression of ribosomal protein RplC. Tigecycline showed a dose-dependent bactericidal activity, exhibited by a strong decrease in proteins expression, particularly of α-haemolysin Hla. Oxacillin and vancomycin inhibit cell-wall synthesis. Treatment with oxacillin led to an extensive lysis of the bacterial cells. Nevertheless, oxacillin increased the expression of virulence factors such as phenol-soluble-modulins (PSMs) and Panton-Valentine Leukocidin toxin (PVL), but not Hla. Therefore, the USA300 strain appeared to have a capacity to adapt itself to subinhibitory concentrations of antibiotics, by regulating its metabolism, redirecting the energy, and biofilm formation.

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Material & Methods

Bacterial strains and reagents.

The *pvl(+)* CA-MRSA *S. aureus* strain USA300 (SCC*mec*IV-ST8)[200] was kindly provided by Dr. DeLeo (National Institutes of Health, Hamilton) and genotyped as able to release the PVL toxin.[146] The strain was grown in tryptic-soy broth. Linezolid, tigecycline, oxacillin and vancomycin were all obtained from Sigma Chemicals Co. (St. Louis, MO). Antibiotic dilutions were made fresh prior to each experiment. *S. aureus* strains ATCC 29213 and ATCC 25923 served as controls for MIC determinations.

Determination of MICs.

Antibiotic MICs were determined for each isolate using the broth microdilution method recommended by Clinical Laboratory Standards Institute (CLSI) in Mueller-Hinton II medium (bioMérieux, Marcy l'Etoile, France),[14] and by E-test® following the manufacturer's instructions (bioMérieux). The medium was inoculated with $5x10^5$ colony-forming units per milliliter (CFU/ml) (equivalent to 0.5 McFarland) and incubated for 18-20 h and until 24 h for vancomycin, at 37 °C without shaking. Experiments were done in triplicate and the results are given in **Supplementary Table S1**. The E-test® method was used for determining vancomycin MICs, because it is considered to be more reliable in predicting treatment response, and also showed higher correlation with population analyses profile-area under the curve (PAP-AUC).[231-233]

Culture conditions for proteomic analysis.

To prepare stock cultures, a single colony of *S. aureus* USA300 was inoculated into 5 ml of sterile tryptic-soy broth and incubated overnight at 37 °C with rotary aeration (200 rpm). 100 µl of the overnight culture was used to inoculate fresh TSB (dilution 1:100, volume ratio 5:1, OD_{600} = 0.04 $\pm$ 0.005), and incubated for 4 h at 37 °C with shaking (250 rpm). The growth was monitored spectrophotometrically until OD_{600} = 0.75 $\pm$ 0.05 (early stationary phase). At this point, concentrations of 0.25 and 0.5 of MICs of each antibiotic were added to 2 ml of culture tubes (final volume and same conditions, volume ratio 5:1). The concentrations used were: 0.25 and 0.5 µg/ml for linezolid, 0.12 and 0.25 µg/ml for tigecycline, 8 and 16 µg/ml for oxacillin, 0.5 and 1 µg/ml for vancomycin, respectively. Cultures with or without antibiotics were re-incubated for 4 h at 37 °C with shaking (200 rpm) until OD_{600} reached at 2 $\pm$ 0.1 (stationary phase). Subsequently, an aliquot of each culture was plated on blood agar to determine CFU/ml. Experiments were performed in triplicate (Supplementary Figure S1.A).

Bacteria were harvested by centrifugation at 2 000 *g* for 10 min at 4 °C. The pellet was washed 3 times with phosphate-buffer saline (PBS) pH 7.4, at 1 000 *g* for 5 min at 4 °C, and subsequently resuspended in 10mM Tris-HCl pH 8.5 with 10% Sodium-dodecyl-sulphate (Tris-HCl-SDS). An equal volume of 0.1 mm glass beads (Biospec Products Inc., Bartlesville, OK) was added to the pelleted cells. The cells were lysed mechanically by bead beating for 40 s in a Ribolyser (Bio101 Savant, Vista, CA) at a speed of 6.4 m/s. The cell extracts were clarified by centrifugation (15 000 *g* for 10 min) at 4 °C. Both the lysate from pellet and the supernatant from soluble culture, were filtered through pore size 0,22 µm (Millipore, Billerica, MA). Both protein extracts were next concentrated by using a 3K Nanosep® Filter (Pall Life Sciences, MI, USA), the yield was assessed with a Direct Detect™ Spectrometer (Millipore, Billerica, MA), and stored at -80 °C until further processing.

SDS-PAGE and in-gel trypsin digestion.

Total protein extracts (20 mg) were added to an application buffer mixture, containing 10 mM dithiothreitol, following the manufacturer's instructions (NuPAGE®Sample Reducing Agilent Invitrogen), and heated for 7 min at 96 °C. Thereafter the proteins were fractionated by SDS-PAGE using a 4-12% gradient, MiniGels NuPAGE® Novex® (Invitrogen) for 35 min at 200 V. SDS-PAGE gels were Coomassie stained using a Colloidal Blue Staining kit (NuPAGE Invitrogen). After staining, each gel lane was subjected to in-gel reduction, alkylation, and tryptic digestion according to protocol used at the Proteomic Unit of University of Bergen (PROBE). In brief, proteins were reduced using 10 mM dithiothreitol for 45 min at 96 °C and alkylated with 55 mM iodoacetamide for 30 min at room temperature in the dark. The reduced and alkylated peptides were digested with sequence grade, modified trypsin (Promega, Madison, WI) at enzyme to protein ratio of 1:50 (w/w) for 16 h at 37 °C in 50 mM NH_4HCO_3, pH 8.0. The reaction was quenched through acidification with 1% trifluoroacetic acid (Fluka, Buchs, Switzerland). The resulting peptide mixture was desalted on reverse phase C_{18} stop and go extraction tips,[234] and diluted in 0.1% trifluoroacetic acid prior to nano-LC-ESI-MS/MS analysis. (Supplementary figure S1.B).

Mass Spectrometry.

All experiments were performed on a Dionex Ultimate 3000 nano-LC system (Dionex, Sunnyvale, CA) connected to linear quadrupole ion trap-Orbitrap-Elite (LTQ-Orbitrap) mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanoelectrospray ion source. For liquid chromatography separation, we used an Acclaim

PepMap 100 capillary column (C_{18}, 2 µm, 100 Å) (Dionex), 15 cm x 75µm inner diameter nanoViper column (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). The flow rate used was 280 nl /min, and the solvent gradient was 8-40% B over 31.5 min and then 40-90% B over 3 min. Elution of very hydrophobic peptides and conditioning of the column were performed during 5 minutes isocratic elution with 90% B and 12 minutes isocratic elution with 5% B respectively. Solvent A was aqueous 2% acetonitrile in 0.1% formic acid, and solvent B was aqueous 90% acetonitrile in 0.1% formic acid.

The eluting peptides were ionized in the electrospray and analyzed by the LTQ-Orbitrap Velos Elite. The mass spectrometer was operated in the data-dependent-acquisition mode to automatically switch between full scan MS and MS/MS acquisition. Instrument control was through Tune 2.7 and Xcalibur 2.2. Survey of full-scan MS spectra (from *m/z* 300 to 2 000) were acquired in the Orbitrap with resolution of R = 240 000 at *m/z* 400 (after accumulation to a target of 1 000 000 charges in the LTQ with maximum allowed ion accumulation time of 300 ms). The 7 most intense eluting peptides above a ion threshold value of 1000 counts, and charge states 2 or higher, were sequentially isolated to a target value of 1E4 and fragmented in the high-pressure linear ion trap by low-energy CID (collision-induced-dissociation) with normalized collision energy of 35 % and wideband-activation enabled. The maximum allowed accumulation time for CID was 150 ms, the isolation width maintained at 2 Da, activation q = 0.25, and activation time of 10 ms. The resulting fragment ions were scanned out in the low-pressure ion trap at normal scan rate, and recorded with the secondary electron multipliers. One MS/MS spectrum of a precursor mass was allowed before dynamic exclusion for 10 s. Lock-mass internal calibration was not enabled.

Sequence database searching.

All acquired data were processed and analyzed using MaxQuant software (version 1.4.1.2)[235] and MS/MS peak lists from individual 60 RAW files were generated. Andromeda engine performed protein identification by searching the data separately against *S. aureus* USA300 protein database, downloaded from UniProt knowledgebase on 2nd May 2014. The database contained incomplete protein records of 4 *S. aureus* USA300 strains; two strains of CA-MRSA (USA300/FPR and USA300/TCH1516) and two strains CA-methicillin sensible (USA300/TCH959 and USA300/ISMMS1). Common contaminants, such as keratin, bovine serum albumin and trypsin were also added to the database. The search parameters used were as follows: enzyme specificity, trypsin/with no proline restriction; maximum missed cleavages, 2; carbamidomethyl (Cys) as fixed modification;

N-acetyl (protein), oxidation (Met), Glu (pyro-Gln), and Gln (pyro-Glu) as variable modifications; first search precursor ion mass tolerance, ± 20 ppm for mass calibration, while a tolerance of 6 ppm was used for the main search after calibration; and MS/MS mass tolerance, 0.5 Da. For protein identification and validation the following parameters were used: 1% peptide and protein false discovery rate (FDR), minimal peptide length was 7 amino acids, and minimal number of unique peptides per protein equalled 1. Proteins were validated statistically based on the score of their individual peptides. All MS/MS identifications of peptides present in entries with reversed sequences, proteins only identified by site and contaminants were discarded. Proteins identified confidently in at least two out of three biological replicates for antibiotics assays, and three out of six biological replicates for untreated control, were included in the subsequent analysis.

Label-free quantification.

Label-free quantification based on the peak area was performed in MaxQuant. Processing evidence files, visualization, and statistical analysis were performed by Perseus (v.1.4.1.3 and v. 1.5.1.6) (Max Plank Institute, Germany).[235] The intensity derived from label-free quantification (LFQ) for each protein was transformed by log2 and next normalized. Data normalization (*N*) was performed by dividing LFQ intensity of every detected protein (row) by LFQ intensities mean of its own biological replicate (column): $\frac{(\text{LFQi of protein } \chi)\text{replicate n}}{\text{LFQi mean of replicate n}}$. The quantitative data were reported with the t-test differences between each antibiotic condition and the untreated-control. Estimation of FDR[236]= 0.05 and S0 = 0 (S0 is a small positive constant ensuring that the variance is independent of protein expression) was used for *q* values for both raw and normalized-transformed LFQ intensities[236], which were imputed in replicated runs using a Gaussian distribution with a width of 0.3 and downshift of 1.8 as the imputation parameters.[237] The differentially expressed proteins were filtered by the following cut-off: *p*-value for t-test was lower than 0.05 and the fold changes expressed like t-test difference. For normalized data without transforming by log2 (*N*-LFQ), the fold change was calculated as ratio of normalized LFQ (*N*-LFQ) intensities means by, the treated samples against untreated control

Bioinformatics Analysis.

To determine the subcellular localization of the proteins, as well as their biological process, molecular function, and other annotations, we used the UniProt access/identification of both CA-MRSA strains, USA300/FPR and USA300/TCH1916, without redundant proteins. All identified proteins were analyzed by using the DAVID

web (Database for Annotation, Visualization and Integrated Discovery) algorithm to evaluate the pathway enrichment. A group enrichment score, which is a ranking of the biological significance of gene groups based on overall EASE (Expression Analysis Systematic Explorer) scores[238] of all enriched annotation terms, was calculated by using the default parameters.[239] The *p* value threshold for pathway enrichment was set to 0.1, and medium classification stringency was chosen. The fold change was establish as the difference higher/lower than $\pm$ 1.0, calculated as the subtracting among enrichment scores (antibiotic – control) for each cluster.

To know the context of network interacting, predict protein-protein interactions,[240] we used STRING 9.1 (Search Tool for the Retrieval of Interacting Genes/Proteins) and Medusa 1.5[241] to show the graphic analysis. NCBI Protein BLAST was used to complete and search the information about the uncharacterized proteins.

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Results & Discussion

To study proteins involved in pathogenesis under different subinhibitory antibiotic concentrations, we established a workflow to treat the cultures in the early stationary growth-phase, and harvest them in the stationary growth-phase. We determined the respective subinhibitory concentrations, as half and quarter MICs of each antibiotic (Supplementary Table S1). The subinhibitory concentrations, subsequently used in the proteomic experiment, reduced the viability of the *S. aureus* USA300 strain for 2 of the 4 antibiotics. This was most pronounced with 0.5 MIC of tigecycline and oxacillin (1-2 $\log_{10}$ reduction). All of the antibiotics resulted in a reduction of the total protein outcomes in the extracts (Supplementary Table S2).

Overview of the proteomic identifications

The proteomic experiment included *S. aureus* USA300 cells grown in the presence of two sub-concentrations of each antibiotic, and a control cultured without any antibiotic. The respective trypsinated cell extracts were subjected to tandem mass spectrometry analysis, and the acquired mass spectra were searched against *S. aureus* USA300 protein database with the MaxQuant/Andromeda software. The peptide matches allowed the identification of 1 284 proteins, all extracts considered (excel file). The number of protein identifications corresponded to approximately 40 % of the 2 600 proteins encoded in the *S. aureus* USA300 genome annotated by Diep.[146] Nine-hundred-and-eighty proteins were present in at least two biological replicas, and therefore considered as valid proteins. The number of identified proteins in each type of extract varied between 418 and 774 proteins, and the identified protein repertoires were compared by Venn diagrams (**Figure 1**). Tigecycline consistently resulted in a lower number of identified proteins in both sub-concentrations. This effect could be explained by a dose-dependent bactericidal activity of tigecycline against *S. aureus.*[242]

To characterize the effects of the different antibiotic conditions, we first retrieved the

cellular localization for the identified proteins from the UniProt database (**Table 1**). Treatment with linezolid and vancomycin preserved the cellular localization distribution of proteins with respect to the control. In samples treated with 0.5 MICs of oxacillin or tigecycline, the numbers of identified membrane proteins were decreased more than 40 %, especially proteins involved in transport processes, whereas extracellular proteins were increased more than 40 %. Extracellular proteins,[30] cell surface proteins or proteins associated with the membrane,[100,243] are often associated with pathogenesis, colonization or antibiotic resistance. Therefore, a loss of transporters could point to a loss of viability of cell wall function, and the great number of extracellular proteins could be associated with an increase in virulence factors and / or a stress response. Second, we performed qualitative analysis by DAVID software program, to predict associated biological processes and metabolic pathway enrichments. As expected, 0.5 MICs gave the most pronounced differences, while 0.25 MICs produced only minor changes, compared to the control. Hence according to the literature, the antibiotic pressure led to a redirection of the cell metabolism by regulation of the energy, amino acid or purine / pyrimidine metabolism pathways, to overcome the influence of the antibiotics[244] (Supplementary Table S3). In addition, changes in the metabolic pathways observed for 0.5 MIC of linezolid was concordant with the study of Bernando *et al.*,[245] and the down-regulation of metabolic pathways observed for tigecycline could be associated with bactericidal activity[242,246] (Supplementary Table S3). On the other hand, linezolid showed a dose-dependent increase in a number of ABC transporters, which could be related to an early stage of developing resistance.[247,248] Whilst, oxacillin increased the number of stress response proteins and pathogenesis proteins.[247] (Supplementary Table S4). Indeed, the treated *S. aureus* USA300 cells had a substantial number of proteins involved in response to stress (Supplementary Figure S2). Therefore, there were considerable changes in the protein profiles in response to 0.5 MICs of the antibiotics. The effects were particularly pronounced for tigecycline and oxacillin.

Figure 1. Proteome comparison in response to antibiotic treatments. Venn Diagrams showing shared and exclusive proteins among control samples and both subinhibitory concentrations (0.25 MIC and 0.5 MIC) of linezolid, tigecycline, oxacillin and vancomycin.

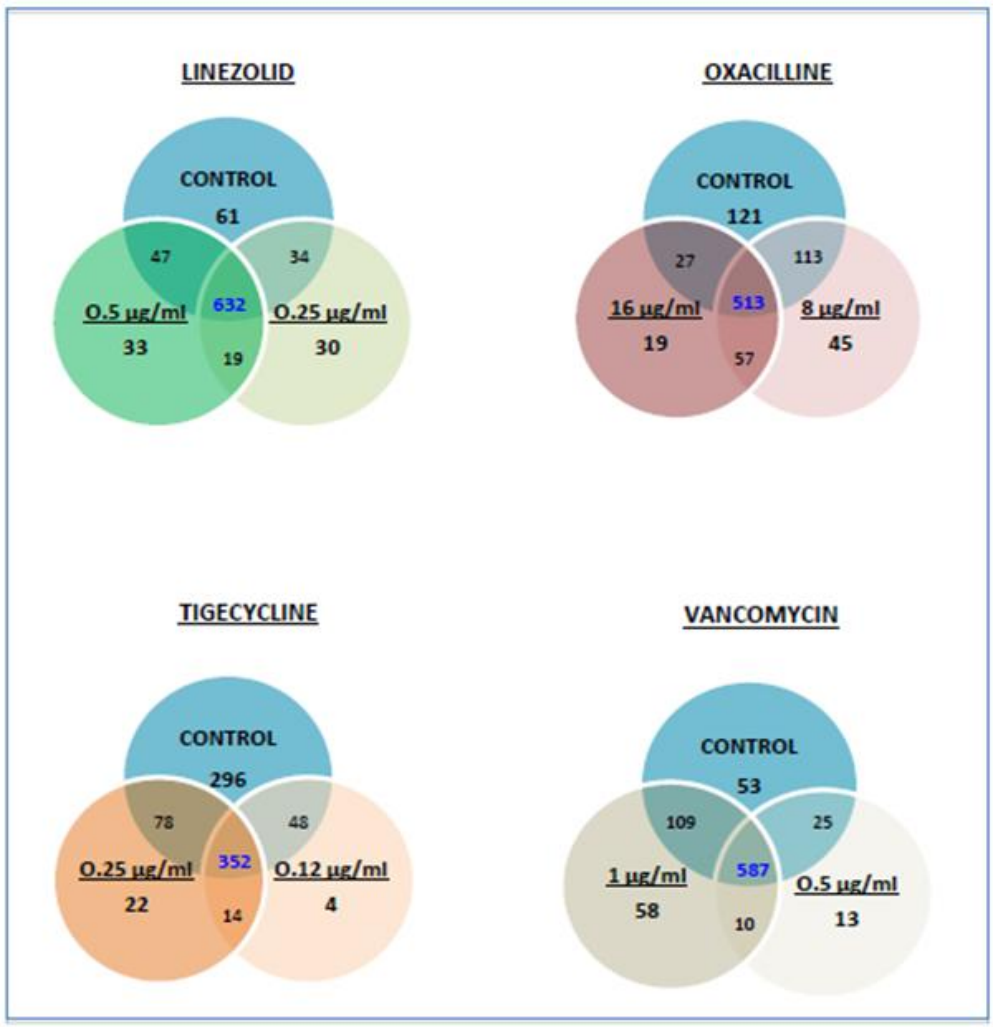


Table 1. Cellular localization distribution of identified proteins in USA300 proteome, under absence (control) or presence of subinhibitory concentrations of antibiotics.

CELLULAR LOCALIZATION	Control	ANTIBIOTIC								Total
		Linezolid		Tigecycline		Oxacillin		Vancomycin		
		0.25 µg/ml	0.5 µg/ml	0.12 µg/ml	0.25 µg/ml	8 µg/ml	16 µg/ml	0.5 µg/ml	1 µg/ml	
CELL WALL	8	6	6	5	4	6	6	7	7	**10**
MEMBRANE	56	49	53	25	21	40	27	45	51	**67**
EXTRACELLULAR	30	31	32	23	33	36	35	30	30	**42**
CYTOPLASM	247	241	245	169	189	239	216	219	258	**291**
UNKNOWED	433	388	395	196	219	407	332	334	418	**570**
Total proteins	**774**	**715**	**731**	**418**	**466**	**728**	**616**	**635**	**764**	**980**

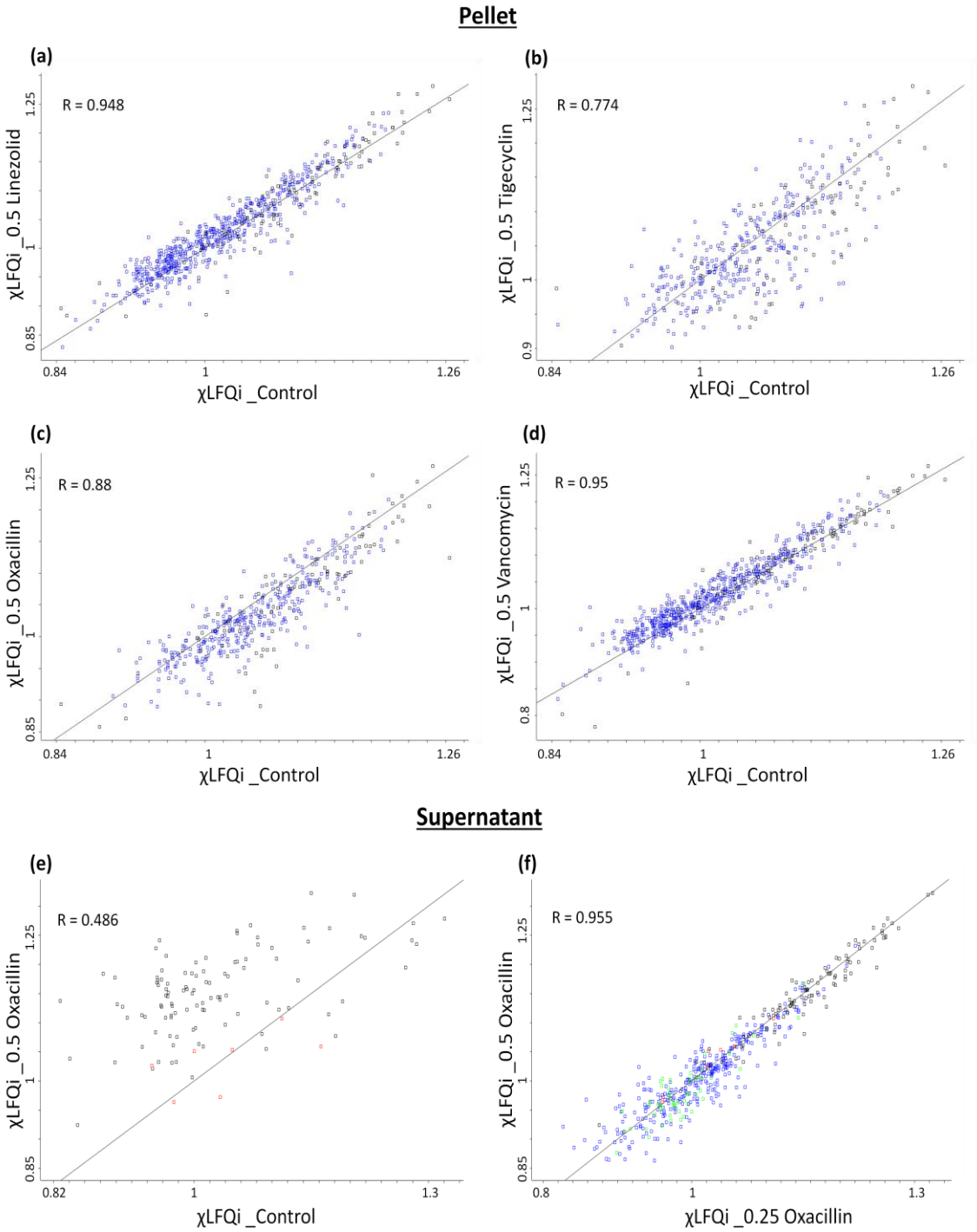
Quantitative analysis of differentially expressed proteins in response to 0.5 MICs

To ascertain that the identified proteins originating from extracts of *S. aureus* USA300 cells showed good correlation, the LFQ intensities of identified proteins were used to calculate Pearson correlation coefficient R, determined by Perseus software (Supplementary figure S3). The scatter plots showed good correlations, comparing each antibiotic condition with the control (**Figure 2, a – d**), or by comparing the two different antibiotic concentrations. The culture supernatants had relatively few identified proteins, except for oxacillin, which resulted in a much higher number of proteins in the supernatant than the other antibiotics (**Figure 2, e – f**). The LFQ intensities of identified proteins in the culture supernatants of the two concentrations of oxacillin, correlated well but poorly to the control. These "extra" proteins found in the oxacillin supernatants, were mainly identified as typical intracellular proteins, inferring extensive bacterial lysis of *S. aureus* USA300 cells.[249]

For the comparative analysis of *S. aureus* USA300 specific response to each antibiotic, we only considered proteins differentially expressed in samples treated with 0.5 MICs. To analyze these differentially expressed proteins, we took into consideration the experiment design and the detection limitations. The differentially expressed proteins not identified under some condition, antibiotic or control, with a normalized LFQ intensities (*N*–LFQ intensity) less than 0.20 (LFQ intensity $< 10^6$), we decided not to consider them. Therefore, among differentially expressed proteins in control, 26, 205, 117 and 21 were not observed with linezolid, tigecycline, oxacillin and vancomycin treatments, respectively, whereas 19, 32, 39 and 17 proteins were differentially expressed with linezolid, tigecycline, oxacillin and vancomycin, respectively, which were not observed in the control. For the differentially expressed proteins in treated and untreated-control *S aureus* USA300 cells, the statistical analysis revealed 4, 37, 24 and 3 proteins showing significant statistical differences for linezolid, tigecycline, oxacillin and vancomycin, respectively (**Figure 3**). We only investigated in depth proteins predicted to

Figure 2. Individual Plots correlation by Pearson coefficient R. Scatter plots showing each Pearson coefficient R, and the correlation between LFQ intensities means (χLFQi) of, (**a–d**) proteins identified in the pellet fraction under 0.5MICs (*y* axis), plotted against proteins identified in the control (*x* axis), and (**e–f**) proteins identified in supernatant fraction under 0.5 MIC of oxacillin (*y* axis), plotted against proteins identified in control and 0.25MIC of oxacillin, respectively (*x* axis). LFQ intensities means were calculated as the average among the six, for the control and three, for antibiotics, replicates.

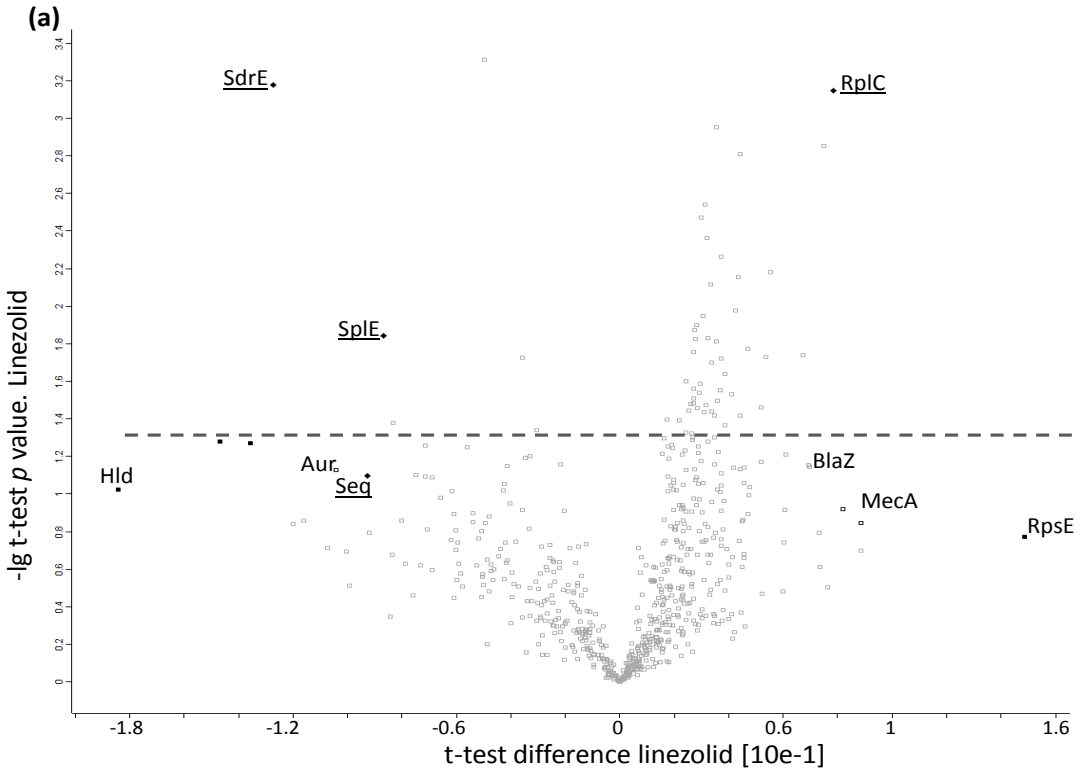
BLUE = proteins from pellet; BLACK = shared proteins between both fractions; RED = proteins from supernatant; GREEN = proteins are not within control.

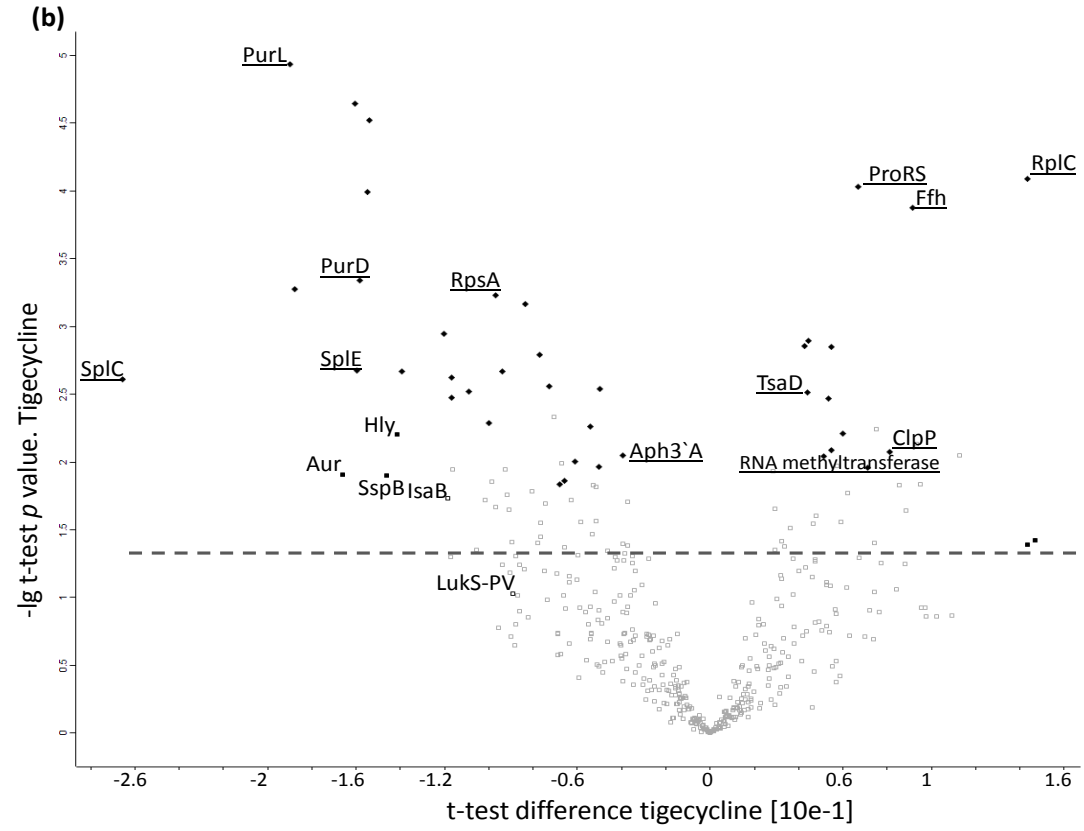


be involved in mechanism of action, pathogenesis and response to stress, including mechanism of resistance. Hence, we could analyze regulatory systems or proteins that regulate virulence factors in *S. aureus* USA300, *e.g.*, repressor CodY[250] or Clp protease family,[86] and proteins involved in pathogenesis such as phenol-soluble-modulins (PsmA), α-haemolysin (Hla/Hly) and PVL (LukF-PV / LukS-PV). Phenol-soluble-modulins, Hla/Hly and PVL are the main virulence toxins characteristic of hypervirulent *S. aureus* USA300.[251] We can also analyze proteins predicted to be involved in mechanism of resistance to vancomycin (RpoB, RpoC),[252] β-lactam family (BlaZ, PBP2a/MecA) or aminoglycosides (AphA(3')III). The differentially expressed proteins in control but not observed with antibiotics are summarized in Table 2, the differentially expressed proteins under antibiotic conditions in Table 3, and the statistical analysis is summarized in Supplementary Table 5 and is shown by volcano plots (Figure 3).

Figure 3. Volcano Plots. Volcano Plots generated by Perseus (v.1.5.1.6) showing the most significant differences for proteins differentially expressed under 0.5MIC-conditions of, (**a**) linezolid, (**b**) tigecycline, (**c**) oxacillin, and (**d**) vancomycin, respect to untreated-control-USA300.
Volcano Plots display both high statistical significance, *p*-value < 0.05 (*y*-axis), and large magnitude fold changes, antibiotic *vs* control (*x*-axis). The dashed black-line shows where *p*-value =0.05 expressed as -log t-test p-value ($\log_{10}$ p-value) = 1.30103, with points above the line $p < 0.05$ and pints below the line having $p > 0.05$. The filled square plot is such that points having a fold-changes less/more than 4x ($4 < x < 0.25$) expressed as ratio antibiotic / control and according to t-test difference [antibiotic - control]. The filled diamond plot is such that points having statistical significance. ♦ Statistical significance proteins. Underline protein name.

Aph3'A: aminoglycoside 3'-phosphotransferase; **Aur**: zinc metalloproteinase aureolysin; **BlaZ**: beta-lactamase; **ClpP**: ATP-dependent Clp protease proteolytic subunit; **Eap**: extracellular adherence protein; **EsaA**: virulence protein; **EzrA:** septation ring formation regulator; **Ffh**: signal recognition particle protein; **FtsZ**: cell division protein; HemG: protoporphyrinogen oxidase; **Hld**: delta-haemolysin; **Hly**: alfa-haemolysin; **IsaB**: immunodominant antigen B; **RpsA**: 30S ribosomal protein S1; **LukF-PV**: Panton-Valentine leukocidin subunit F; **LukS-PV**: Panton-Valentine leukocidin subunit S; **Mec**A: pencillin binding protein 2a (PBP 2a); **PheT**: phenylalanine--tRNA ligase beta subunit; **ProRS**: Proline-tRNA ligase; **PurD**: phosphoribosylamine-glycine ligase; **PurL**: phosphoribosylformylglycinamidine synthase 2; **RplC:** 50S ribosomal protein L3; **RplE**: 50S ribosomal protein L5; **RpoB**: DNA-directed RNA polymerase subunit beta; **RpoC**: DNA-directed RNA polymerase subunit beta; **RpsE**: 30S ribosomal protein S5; **RpsJ:** 30S ribosomal protein S10; **SarA**: Staphylococcal accessory regulator A; **SdrE**: serine-aspartate repeat-containing protein E; **Seq**: enterotoxin Q; **SodA:** superoxide dismutase A; **SplC**: serine protease C; **SplE**: serine protease E; **SplF**: serine protease F; **Ssb**: single-stranded DNA-binding protein; **SspB**: C47 family staphopain B; **SspC**: I57 family staphostatin B; **TsaD**: tRNA N6-adenosine threonylcarbamoyltransferase.


(a)
SdrE
RplC
SplE
Hld
Aur
Seq
BlaZ
MecA
RpsE
-lg t-test p value. Linezolid
t-test difference linezolid [10e-1]
-1.8
-1.2
-0.6
0
0.6
1
1.6


(b)
PurL
RplC
ProRS
Ffh
PurD
RpsA
SplC
SplE
TsaD
Hly
ClpP
Aph3`A
Aur
RNA methyltransferase
SspB
IsaB
LukS-PV
-lg t-test p value. Tigecycline
t-test difference tigecycline [10e-1]
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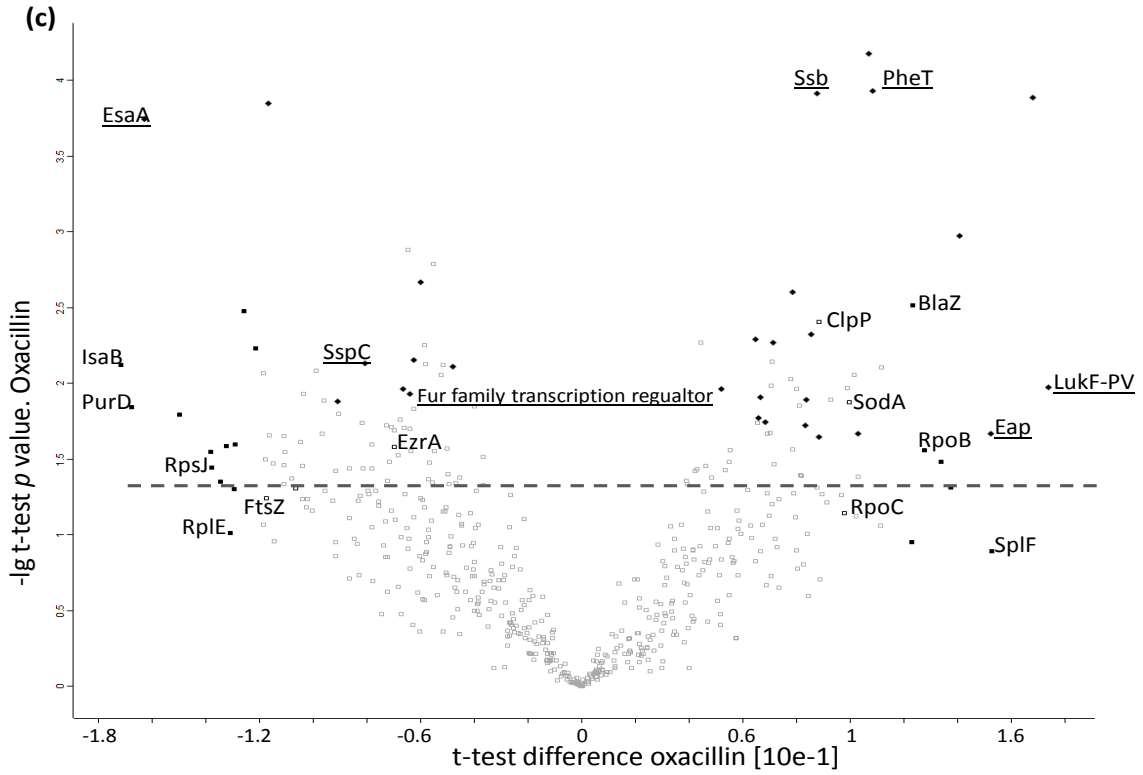
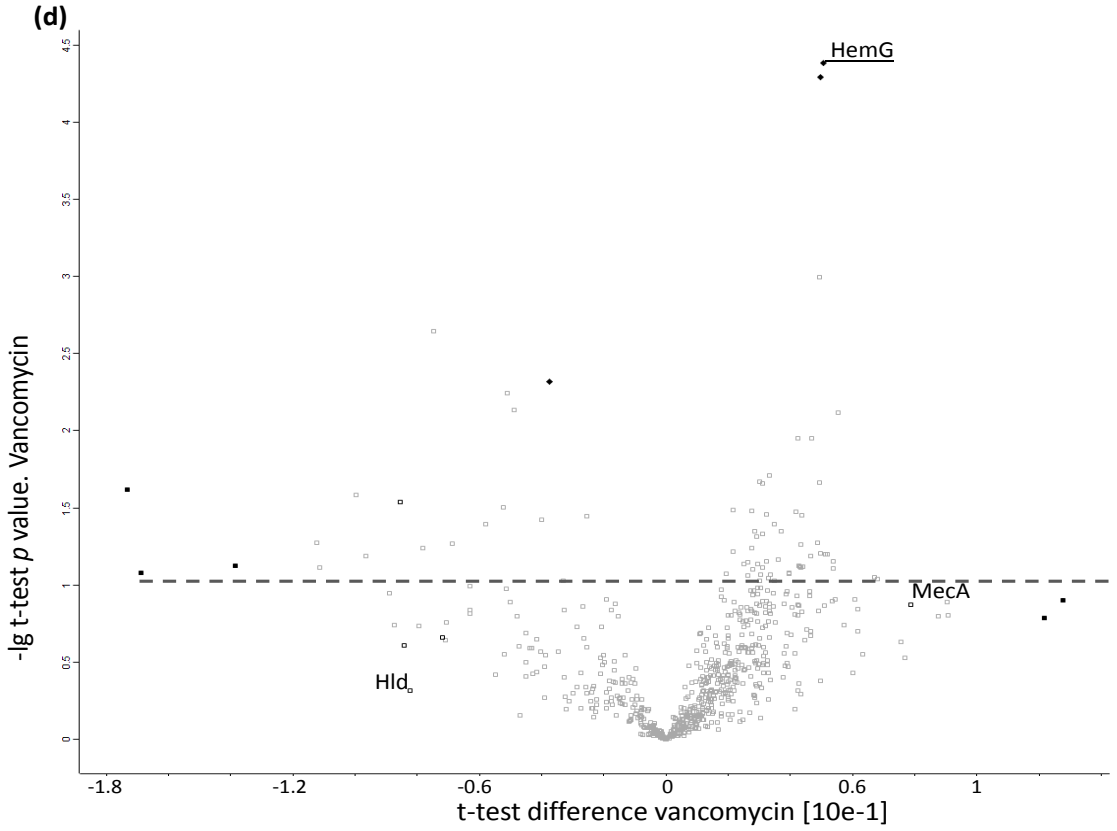
-2
-1.6
-1.2
-0.6
0
0.6
1
1.6





Mechanism of action

Mechanism of action

The protein synthesis inhibitors, linezolid and tigecycline, showed a significant increase in the expression of RplC (50S L3) (**Figure 3a – b**, Supplementary Table S5) assembly initiator protein involved in the initiation first step, which has also been implicated in resistance to linezolid.[253] Tigecycline further showed a significant increase in the expression of other proteins involved in protein synthesis, such as proline–tRNA–ligase ProS and RNA–methyl–transferase (**Figure 3b**, Supplementary Table S5). This more significant increase with tigecycline was concurrent with the lower expression of ribosomal proteins, *e.g.,* RplP (**Table 2**). The cell wall inhibitors, oxacillin and vancomycin, did not show significant statistical differences in the proteins expression involved in cell wall processes (**Figure 3c – d**, Supplementary Table S5). Nevertheless, oxacillin decreased the expression of FtsZ and its negative regulator EzrA (**Figure 3c**, Supplementary Table S5), predicted to be involved in cell division, and proteins such as DltD or MurF (**Table 2**), involved in cell wall biogenesis / degradation, as well as the organelle–organization crucial in chromosome dynamics Smc,[254] were not observed (**Table 2**). This was expected considering that linezolid and tigecycline have effect on proteins synthesis, and oxacillin on cell wall synthesis.

Virulence

We observed that approximately 1 % of differentially expressed proteins in the control with a *N*–LFQ intensity more than 0.5, were not observed with linezolid or tigecycline treatments (**Table 2**), and the statistical analysis showed a decreased differential expression of virulence, especially with linezolid and tigecycline (**Figures 3a – b** respectively, Supplementary Table S5). In samples treated with linezolid, serine proteases SplC and Ear protein were not observed, while another serine protease SplE, enterotoxin Seq and haemolysin Hld were significantly less expressed. In samples treated with tigecycline, Hld, Ear protein, virulence proteins EsaA and EsxA, and serine protease SplA were not observed, while SplC and SplE were significantly less expressed. The main

virulence factors Hla/Hly and LukS-PV (PVL)[255] also showed a lower expression with tigecycline, besides other minor virulence factors (Aur, IsaB, SspB). In samples treated with oxacillin, only EsaA and SspC significantly, and minor virulence factors (IsaB, SspB) showed a lower expression (**Figure 3c**, Supplementary Table S5). By contrast, an highest expression of enterotoxin Sek was observed in samples treated with tigecycline and oxacillin (**Table 3**). In addition, in samples treated with oxacillin, the main virulence factor of *S. aureus* USA300 LukF-PV (PVL)[30] was significantly more expressed (**Figure 3c**, Supplementary Table S5), likewise leukocidin LukDE (*N*-LFQ intensity $\leq$ 0.20) and SCIN (**Table 3**) were more highly expressed. In summary, the protein synthesis inhibitors, linezolid and tigecycline, were superior inhibitors of virulence proteins, and the uniques with a effect on serine proteases, toxins and haemolysins, which play a relevant role in the pathogenesis of *S. aureus*.

Response to stress

Biofilm formation. We observed that most of proteins predicted to be involved, direct or indirect mode, in biofilm formation were highly expressed under antibiotics conditions. In fact, FnbA was highly expressed in response to linezolid and tigecycline (**Table 3**); FnbB, coagulase Coa (**Table 3**) and SdrD (*N*-LFQ intensity = 0.30) in response to tigecycline; ClfA was highly expressed in response to tigecycline and oxacillin (**Table 3**), and Eap showed a higher expression with oxacillin (**Figure 3c,** Supplementary Table S5). Unexpectedly, one of virulence factor for *S. aureus* USA300 also involved in biofilm formation, PsmA1, was more highly expressed only in response to oxacillin (**Table 3**). In contrast, linezolid was the unique antibiotic that significantly decreased the SdrE expression (**Figure 3a**, Supplementary Table S5). Therefore, the increment in the number of proteins involved in response to stress by biofilm formation was superior for tigecycline and oxacillin, according to increased of the antibiotic pressure carried out for these antibiotics.

Mechanism of resistance. The proteins involved in the mechanism of resistance to β-lactam antibiotics were higher expressed as response to oxacillin pressure as expected, though also as response to linezolid and vancomycin pressure. Oxacillin and linezolid increased the expression of BlaZ (**Figure 3a – c**, Supplementary Table S5), linezolid and vancomycin increased the Pbp2a/MecA expression (**Figure 3a – d**, Supplementary Table S5), and with linezolid, even Ppb3 was highest expressed (*N*-LFQ intensity = 0.24). Both proteins, MecA and Pbp3, are involved in the resistance to all β-lactam antibiotics, thus it might indicate that linezolid confer an increased resistance to this antibiotic family, at least with this sub-concentration. The resistance to glycopeptides was only increased with oxacillin by the higher expression of $RpoB^{256}$ (**Figure 3 c**, Supplementary Table S5). The resistance to aminoglycosides was only reduced with tigecycline by the significantly lower expression of AphA (3´)III (**Figure 3b**, Supplementary Table S5).

Endopeptidases Clp family. This heterogeneous Clp family, highly conserved among eubacteria, has a dual role, as it both eliminates stress-damaged proteins, as well as ensures the timely degradation of major stress regulators.[86] In our study, endopeptidases Clp were differentially expressed according to antibiotic and subinhibitory concentration, especially ClpP. Endopeptidase ClpP was significantly more expressed with tigecycline and more expressed with oxacillin (**Figure 3b – c,** Supplementary Table S5) whereas in samples treated with linezolid and vancomycin (Supplementary Table S5) was not changed, likely according with the cell density and antibiotic pressure. Hence, we support that Clp endopeptidases play a key role in regulatory network in response to stress and pathogenesis, and furthermore, ClpP could also have a key role in the *S. aureus* USA300 regulation, by interacting with regulatory systems, for the coordinated response against antibiotic pressure.

Table 2. Differentially expressed proteins in untreated-control USA300, which were not observed in 0.5MIC-treated USA300. Proteins summarized to be associated with mechanism of action of antibiotics, response to stress or pathogenesis. Normalized LFQ intensities, not transformed by log_2, represented as the mean among the six (untreated) or three replicates (treated), and more than 0.2. (see excel file SI002 for more information).

Num id	Protein Name	Control	ANTIBIOTIC				Biological Category Group
			Linezolid 0.5 µg/ml	Tigecycline 0.25 µg/ml	Oxacillin 16 µg/ml	Vancomycin 1 µg/ml	
1076	Serine protease **SplC** (EC 3.4.21.-)	**20.33**		0.16	1.29	15.40	2
1078	Serine protease **SplA** (EC 3.4.21.-)	**3.15**	0.29		1.07	2.15	2
873	Probable dual-specificity RNA methyltransferase **RlmN** (EC 2.1.1.192)	**1.71**	1.48			1.30	3
1257	30S ribosomal protein S16 **RpsP**	**1.09**	0.77		0.79	0.66	3
1101	Serine-protein kinase **RsbW** (EC 2.7.11.1) (Anti-sigma-B factor)	**1.01**	0.91		1.28	1.09	3
796	HTH-type transcriptional regulator **SarR** (Staphylococcal accessory regulator R)	**0.92**	0.97		1.25	0.79	3
1197	50S ribosomal protein L16 **RplP**	0.71	1.00		0.65	1.05	3
1195	Redox-sensing transcriptional repressor **Rex**	0.59	0.69		0.39	0.74	3
1000	RNA polymerase sigma factor SigA **RpoD**	0.51	0.62		0.56	0.67	3
314	UvrABC system protein A (**UvrA** protein) (Excinuclease ABC subunit A)	0.36	0.36			0.42	3
61	Probable DNA-directed RNA polymerase subunit delta (RNAP delta factor) **RpoE**	0.33	0.33		0.30	0.25	3
632	Fur family transcriptional regulator	0.26			0.07	0.28	3
313	UvrABC system protein B (Protein **UvrB**) (Excinuclease ABC subunit B)	0.22	0.23			0.30	3
781	Peptidoglycan pentaglycine interpeptide biosynthesis protein **FemB** (EC 2.3.2.16)	0.70	0.56		0.64	0.86	6
1107	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase (EC 6.3.2.10) **MurF**	0.39	0.45			0.56	6
520	D-alanine--poly(phosphoribitol) ligase subunit 2 (EC 6.1.1.13) (D-alanyl carrier protein) **DltC**	0.35	0.31			0.55	6
619	Methicillin resistance factor **FemB**	0.31	0.31	0.25		0.77	6
1185	Beta-lactamase regulatory protein (Fragment) **BlaR**	0.28	1.73		0.43	0.40	6
1105	Alanine racemase (EC 5.1.1.1) **Alr**	0.21	0.19		0.23		6
1397	Delta-hemolysin **Hld**	**47.83**	7.07		36.95	31.06	7
701	Probable thiol peroxidase (EC 1.11.1.-) **Tpx**	0.79	0.64		1.10	0.62	7
1047	Putative thioredoxin	0.75	0.41		0.29	0.59	7

24	Bacterioferritin comigratory protein (EC 1.11.1.-) **Bcp**	0.57	0.43		0.39	0.45	7
69	General stress protein 20U (EC 1.16.3.1), **Dps** family stress protein	0.52	0.39		1.64	0.54	7
1088	Possible leukocidin subunit	0.37	0.34	0.78		0.63	7
145	Transcriptional regulatory protein **WalR**	0.32	0.23		0.14	0.34	7
65	S-ribosylhomocysteine lyase (EC 4.4.1.21) Autoinducer-2 production protein **LuxS**	0.31	0.33			0.44	7
284	Histidine protein kinase **SaeS** (EC 2.7.13.3)	0.28	0.29			0.49	7
167	Superoxide dismutase [Mn/Fe] 2 (EC 1.15.1.1) **SodM**	0.27	0.21		0.46	0.09	7
1360	Putative septation protein **SpoVG**	0.26	0.32		0.58	0.19	7
629	Staphylococcal respiratory response protein **SrrA**	0.25	0.21		0.24	0.27	7
363	I57 family staphostatin B **SspC**	0.23			0.04	0.22	7
591	Glutathione peroxidase **Bsa**	0.23			0.68	0.45	7
887	Chromosome partition protein **Smc**	0.23	0.22	0.19		0.19	7
1079	Uncharacterized protein. Probabey beta-lactamase.	**15.36**	0.61		4.51	12.50	
402	Virulence protein **EsaA**	**8.27**	6.02		0.70	5.43	
751	Uncharacterized protein (1)	**2.12**	6.79		0.30		
509	**Ear** protein	**1.26**			1.19	3.05	
14	UPF0342 protein SAUSA300_1795. UPF0342 protein USA300HOU_1838 (2)	**1.17**			0.87	0.63	
401	ESAT-6 family virulence protein **EsxA**	**0.84**	0.13		1.51	0.70	
1004	Endoribonuclease **YbeY** (EC 3.1.-.-)	0.49	0.08			0.07	
521	D-alanine-activating enzyme/D-alanine-D-alanyl. D-alanine transfer protein **DltD**	0.48	0.41			0.52	
571	Fibrinogen-binding protein	0.34	1.27	0.68		0.36	
865	Fibrinogen-binding protein **FbpA**	0.24	0.15		0.42	0.17	

(1) Probably regulatory protein involved in competence development and sporulation [Replication, recombination and repair; Signal transduction mechanisms] Cytoplasmic (Probability = 0.75).
(2) Putative exported protein, surface protein, putative toxin, beta-grasp domain protein. The protein domain family has been found in a wide range of extracellular matrix proteins truncated MHC class II analog protein, partial.

Table 3. Highly expressed proteins only under 0.5MICs of each antibiotic. Normalized LFQ intensities, not transformed by log_2, represented as the mean among the three replicates, and higher than 0.75. (see excel file SI003 for more information).

Num Id	Protein Name	ANTIBIOTIC				Biological Category Group	KEGG_PATHWAY
		Linezolid 0.5 µg/ml	Tigecycline 0.25 µg/ml	Oxacillin 16 µg/ml	Vancomycin 1 µg/ml		
472	CHAP domain family. Possible LysM domain protein	**1.45**	0.38			1	
527	Chitinase-related protein (EC 3.2.1.14)		**3.29**			1	Amino sugar and nucleotide sugar metabolism
1164	Ornithine carbamoyltransferase (OTCase) (EC 2.1.3.3) **ArcB ArgF**		**1.83**			2	Arginine and proline metabolism
123	Fibronectin-binding protein B **FnbB**		**3.93**			6	
124	Fibronectin-binding protein A **FnbA**	**0.79**	**1.46**			6	
488	Clumping factor A **ClfA**		**1.59**	0.13		6	
503	Staphylococcal enterotoxin K **Sek**		**0.91**	0.72		7	
741	Staphylococcal complement inhibitor (SCIN) **Scn**			**2.71**		7	
379	Staphylocoagulase (EC 3.4.23.48) **Coa**		**0.85**				
413	Putative surface protein		**6.80**				
421	Phenol-soluble modulin alpha 1 peptide **PsmA1**			**8.33**			
604	UPF0154 protein SAUSA300_1240, USA300HOU_1280 (1)	**0.79**			**0.85**		

(1) Putative exported protein. Cytoplasmic Membrane (Probability = 0.955). Only identified in supernatants.

Comparative analysis of *S. aureus* USA300 strain in response to each antibiotic.

Linezolid

Linezolid showed a dose-dependent effect reflecting bacteriostatic activity, which also led to higher cell density (UFC /ml) (Supplementary Table S2). This higher cell density could explain that the expression of AgrC[257] (excel file) was only observed under linezolid and control conditions. Thus, this AgrC expression could also support the lower biofilm formation observed in response to linezolid pressure, compared with tigecycline and oxacillin, because AgrC is the receptor of the global regulatory system *agr*-locus that allows bacteria to adjust gene expression[258] in response to cell density, interconnecting metabolism and virulence gene expression.[41,44] Linezolid at 0.25 MIC did not bring about major changes in the protein expression, however 0.5 MIC inhibited the synthesis of proteins at initiation step. Therefore, aforementioned virulence factors involved in pathogenesis, such as enterotoxins, serine proteases and haemolysins were decreased, and even the expression of main virulence factors PVL and especially Hla/Hly[255] were a little less expressed under higher 0.5MIC (Supplementary Table S5).

Tigecycline

Tigecycline also inhibited the synthesis of proteins at initiation and elongation steps. One of the key factors that distinguished tigecycline from the other conditions was a strong down-regulation of protein expression, which resulted in a reduction of observed proteins between 55 - 40 % (0.25 - 0.5 MIC, respectively). This affected mainly those proteins expressed by low LFQ intensities in the control (*N*-LFQ intensity < 0.10), such as nucleoproteins or transporters. Notably with 0.5 MIC treatment, this reduction affected to two-component system such as SarR, critical components of the transcription such as RpoD/SigA, or endoribonuclease such as YbeY (Table 2). Unexpectedly, RsbW anti-sigma-factor (Table 2) and likely RsbU-sigma-factor by extension (control *N*-LFQ intensity = 0.17) were also not observed. This inactivation of the sigmaB could explain that proteins involved in response to changes in cellular redox state (*e.g.*, Rex),

homeostasis (*e.g.*, Tpx) or SOS system, were neither observed (Table 2). In addition to this microbial response to stress, SigmaB operon has also an indirect impact on the *agr* 'quorum sensing' system,[74] demonstrating its role in virulence as a response to stress.[75] Hence, this fact could have also contributed in the lower expression of Hla/Hly and PVL (Figure 3b), together with other factors, such as the reduced expression of RNApol/RpoE (Table 2), which also plays an important role in Hla/Hly and PVL regulation.[259] On the other hand, these findings also support the possibility that tigecycline can have a bactericidal activity as suggested.[246] This bactericidal activity can be demonstrated by lysis and kill of *S. aureus* USA300 cells due to, i) not detection of several two-component and regulatory system, ii) lower expression of proteins involved in cell wall cycle, iii) the increased expression of proteins involved in the biofilm formation, which helps bacteria to escape antibiotic-induced killing;[260] and iv) the significant decrease in cell density (CFU / ml)[246] (Supplementary Table S2). Therefore, subinhibitory concentrations of tigecycline could be beneficial for treatment of infections by *S. aureus* USA300, due to the lower expression of main virulence factors, Hla/Hly and PVL[255], and its probable bactericidal activity.

Oxacillin

The key factor of oxacillin was that both subinhibitory concentrations had an effect on cell wall, which could have achieved the observed cell wall lysis on *S. aureus* USA300 cells, as a result of the antibacterial activity of the both oxacillin sub-concentrations. However, the reduction over 15 % with 0.5 MIC, in respect to treatment with 0.25 MIC, on the *S. aureus* USA300 proteome showed a stronger effect of this 0.5 MIC, including a great effect on cell wall processes. Contrary to the expected, the reduced expression of proteins, with 0.5 MIC, were harmful to ribosomal proteins, helicases or nucleases such as YbeY (Table 2), and even aforementioned ABC transporters. We also observed that there were not a higher expression of proteins involved in resistance to β-lactams (*e.g.*, MecA or PBPs). This was interpreted as a not selection to high level resistance to β-

lactams (homotypic resistance, HoR),[261,262] despite the fact that, the increased TCA activity and production of acetyl-CoA (Supplementary Table S4) would support the selection of heterogeneous expression resistance (HeR) towards the high level expression (HoR), to promote survival in the presence of β-lactam antibiotics.[261–263] Since the understanding of this selection remains to be completed, we cannot know if these results could be due to the sub-MICs killing the HoR populations, or it could even be related with the overexpression of ClpP protease[263] (**Figure 3c**). Anyhow, under oxacillin conditions, the biofilm formation was more assisted due to higher expression of PSM[105] and other proteins involved in biofilm formation. It further increased LukF-PVL[30] significantly. Both facts would advise against its use for infections by PVL producer strains.

Vancomycin

In samples treated with vancomycin, we observed very few changes and most of them common to the other antibiotics. The vancomycin sub-concentrations showed poor antibacterial activity, and only the higher dose showed some effect on the cell wall synthesis. In samples treated with 0.5MIC, SepF was barely expressed (*N*-LFQ intensity = 0.12) and D-Ala pathway was also slightly down-regulated (Supplementary Table S4), only for this condition. This down-regulation could have led the bacteria towards an increased of peptidoglycan biosynthesis, and consequently increasing the synthesis of proteins. These observations could support the significantly increased of HemG only for this 0.5MIC (**Figure 3d**, Supplementary Table S5). The vancomycin sub-concentrations had not effect on the virulence factors.

Non-specific response to antibiotic pressure.

Some of the observed changes in protein expression appear to be more a non-specific responses due to the disruption of the environment of *S. aureus* USA300 by the subinhibitory antibiotic concentrations. Concerning proteins differentially expressed in at least 3 of the 4 highest sub-concentrations (0.5 MIC) of the antibiotics, we found 13

proteins highly expressed and 96 proteins not expressed. Among the highly expressed proteins under antibiotic conditions (**Table 4**), approximately 40 % have been documented to be related with virulence or response to stress, including colonization and biofilm formation. For example, PstS has been involved in a reversible adaptation to antibiotic stress to survive in the presence of β-lactams,[264] and by extension, in our study to other antibiotics. In addition, IsdA protein and ClfB are involved in the cell adhesion and biofilm formation,[265] and are overexpressed under restricted environments of iron[266] and calcium[265] respectively. Hence, the common bacterial response against antibiotic pressure was the higher increase of response to stress and biofilm-formation,[265,267] according to the literature. The differentially expressed proteins in control not observed under antibiotic conditions, close to 80 % and only 3 % had *N*-LFQ intensities less than 0.20 and more than 0.5, respectively (data not shown). The haemolysin HlgA was the unique virulence protein highly expressed in control (*N*-LFQ intensity = 14.32) that was not observed with linezolid, tigecycline and oxacillin. Aforementioned AgrC (control *N*-LFQ intensity = 0.20) was not observed with tigecycline, oxacillin and vancomycin treatments, probably owing to lower cell density (CFU / ml) observed due to bactericidal activity of these antibiotics (Supplementary Table S2). Another EssB/C system (control *N*-LFQ intensities = 0.23 / 0.17) involved in the establishment of infection in the host and dispensable for laboratory growth,[268] were not detected with any treatment, as expected.

Table 4. Differentially expressed proteins only under 0.5MICs of all or three antibiotics. Normalized LFQ intensities, not transformed by log_2, represented as the mean among the three replicates. (see excel file SI003 for more information).

Num Ids	Protein Name	ANTIBIOTIC				Biological Category Group	KEGG_PATHWAY
		Linezolid 0.5 µg/ml	Tigecyclin 0.25 µg/ml	Oxacillin 16 µg/ml	Vancomycin 1 µg/ml		
338	3-oxoacyl-[acyl-carrier-protein] synthase 3 (EC 2.3.1.180) FabH	0.24	0.28	0.16	0.26	1	Fatty acid biosynthesis
728	Nicotinamidase (EC 3.5.1.19)	0.09		0.20	0.19	1	
132	tRNA uridine 5-carboxymethylaminomethyl modification enzyme **MnmG** (Glucose-inhibited division protein A)	0.21	0.67	0.54	0.20	3	
1013	Chaperone protein **DnaJ**	0.11	0.36	0.21	0.15	3	
1351	DNA topoisomerase 1 (EC 5.99.1.2) **TopA**		0.20	0.15	0.15	3	
808	D-lactate dehydrogenase (EC 1.1.1.28) **Ddh**		**2.58**	0.58	0.18	4	Pyruvate metabolism
919	Phosphate-binding protein **PstS** (PBP);Phosphate ABC superfamily ATP binding cassette transporter, binding protein	0.64	**2.51**	0.29		5	ABC transporters. Two-component system
303	Transferrin receptor. Iron (Fe+3) ABC superfamily ATP binding cassette transporter, binding protein	0.10	0.29	0.26		5	ABC transporters
1163	Clumping factor B **ClfB**	**1.20**	**3.50**	**0.43**		6	
560	Iron-regulated surface determinant protein A (Fur-regulated protein A) (Staphylococcal transferrin-binding protein A) **IsdA**	**0.57**	**0.99**	**1.82**		7	
1173	Putative surface anchored protein. LPXTG family cell wall surface anchor protein **SasF**	**1.63**	**1.25**	**0.54**			
743	Staphylokinase **Sak**	**3.66**	**4.74**	**1.59**			
1182	Uncharacterized protein (1)	**2.62**	**2.02**	**1.02**			

(1) Putative immunity protein/bacteriocin. Only identified in supernatants.

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Conclusions

S. aureus has a marked ability to adapt to different environments by modulation its central metabolism according to changing conditions, *in vitro* and *in vivo*. The adaptive biological response to able to survive, is led by regulatory systems, such as the quorum-sensing. Nevertheless, to understand the differential protein expression as response to antibiotic pressure of *S. aureus* USA300 strain, it would be necessary a specific regulatory network. We think that, the Clp protein family could contribute to antibiotic adaptation, and probably can interplay pathogenicity and response to antibiotic pressure. This response of *S. aureus* USA300 strain, to antibiotic pressure of subinhibitory concentrations, was led towards greater expression of proteins involved in biofilm formation, especially with tigecycline and oxacillin treatments.

The higher dose were superior and showed an effect on the proteins involved in each mechanism of action. The protein synthesis inhibitors, linezolid and tigecycline, inhibited the expression of several virulence factors involved in pathogenesis, such as serine proteases and haemolysins. The 0.5 MIC of linezolid further significantly decreased the expression of enterotoxin Seq and sialoprotein SdrE, which can be an advantage for clinical treatment of CA-MRSA infections such as osteomyelitis, bacteremia and even endocarditis. Tigecycline was superior against *S. aureus* USA300 using both subinhibitory concentrations, and showed a decreased expression of the main virulence factor of *S. aureus* Hla, as well as of PVL. Tigecycline was also considered to show a bactericidal activity. The sub-concentrations of oxacillin were efficient against *S. aureus* USA300, though the higher expression of virulence factor involved in the pathogenesis such as PSMs and Luk-F (PVL), more than the possible selection of homotypic-resistant MRSA, would reject its clinical used. The vancomycin had not effect over the virulence factors and was the less efficient. Hence, this study provides for the proteomic changes in a CA-MRSA USA300 strain and an insight in cellular response to different sub-concentrations of several antibiotics. All these findings might ultimately be useful for further study in a clinical setting.

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Supplementary data

Quantitative Proteomic Analysis of Community-Acquired Methicillin-Resistant *Staphylococcus aureus* USA300 in Response to Subinhibitory Concentrations of Antibiotics

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Supplementary Information

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Table S1. *Minimal Inhibitory Concentration (MICs) of antibiotics tested against USA 300*. **Figure S1**. *Schematic diagram of sample preparation (A) culture and (B) digestion*, for proteomics analysis by LFQ. **Table S2**. *CFU/ml and amount of protein (means) of sub-MICs tested against USA 300*. Cultures were incubated until optical density OD_{600} reached at 2 $\pm$ 0.1 (stationary phase) and the aliquots were plated on blood-agar. The proteins extracts were previously concentrated and 20 µg of protein were loaded and fractionated by SDS-PAGE. **Table S3.** *Pathway enrichment study* by DAVID with a score more than 2.5. Annotation coverage of 45%. Up- or down- regulation of metabolic pathways was defined by score enrichment values from DAVID web for each pathway cluster using the score value for the antibiotic condition minus the score value of the control (antibiotic - control). Differences between $\pm$ 1 was considered not significant**. Table S4.** *Biological Process distribution of identified proteins (absolute values).* Definition of Biological Category group in the left. The GO annotations coverage was 75%. **Figure S2***. Stress Profile Scatter Plot..* Profile scatter-plot for differentially expressed proteins involved in pathogenesis and response to stress identified for 0.5MICs. LFQ intensities normalized without transforming, represented as the mean among the three (treated samples) and six biological replicates (control). n= number of identified proteins, χ LFQ i = LFQ intensities means. **Figure S3**. *Plots correlation by Pearson coefficient R.* MultiScatter Plot showing the correlation between LFQ intensities means of identifiend proteins for each subinhibitory concentration of every antibiotic, plotted against LFQ intensities means of identifiend proteins for the control, and their corresponding Pearson correlation R, for (a) pellet cells, and (b) supernatant cells. BLUE = proteins from pellet; BLACK = shared proteins between both fractions; RED = proteins from supernatant; GREEN = proteins are not within control. P = pellet; S = supernatant; Ctrl = CONTROL; LNZ= linezolid; TIG= tigecycline; OXA= oxacilline; VAN= vancomycin. **Figure S4**. *HeatMap and detail of the most relevant differences between treated-USA300 and control-USA300*. The HeatMap was generated by Perseus software, it shows the LFQ intensities means of the untreated-control USA300 and the treated USA300 (columns), and the proteins names (rows) clustered following the instructions from Perseus. The row cluster was defined as $\leq$ 20. The row color bar show the untreated-control USA300´s cluster. The used parameters were the euclidean distance linkage as mean, one preprocess with k-means and number the cluster 300 (parameters by default) to the imputed LFQ intensities and without the preprocess to no imputed LFQ intensities. LFQ intensities means calculate as the average among six (control) or three replicates (antibiotic). Ctrl= control; LNZ= linezolid; TIG= tigecycline; OXA= oxacilline; VAN= vancomycin. Bars of LFQ intensities and GREY = undetectable protein. **Table S5**. *Statistical significance and fold change of differentially expressed*

proteins for 0.5 MICs. Proteins summarized to be associated with mechanism of action of antibiotics or pathogenesis Statistical analysis performed by Perseus v.1.4.1.3. Fold-change [2] (Antibiotic / Control) calculated as [(Antibiotic mean / Control mean)] for each protein individually, and [1] log t-test *p*-value = ($\log_{10}$ *p*-value)*(−1). absolute value = 1.30103. (See excel file SI004 for more information).

Excel file SI_001. This file includes a list of the 1284 identified proteins and their LFQ intensities, as well as the Protein ID, total number of unique peptides, sequence coverage, posterior error probability (PEP) score from MaxQuant. Excel file SI_002. Proteins uniquely expressed in untreated USA300 but not observed in treated-control-USA300. LFQ intensities normalized without transforming, represented as the mean among the six biological replicates. Excel file SI_003. Proteins uniquely expressed in treated USA300 but not observed in untreated-control-USA300. LFQ intensities normalized without transforming, represented as the mean among the three biological replicates. Excel file SI_004. *Statistical significance proteins*. Statistical analysis performed by Perseus v.1.4.1.3 where log t-test *p*-value = ($\log_{10}$ *p*-value)*(−1), absolute value = 1.30103 and the fold-change as *t*-test difference (antibiotic – control).

FIGURES

Figure S1. Schematic diagram of sample preparation (**A**) culture and (**B**) digestion, for proteomics analysis by LFQ. TSB= Tripticase-soy broth. LNZ= linezolid; TIG= tigecycline; OXA= oxacilline; VAN= vancomycin.

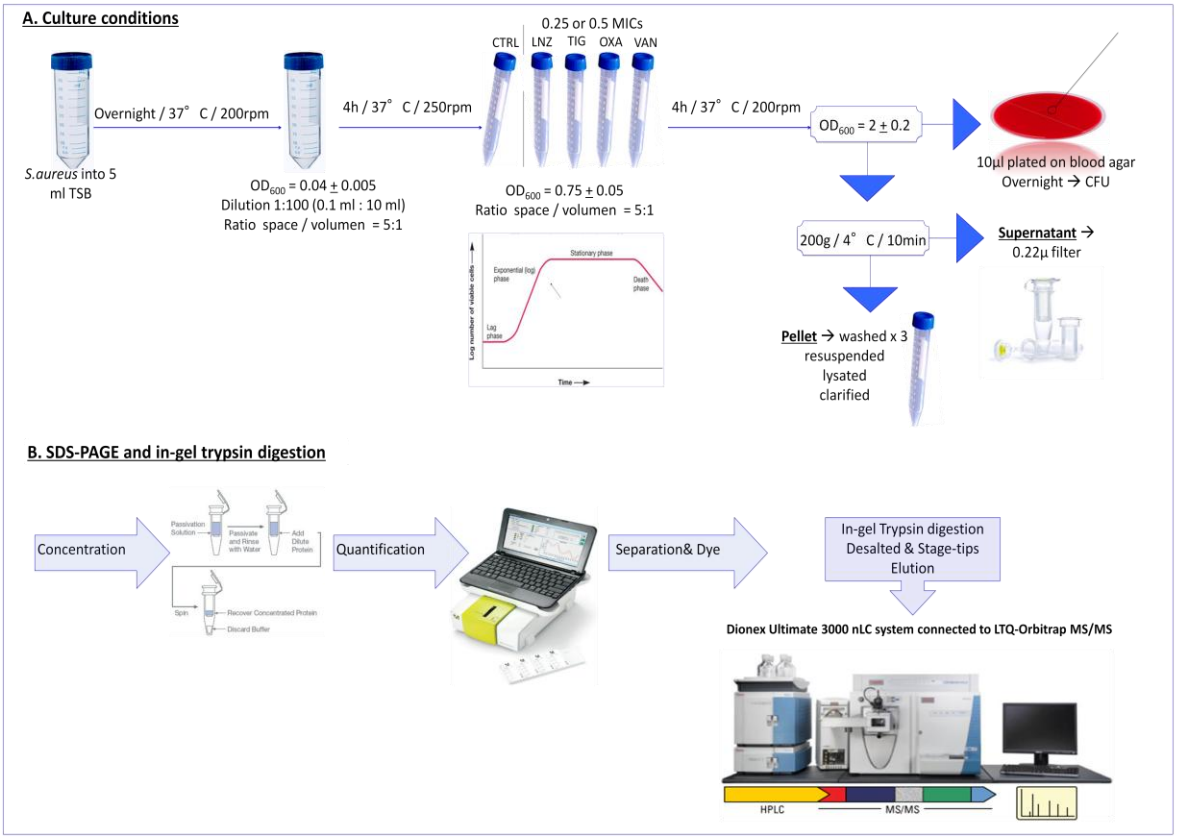


Figure S2. Stress Profile Scatter Plot under 0.5MICs.

χ LFQ i = LFQ intensities means.

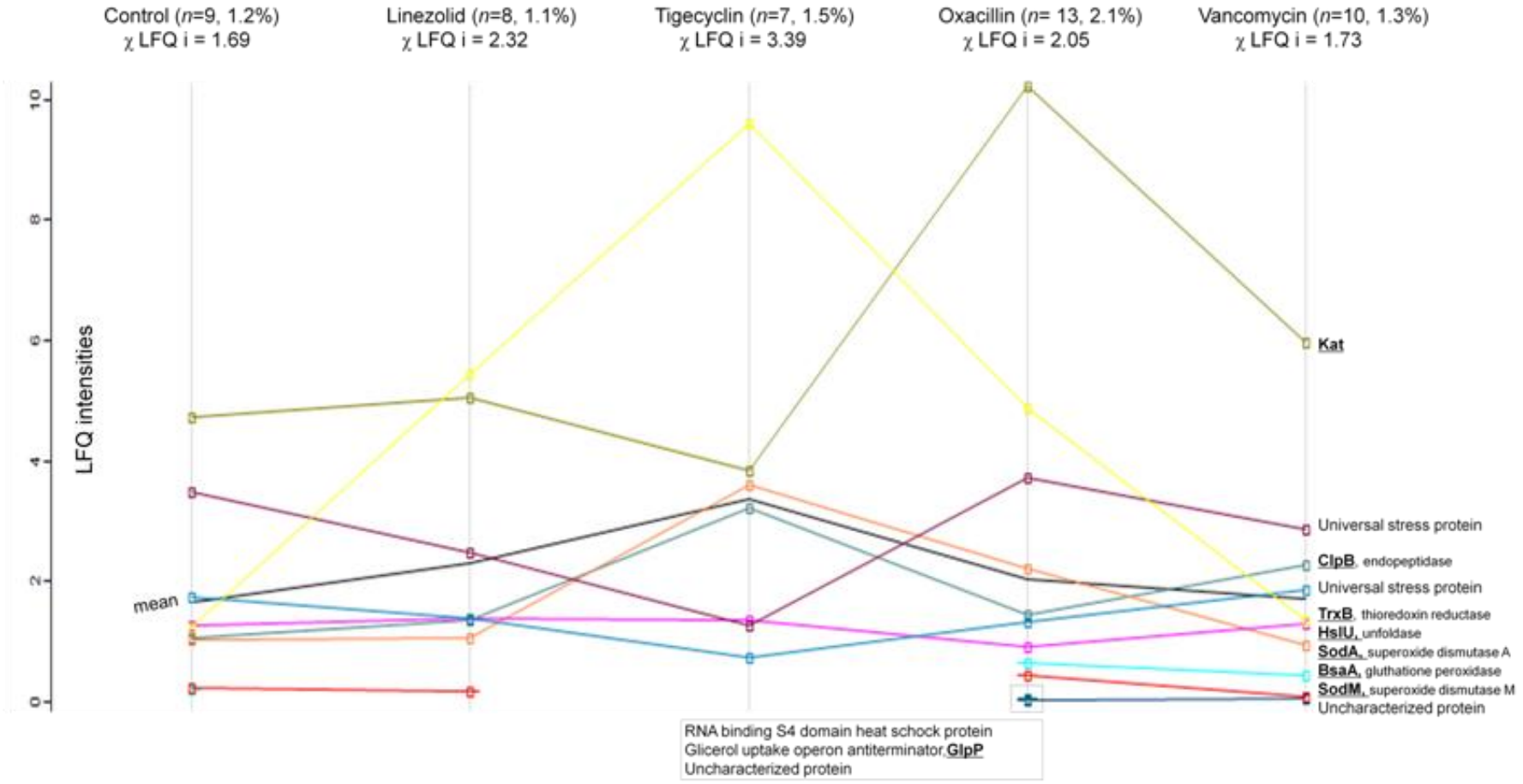
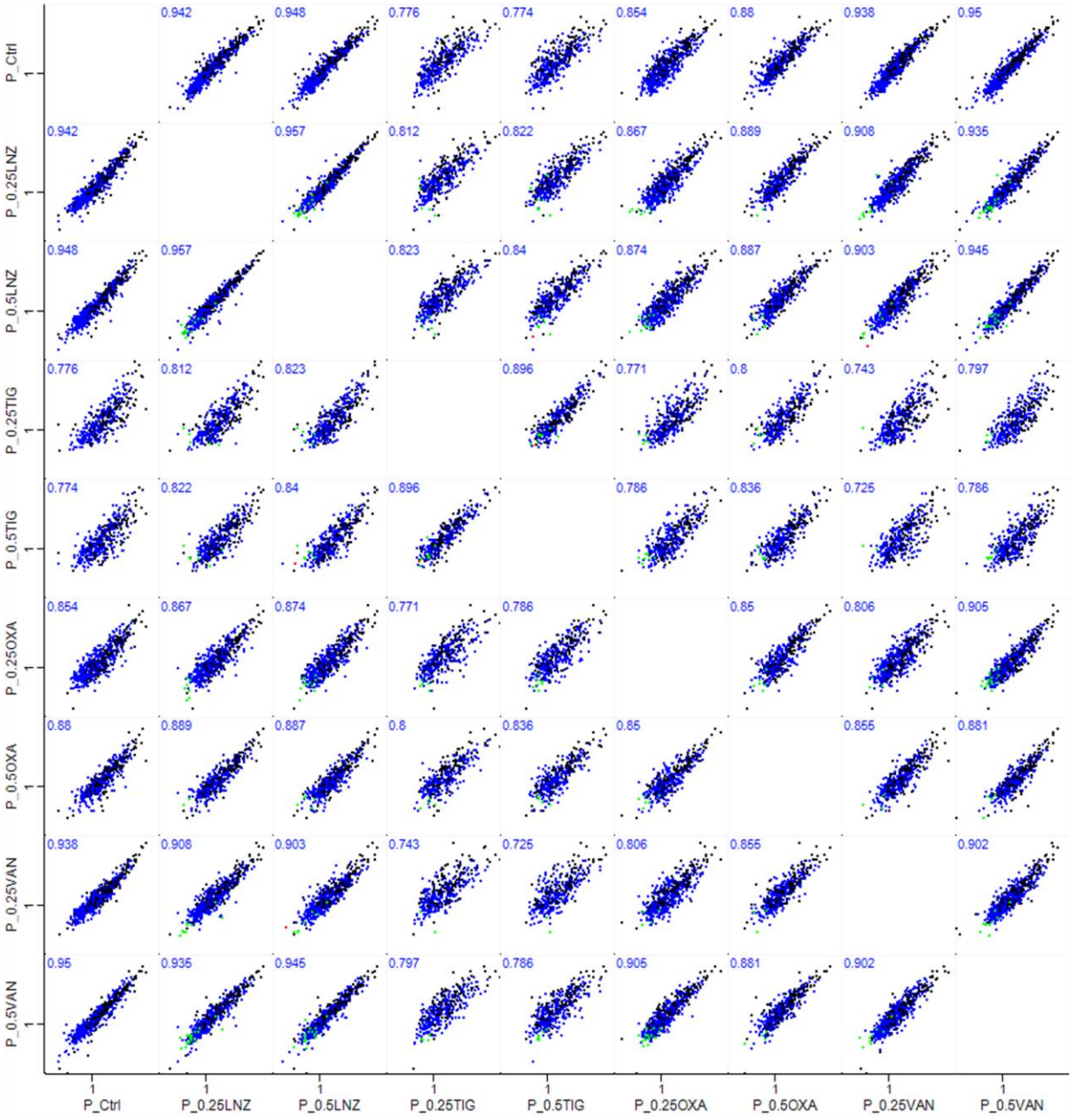


Figure S3. Plots correlation by Pearson coefficient R.

A. Pellet



BLUE = proteins from pellet; BLACK = shared proteins between both fractions; RED = proteins from supernatant; GREEN = proteins are not within control.

P = pellet; S = supernatant; Ctrl = CONTROL; LNZ= linezolid; TIG= tigecycline; OXA= oxacilline; VAN= vancomycin

B. Supernatant

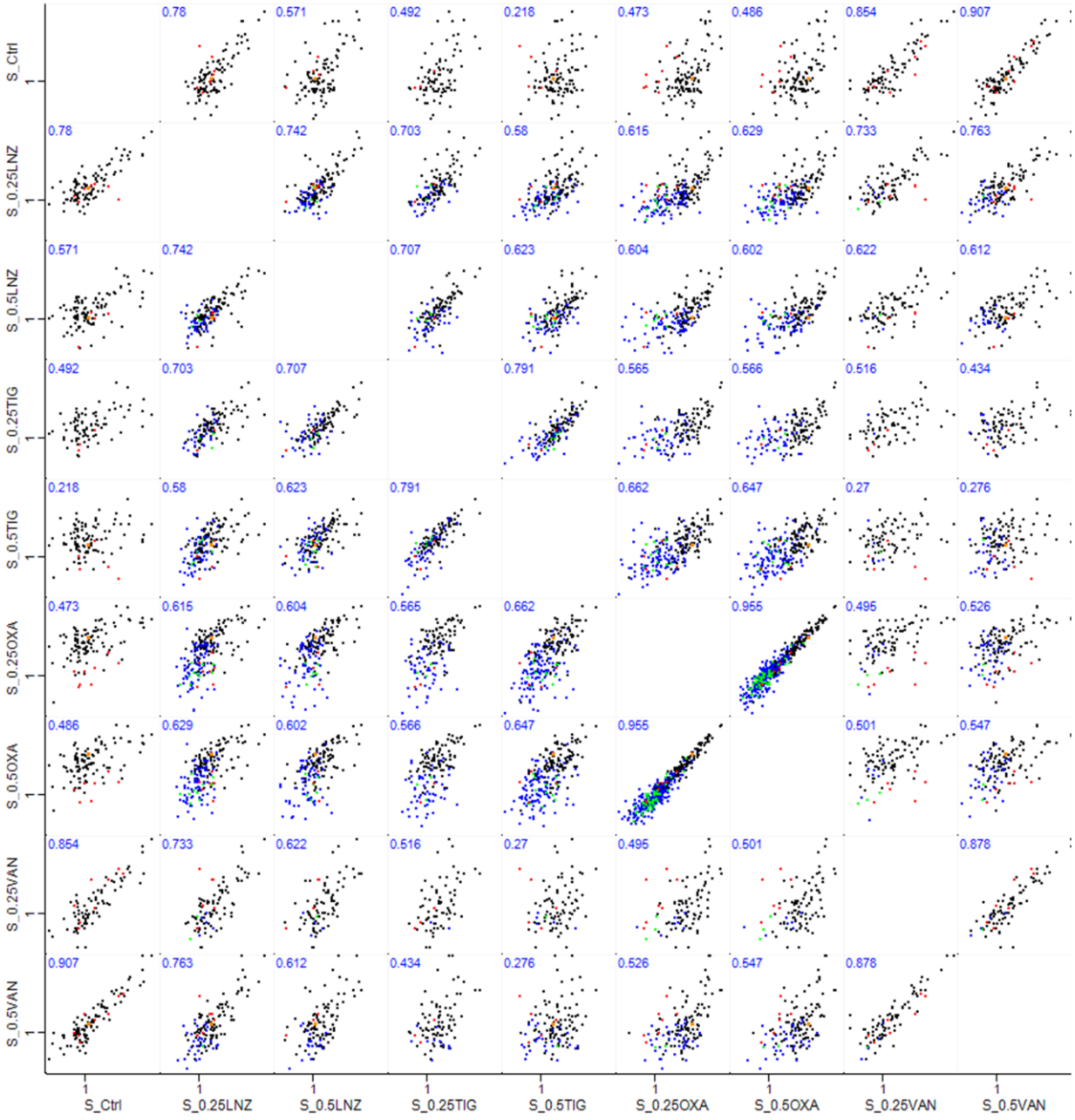
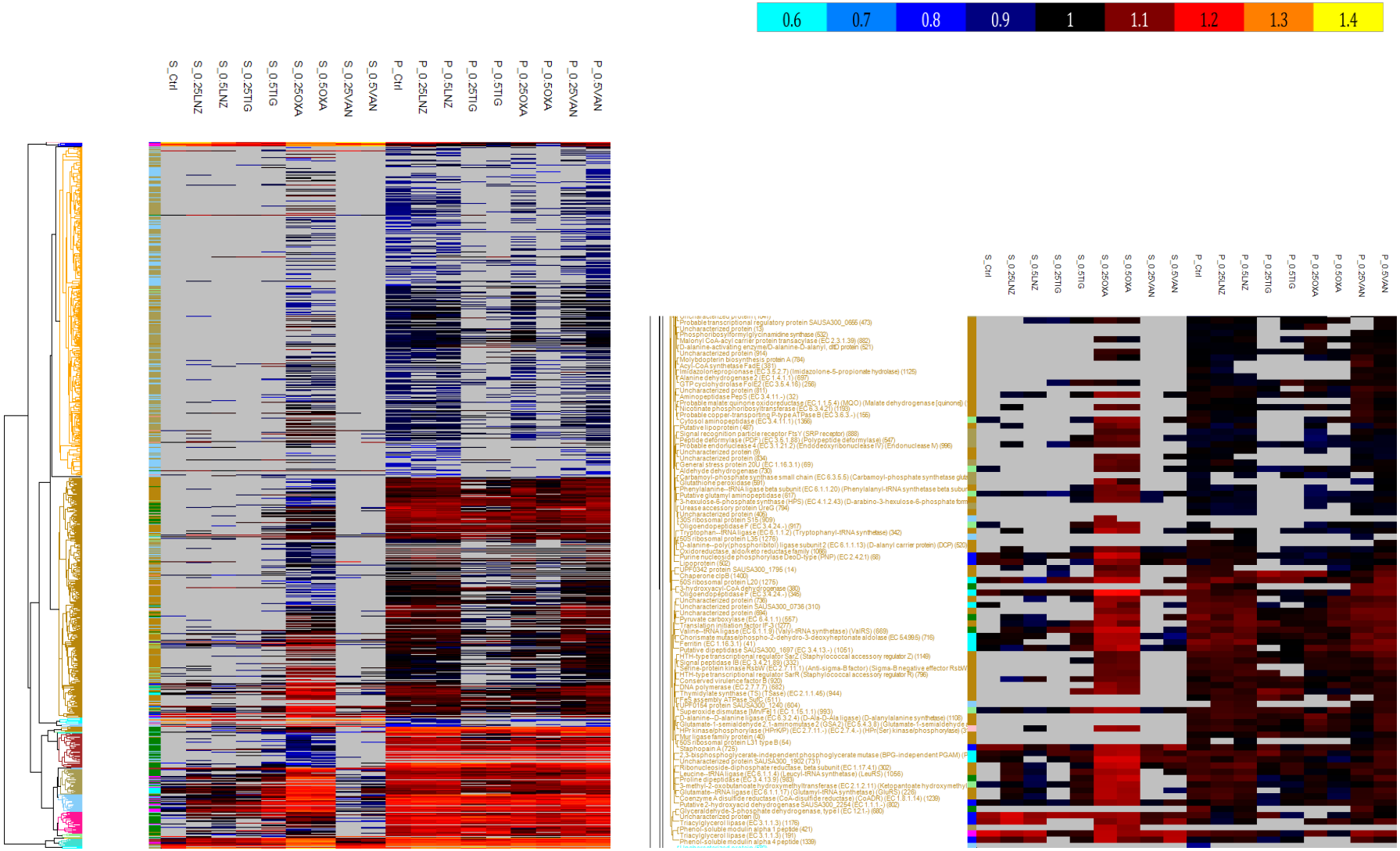


Figure S4. Heat Map and detail of the most significants changes with respect to untreated-control-USA300.



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TABLES

MATERIAL AND METHODS

Table S1. MICs of antibiotics tested against USA 300.

	MIC (mg/L) in MH-II medium	
	Microdilution*	E-test
Linezolid	1	1
Tigeciclyn	0.5	0.5
Oxacillin	32	16
Vancomycin	0.5	2*

*MICs used to this study except the vancomicyn where the MICs by E-test was used.

RESULTS AND DISCUSSION

Table S2. CFU/ml and amount of protein (means) of sub-MICs tested against USA 300.

	Concentration (µl/ml)	CFU/ml (mean)	Amount (gr) of protein (mean)	
			400 µl of pellet	1 ml of supernatant
Linezolid	0.25	1.54E+11	1	3.6
	0.5	1.58E+11	0.65	1.87
Tigecycline	0.125	8.68E+09	0.5	2.6
	0.25	6.77E+09	0.44	1.9
Oxacillin	16	2.57E+10	1.1	1.9
	32	7.77E+09	1.1	1.8
Vancomycin	0.5	2.77E+11	1.2	2.2
	1	1.05E+10	0.74	2.75
Control		**3.52E+11**	1.2	2.25

Table S3. Pathway enrichment study by DAVID with an annotation coverage of 45%.

	ANTIBIOTIC							
	Linezolid		Tigecycline		Oxacillin		Vancomycin	
Pathway Cluster	0.25 µg/ml	0.5 µg/ml	0.12 µg/ml	0.25 µg/ml	8 µg/ml	16 µg/ml	0.5 µg/ml	1 µg/ml
Ribosome								
Purine metabolism								
Aminoacyl-tRNA biosynthesis								
Pyrimidine metabolism								
Glycolysis / Gluconeogenesis								
Citrate cycle (TCA cycle)								
Pyruvate metabolism / Propanoate metabolism								
Peptidoglycan biosynthesis								
Amino sugar and nucleotide sugar metabolism								
Oxidative Phosphorylation								
Fatty acid biosynthesis								
Nucleotide excision repair			ND	ND		ND		
D-Alanine metabolism								

ND = Enrichment score No determined

Table S4. Biological Process distribution of identified proteins (absolute values).

Biological Category group	BIOLOGICAL PROCESS		Control	Linezolid		Tigecycline		Oxacilllin		Vancomycin		Total
				0.25 µg/ml	0.5 µg/ml	0.12 µg/ml	0.25 µg/ml	8 µg/ml	16 µg/ml	0.5 µg/ml	1 µg/ml	
1	METABOLISM	CARBOHIDRATES	41	40	40	28	31	41	38	39	39	46
		LIPID-FATTY AC MP	17	18	17	9	11	15	17	14	18	21
		CATABOLIC MP	2	3	3	1	2	2	2	3	2	4
		BIOSYNTHETIC MP	8	6	6	5	4	8	6	6	8	10
		MP	31	28	28	14	13	31	23	26	31	41
		PRIMARY MP	10	7	8	5	5	7	7	9	9	11
2		AMINO ACIDS & DERIVATIVES MP	44	42	39	18	24	38	31	36	48	56
		PROTEINS MP	49	45	43	28	33	43	45	38	44	56
3		NUCLEOTIDE, NUCLEOSIDE AND NUCLEIC ACID MP	56	52	56	30	36	57	46	44	63	71
		DNA MP	17	16	17	11	11	22	14	12	21	27
		REPLICATION	13	11	15	10	8	16	10	11	17	20
		TRANSCRIPTION	25	21	22	14	10	23	22	20	27	37
		TRANSLATION	80	78	82	64	70	77	69	77	79	84
4		OXIDO-REDUTION	26	21	27	12	15	22	19	18	28	33
		GENERATION OF PRECUSOR METABOLITES &ENERGY	22	22	22	19	18	21	18	22	22	22
		OTHERS (Vit, CoEnz, Cofactor & others)	23	22	20	10	15	30	23	20	25	32
5	TRANSPORT	Metal IonTransport	12	12	16	7	7	10	8	11	9	17
		Others	25	20	21	5	6	15	11	19	22	30
6	CELL-CELLULAR MP	CELL ORGANIZATION & BIOGENESIS	32	29	32	21	19	29	25	26	28	33
		CELL CYCLE, CELL ADHESION & CELL DIVISION	8	3	7	5	8	6	5	4	6	14
7		CELL DEATH, CELL KILLING & REPRODUCTION	11	10	10	8	8	12	11	10	12	14
		SIGNAL TRANSDUCTION	8	7	7	3	3	7	7	6	10	10
		CELLULAR PROCESS	6	6	6	1	1	5	3	3	6	6
		CELLULAR HOMEOSTASIS	8	8	8	3	3	9	8	7	8	9
		CELLULAR COMPONENT ORGANIZATION	1	1	1	1	1	1	0	1	1	1
	RESPONSE	TO STRESS	9	10	8	7	7	13	12	8	10	13
		TO BIOTIC STIMULUS	0	0	0	0	0	1	1	0	0	1
	PATHOGENESIS		8	8	8	6	9	10	12	8	8	12
-	UNKNOWN		182	169	162	73	88	157	123	137	163	249
TOTAL			**774**	**715**	**731**	**418**	**466**	**728**	**616**	**635**	**764**	**980**

Table S5. Statistical significance and fold change of differentially expressed proteins for 0.5 MICs.

Num Ids	Protein Name	ANTIBIOTIC												Biological Category Group	KEGG_PATHWAY
		linezolid			tigecyclin			oxacillin			vancomycin				
		(1)	log t-test *p*-value[2]	Fold-change[3]	(1)	log t-test *p*-value[2]	Fold-change[3]	(1)	log t-test *p*-value[2]	Fold-change[3]	(1)	log t-test *p*-value[2]	Fold-change[3]		
(1) Statistically Significant Proteins															
	ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92)														
320	Endopeptidase **ClpP**		0.03	2.41	+	**1.96**	**4.63**		**2.41**	**2.87**		0.01	0.78	2	
363	I57 family staphostatin B **SspC**		NaN	NaN		NaN	NaN	+	**2.13**	**0.16**		0.15	0.98	2	
1074	Serine protease **SplE** (EC 3.4.21.-)	+	**1.84**	**0.39**	+	**2.68**	**0.05**		0.05	0.43		0.20	0.62	2	
1076	Serine protease **SplC** (EC 3.4.21.-)		NaN	NaN	+	**2.61**	**0.01**		1.18	0.06		0.05	0.76	2	
1096	tRNA N6-adenosine threonylcarbamoyltransferase (EC 2.3.1.-) **TsaD**		NaN	NaN	+	**2.52**	**1.75**		NaN	NaN		0.89	1.41	2	
1256	Signal recognition particle protein (Fifty-four homolog) **Ffh**		2.15	1.58	+	**3.88**	**6.03**		0.20	1.56		0.40	1.06	2	Protein export. Bacterial secretion system
206	Single-stranded DNA-binding protein **Ssb**		0.31	1.00		NaN	NaN	+	**3.92**	**4.11**		0.16	0.49	3	DNA replication. Mismatch repair. Homologous recombination
229	RNA methyltransferase, TrmH family, group 3		1.21	1.06	+	**2.04**	**2.79**		0.79	3.31		1.31	1.45	3	
534	Phosphoribosylformylglycinamidine synthase 2 (EC 6.3.5.3) **PurL**		0.26	0.68	+	**4.93**	**0.07**		0.60	0.73		0.14	0.82	3	Purine metabolism
538	Phosphoribosylamine--glycine ligase (EC 6.3.4.13) **PurD**		0.02	0.87	+	**3.34**	**0.12**		1.84	0.39		0.02	0.97	3	Purine metabolism
564	Phenylalanine-tRNA ligase / tRNA synthetase beta subunit (EC 6.1.1.20) **PheT**		0.14	0.90		0.35	1.72	+	**3.93**	**4.76**		0.29	1.06	3	Aminoacyl-tRNA biosynthesis
632	Transcriptional regulator, Fur family		NaN	NaN		NaN	NaN	+	**1.93**	**0.26**		0.20	1.04	3	
901	Proline--tRNA ligase (EC 6.1.1.15) (Prolyl-tRNA synthetase) **ProRS**		1.30	1.23	+	**4.03**	**3.91**		0.35	1.66		0.69	2.21	3	Aminoacyl-tRNA biosynthesis
972	30S ribosomal protein S1 **RpsA**		0.64	0.54	+	**3.23**	**0.36**		1.42	0.34		0.28	0.66	3	Ribosome
1204	50S ribosomal protein L3 **RplC**	+	**3.15**	**10.99**	+	**4.09**	**26.97**		0.48	11.77		0.13	1.40	3	Ribosome
2	Protoporphyrinogen oxidase (EC 1.3.3.4) **HemG**		NaN	NaN		NaN	NaN		NaN	NaN	+	**4.38**	**1.96**	4	Porphyrin and chlorophyll metabolism
253	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein **SdrE**	+	**3.18**	**0.04**		0.50	0.62		0.11	0.54		0.55	0.33	6	

504	Staphylococcal enterotoxin Q **Seq**	+	**1.10**	**1.03**		0.25	1.84		0.08	0.88		0.07	1.39	7	
622	Panton-Valentine leukocidin, **LukF-PV**		0.08	0.96		0.04	0.59	+	**1.98**	**1.94**		0.40	0.58	7	
337	Possible extracellular adherence protein **Eap**		0.08	1.34		0.21	0.95	+	**1.67**	**2.07**		0.42	0.53		
402	Virulence protein **EsaA**		0.30	0.73		NaN	NaN	+	**3.75**	**0.08**		0.60	0.66		
1187	3'5'-aminoglycoside phosphotransferase (Aminoglycoside 3'-phosphotransferase) (EC 2.7.1.95) **Aph(3')III**		0.71	0.60	+	**1.83**	**0.57**		0.78	0.65		1.20	1.93		
Not statistically Significant Proteins															
1166	Zinc metalloproteinase aureolysin (EC 3.4.24.29) **Aur**		**1.13**	**0.15**		**1.91**	**0.08**		0.48	0.26		0.63	2.13	2	
1184	Beta-lactamase **BlaZ**		**1.15**	**4.44**		0.82	1.47		**2.52**	**2.76**		0.91	1.14	2	beta-Lactam resistance. Two-component system
236	DNA-directed RNA polymerase subunit beta (RNAP subunit beta) (EC 2.7.7.6) **RpoB**		0.48	1.39		0.93	2.28		**1.48**	**2.44**		0.12	0.88	3	Purine metabolism. Pyrimidine metabolism. RNA polymerase
237	DNA-directed RNA polymerase subunit beta' (RNAP subunit beta') (EC 2.7.7.6) **RpoC**		0.36	1.48		0.72	1.83		**1.14**	**2.22**		0.24	1.03	3	Purine metabolism. Pyrimidine metabolism. RNA polymerase
452	Transcriptional regulator **SarA** (Staphylococcal accessory regulator A)		**0.84**	**2.10**		0.35	1.95		**1.12**	**4.89**		0.07	1.27	3	
769	30S ribosomal protein S5 **RpsE**		**0.77**	**2.88**		**0.19**	**3.40**		0.04	2.04		0.20	1.19	3	Ribosome
1205	30S ribosomal protein S10 **RpsJ**		0.68	0.67		0.04	1.61		**1.45**	**0.51**		0.47	1.10	3	Ribosome
149	**MecA** (Pbp2a) (Pencillin binding protein 2a (PBP 2a, methicillin resistance determinant MecA, transpeptidase); (EC 3.4.16.4)		**0.92**	**2.28**		0.18	1.19		0.07	0.97		**0.87**	**2.25**	6	beta-Lactam resistance
364	Cysteine protease (EC 3.4.22.48);C47 family staphopain B **SspB**		0.31	0.54		**1.90**	**0.05**		0.71	0.10		0.25	0.24	7	
574	Alpha-haemolysin **Hly**		0.56	0.42		**2.20**	**0.07**		0.45	0.22		0.16	1.17	7	
623	Panton-Valentine leukocidin, **LukS-PV**		0.63	0.71		**1.03**	**0.35**		0.48	0.48		0.07	1.11	7	
704	Septation ring formation regulator **EzrA**		0.98	1.26		0.43	0.95		**1.58**	**0.50**		0.00	0.89	7	
993	Superoxide dismutase [Mn/Fe] 1 (EC 1.15.1.1) **SodA**		0.07	1.05		**1.29**	**3.48**		**1.88**	**2.15**		0.27	0.92	7	
1252	Cell division protein **FtsZ**		1.55	1.61		0.71	1.31		**1.24**	**0.66**		0.10	0.93	7	
1397	Delta-haemolysin **Hld**		**1.03**	**0.15**		NaN	NaN		0.12	0.77		0.32	0.65	7	
1167	Immunodominant staphylococcal antigen B **IsaB**		0.07	0.69		**1.73**	**0.18**		**2.12**	**0.14**		0.08	0.91		

(1) Statistically significant proteins = +

(2) log t-test p-value = (log10 p-value)*(-1), absolute value = 1.30103

(3) Fold-change (Antibiotic / Control) calculated as [(Antibiotic mean / Control mean)] for each protein individually. Values < 0.5 are down-regulated proteins and values > 2 are up-regulated proteins.

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Annexed: ***Identification and antimicrobial susceptibility of methicillin-resistant Staphylococcus aureus (MRSA) clones.***

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JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2008, p. 4114–4115 Vol. 46, No. 12
0095-1137/08/$08.00+0 doi:10.1128/JCM.01020-08

Evaluation of the Vitek 2 AST-P559 Card for Detection of Oxacillin Resistance in *Staphylococcus aureus*▽

Free PMC article available : http://jcm.asm.org.pva.uib.no/content/46/12/4114

PMID:18945834 [PubMed - indexed for MEDLINE] PMCID: PMC2593277

Abstract not available.

"Staphylococcus aureus strains with resistance to methicillin or oxacillin (MRSA) represent one of the main nosocomial pathogens at present. MRSA infections are clearly associated with higher mortality and economic cost than those caused by methicillin-susceptible S. aureus (1). In Spain, the prevalence of methicillin resistance among S. aureus isolates has increased since the 1990s and in some cases has now reached levels higher than 30% (2).

A total of 301 S. aureus strains were evaluated (51 mecA negative and 250 mecApositive as determined by PCR). S. aureus ATCC 29213 was used as a negative control. Molecular typing of the X region of the spa gene was done with the 250mecA-positive isolates (4), and these were then grouped into a spa clonal complex (BURST.Ridom StaphType software), with 4 types more prevalent than the others (t02, 23.6%; t18, 22%; t67, 17.6%; and t12, 16.3%)."

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J Med Microbiol. 2012 Feb;61(Pt 2):305-7. doi: 10.1099/jmm.0.036889-0. Epub 2011 Oct 6.

JMM Correspondence

Identification of international circulating lineages of meticillin-resistant *Staphylococcus aureus* in the north of Spain and their glycopeptide and linezolid susceptibility

PMC Article available : http://jmm.sgmjournals.org.pva.uib.no/content/61/2/305

PMID:21980043 [PubMed - indexed for MEDLINE

Abstract not available

"The aim of the present study was to determine the circulating clones in the north of Spain and their antimicrobial susceptibility. A total of 455 MRSA isolates were collected in 16 Spanish hospitals over a 4-month period (February-May 2009) and included in this analysis. One sample was taken per patient, and the isolates were mainly from the respiratory tract and wounds.

In conclusion, this study highlights the predominance of spa*-CC067 and ST-CC5 (*spa*-t067 and t002) and of SCC*mec *IV in north Spain hospitals, the utility of* spa*-typing in short-term epidemiological surveillance, and a high prevalence (91.4%) of MRSA isolates with a 'borderline' MIC for VAN (>1 mg l^{-1}), which has been associated with a poor response to this antibiotic."*

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Material & methods

1 Identification of clones of MRSA. Antimicrobial susceptibility.

1.1 Bacterial strains and reagents.

MRSA strains were collected in different Spanish hospitals from the north of Spain: University Hospital Complex of A Coruña (CHUAC), Ourense (CHOU), Vigo (CHUVI), University Clinical Hospital of Santiago de Compostela (CHUS), Hospital of Coast (Burela, Lugo), Architect Marcide Hospital (Ferrol, La Coruña), Xeral Calde University Hospital (Lugo), Civil Hospital of Basurto (Bilbao), General Yagüe (Burgos), Hospital Complex of Leon, Bierzo Hospital (Ponferrada, Leon), Virgen del Camino Hospital (Pamplona), Donostia Hospital (San Sebastian), Marques de Valdecilla University Hospital-IFIMAV (Santander), Hospital of Soria, Hospìtal of Sierrallana (Torrelavega, Burgos), University Clinical Hospital of Valladolid.

S. aureus strains ATCC 29213 and ATCC 25923 were used as controls for MIC determinations, and Mu3 and Mu50 kindly provided by Dr. Rafa Cantón (Ramon y Cajal Hospital, Madrid) were used as control for glycopeptide resistance.

1.2 Determination of MICs.

All strains were tested by microdilution VITEK-2 with AST-P559/-588 cards (bioMérieux, Marcy l'Etoile, France). The sensitivity to ciprofloxacin, cotrimoxazole, clindamycin, erythromycin, gentamicin, rifampicin and mupirocin were confirmed by disk diffusion and the results were interpreted according to the rules of the Clinical Laboratory Standards Institute (CLSI)[14]. The sensitivity to linezolid and glycopeptides, vancomycin and teicoplanin were confirmed by E-test.

1.3 Molecular characterization.

1.3.1 DNA extraction.

A loopful of material from an overnight culture (grown on blood agar plates) was suspended in 0.5 ml sterile H_2O. The suspension was heated to 95°C for 10 min. After centrifugation at 14 000rpm for 5 min, the supernatant was used for PCRs.

1.3.2 PCR conditions and sequencing

The primers used for each PCR and the conditions, initial denaturation, cycles/T°/time and final extension, are summarized in **Table 1.3**. Reactions were performed in a 25-µl volume with buffers and ECOTaq DNA polymerase from Ecogen. PCR products were purified with Montage®-PCR Filter Units from Millipore and sequenced with PCR forward or reverse primers, with BigDye® Terminator v1.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems). DNA sequences were obtained with an ABI PRISM 3100 Avant sequencer (Applied Biosystems). The chromatogram files were assembled and edited using Chromas Pro.

1.3.3 Spa typing and classification.

The variable X region of the spa gene was amplified by PCR with the primers 1095Fow and 1517Rev.[269] In case the sample did not amplificate, another reverse primer (5'-GAACAACGTAACGGCTTCATCC-3') was used with the same forward primer and conditions. *Spa ty*pes were determined with specific software (www.seqtools.com) developed for this study, using the Ridom nomenclature. The new types we found were submitted at the Ridom Spa Server (www.spa.ridom.de).

Spa-types with similar repeat profiles were clustered into different groups (*spa*-CC) with calculated cost between members of a group ≤ 6. *Spa*-types shorter than 3 repeats were excluded from analysis. This was kindly performed by Dag Harmsen using the BURP clustering of the Ridom Staph Type software (www.ridom.de/staphtype).

1.3.4 Multilocus sequence typing (MLST) and classification.

MLST was performed to one representative strain of each spa type.[270] The *S. aureus* MLST scheme uses internal fragments of the following seven house-keeping genes: *arc*C, *aro*E, *glp*F, *gmk* , *pta*, *tpi* and *yqi*L. PCR amplification was carried out using an annealing temperature of 55°C. The same primers were used for amplification and sequencing. Sequence types (ST) and MLST-clonal complex (ST-CC) were determined with the database accessible via internet (www.mlst.net) where 7 numbers depicting the allelic profile defined a ST.

1.3.5 Methicillin resistance and Panton Valentine leukocidin.

The detection of PVL virulence genes and simultaneous confirmation of methicillin-resistant staphylococci were performed with the multiplex PCR[271] to all the isolates. It was performed containing 0.08 and 0.24 µM for the primers specific for the *luk*S/F-PV,

and *mec*A genes, respectively. The PCR produced distinct bands, corresponding to their molecular sizes (433bp and 310bp for the *luk*S/F-PV, and *mec*A genes, respectively).

1.3.6 SCCmec typing.

The method described by Lencastre *et al.*,[272,273] was used for the investigation of SCC*mec* type in the *mecA*-positive isolates.

1.3.7 Agr locus PCR and sequencing.

The characterization of agr locus was realized in at least two representative strain of each type *spa*. It characterized the type of *agr* by means of amplification and later sequencing of the *agr*C.[274] The sequencing made with BigDye Terminator v1.1 Cycle Sequencing Ready Reaction kit, using an Abi Prism 3100-Avant (Applied Biosystems).

1.3.8 Aminoglycosides resistance.

All study strains were tested for the presence of genes encoding key enzymes that confer resistance to aminoglycosides. It was conducted by PCR-multiplex for the *ant(4´)-Ia*, *aph(3´)-IIIa* and *aac(6´)-Ie+aph(2´´)* genes.[275]

Table 1.3. Primers used for this study & PCR conditions.

GEN	SIZE(pb)	PRIMERs (5'→3')		PCR conditions
mecA	310	mecA1	GTAGAAATGACTGAACGTCCGATAA	**Methicillin resistance and PVL** 80ºC 10min **35x** [94ºC 45s + 60ºC 45s + 72ºC 1min 30s] 72ºC 10 min
		mecA2	CCAATTCCACATTGTTTCGGTCTAA	
pvl	433	Luk-pvl-1	ATCATTAGGTAAAATGTCTGGACATGATCCA	
		Luk-pvl-2	GCATCAAGTGTATTGGATAGCAAAAGC	
Spa-X	30-500	1095-F	AGACGATCCTTCGGTGAGC	
		1517-R	GCTTTTGCAATGTCATTTACTG	
Aac(6´)-Ie+aph(2´)	~220	Aac-2022	CCAAGAGCAATAAGGGCATACC	**Aminoglycosides resitance.** 94ºC 5 min **35x** [94ºC 20s + 55ºC 60s + 72ºC 50s] 72º C 10 min
		Aac-2369	CACACTATCATAACCATCACCG	
Ant(4´)-Ia	172	Ant-605	CTGCTAAATCGGTAGAAGC	
		Ant-777	CAGACCAATCAACATGGCACC	
Aph(3´)-III	268	Aph-329	CTGATCGAAAAATACCGCTGC	
		Aph-597	TCATACTCTTCCGAGCAAAGG	
I, J1 region	495	CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	**SCCmec typing** 94ºC 5min **30x** [94ºC 30s + 53ºC 30s + 72ºC 1min] 72ºC 8min
		CIF2 R2	ATTTACCACAAGGACTACCAGC	
V, ccr complex	449	ccrC F2	GTACTCGTTACAATGTTTGG	
		ccrC R2	ATAATGGCTTCATGCTTACC	
III, J3 region	414	RIF5 F10	TTCTTAAGTACACGCTGAATCG	
		RIF5 R13	ATGGAGATGAATTACAAGGG	
V, J1 region	377	SCCmec V J1 F	TTCTCCATTCTTGTTCATCC	
		SCCmec V J1 R	AGAGACTACTGACTTAAGTGG	
I, II, IV, and VI, J3 region	342	dcs F2	CATCCTATGATAGCTTGGTC	
		dcs R1	CTAAATCATAGCCATGACCG	
II and IV, ccr complex	311	ccrB2 F2	AGTTTCTCAGAATTCGAACG	
		ccrB2 R2	CCGATATAGAAWGGGTTAGC	
II, J1 region	284	kdp F1	AATCATCTGCCATTGGTGATGC	

		kdp R1	CGAATGAAGTGAAAGAAAGTGG	
III, J1 region	243	SCCmec III J1 F	CATTTGTGAAACACAGTACG	
		SCCmec III J1 R	GTTATTGAGACTCCTAAAGC	
II and III, mec complex	209	mecI P2	ATCAAGACTTGCATTCAGGC	
		mecI P3	GCGGTTTCAATTCACTTGTC	
Internal positive control	162	mecA P4	TCCAGATTACAACTTCACCAGG	
		mecA P7	CCACTTCATATCTTGTAACG	
arcC	456	arcC-up	TTG ATT CAC CAG CGC GTA TTG TC	**Multilocus sequence typing (MLST)** 94ºC 10min **30x** [94ºC 30s + 55ºC 30s + 72ºC 30s] 72ºC 10min
		arcC-dn	AGG TAT CTG CTT CAA TCA GCG	
aroE	456	aroE-up	ATC GGA AAT CCT ATT TCA CAT TC	
		aroE-dn	GGT GTT GTA TTA ATA ACG ATA TC	
glpF	465	glpF-up	CTA GGA ACT GCA ATC TTA ATC C	
		glpF-dn	TGG TAA AAT CGC ATG TCC AAT TC	
gmk	429	gmk-up	ATC GTT TTA TCG GGA CCA TC	
		gmk-dn	TCA TTA ACT ACA ACG TAA TCG TA	
pta	474	pta-up	GTT AAA ATC GTA TTA CCT GAA GG	
		pta-dn	GAC CCT TTT GTT GAA AAG CTT AA	
tpi	402	tpi-up	TCG TTC ATT CTG AAC GTC GTG AA	
		tpi-dn	TTT GCA CCT TCT AAC AAT TGT AC	
yqiL	516	yqiL-up	TTT GCA CCT TCT AAC AAT TGT AC	
		yqiL-dn	CGT TGA GGA ATC GAT ACT GGA AC	
agrI	106	agrI-F	CCAGCTATAATTAGTGGTATTAAGTACAGTAAACT	**Agr locus** 95ºC 10min **30x** [95ºC 15s + 60ºC 1min + 72ºC 1min] 72ºC 10minutos
		agrI-R	AGGACGCGCTATCAAACATTTT	
agrII	180	agrII-F	CAATAGTAACAATTTTAGTGACCATGATCA	
		agrII-R	GCAGGATCAGTAGTGTATTTTCTTAAAGTT	
agrIII	80	agrIII-F	CATTATAACAATTTCACACAGCGTGTT	
		agrIII-R	GCAAGTGCATAAGAAATTGATACATACA	
agrIV	128	agrIV-F	GAGTTCTCAAAAAGATTAGCTCATCATATC	
		agrIV-R	TAGCTTCATCCGAGTTTATTTGAGAAT	

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Discussions & Conclusions

Identification and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) clones.

- **2005**. In this study, the most prevalent clone was the pandemic New York / Japan (t002, ST5-II), which was associated to MLSBc resistance. The percentage of Iberian clone (t051, ST241-I) was significantly low, likely in order to favour other clones more transmissible, such as the EMRSA-16 (t018, ST36-II). This EMRSA-16 clone had been previously described in United Kingdom in 90s and reported to be the major clone in two hospitals in Vigo during 1997-2005, in fact our findings were very similar to published by Potel *et al.*[276] The EMRSA-16 was also associated to mupirocin and MLSbc resistance. The Brazilian clone (t037, ST239-II), probably imported from Brazil in 90s, which also became one of major clones in hospitals in Vigo,[276] represented 2 % of isolates and was associate to cotrimoxazole resistance.

- **2009.** In this study, the most prevalent clone was the comunitary t067, ST125-IV,[223] followed for the another comunitary clone, the Pediatric clone (t002, ST5-IV), which has been associate to *ermC* plasmid. The prevalence of spa type t008, SCC*mec* IV was high, however all of them were PVL-*negative*, thus the virulent USA300 clone or USA-like clones were not detected. More than 80 % of isolates belonged to sequencing type - clonal complex ST-CC5, and even though the sample size and number of isolates were bigger, there was a great homogeneity of clonal distribution.

- **Clonal evolution over the time**: (i) the Iberian clone was replaced for other clones, such as (ii) EMRSA-16 clone (ST36-II), which became the prevalent clone until 2005, to be also replaced. (iii) The comunity t067, ST125-IV got to replace this pandemic clone and became the major clone in 2009. (iv) The New York/Japan (ST5-II), reported in a northern Spanish in 2005, was replaced by pediatric clone (ST5-IV). Therefore, (v) CA-MRSA replaced to HA-MRSA, and increased its distribution mainly towards the ST-CC5, getting so one more homogeneity in clonal distribution. (vi) The spa-clonal complex CC067 and ST-CC5 were kept over the time like the most prevalent clonal complex.

- **Resistance evolution over the time:** (i) ciprofloxacin resistance was remained, however (ii) tobramycin resistance, mainly associated to *ant (4`)* gen, decreased more than 40 % in 2009 study respect to 2005, and (iii) erythromycin resistance, mainly associated to *mrs(A)* and *mrs(B)*, and clindamycin resistance, associated to *ermC* and *ermA,* decreased more than 30 and 50%, respectively; (iv) the susceptibility to cotrimoxazole decreased, even disappeared, together with the extinction of the Brazilian clone; and (v) for the rest of antibiotics, the susceptibility was maintained in both studies, including the CMI_{90} for linezolid and vancomycin (1 µg and 2 µg / ml, respectively).

In summary, the epidemiology and antibiotic susceptibility of MRSA can switch over the time, especially in favour the clones more transmissible and more able to adapt. In the last decade, it has been observed a global change in the epidemiology of MRSA,[277] towards a higher distribution and uniformity of CA-MRSA clones, which are more transmissible and also more susceptible. Thus, the movement and displacement of clones observed in our study, from a multidrug-resistance HA-MRSA in favour of CA-MRSA, contributed to the reduction of observed resistance, as well as being consistent with to global change.

Since, the genotype can determine the pathogenicity and antibiotic susceptibility pattern of the bacteria, the application of both sequencing techniques can be especially useful in epidemiological studies. In fact, the application of MLST is very useful in long-term studies and the spa-typing is more useful in short-term studies.

Resumen Castellano

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DEPARTAMENTO DE MICROBIOLOXÍA

TESE DE DOUTORAMENTO

Community-Acquired Methicillin Resistant Staphylococcus aureus: Effects Of Subinhibitory Concentrations of Antibiotics on the Proteome Profile USA300. Pathogeneses and susceptibility.

Staphylococcus aureus resistente a meticilina Adquirida en la comunidad: Efectos de concentraciones subinhibitorias de antibióticos en el Perfil proteómico de USA300. Patogénesis y susceptibilidad.

Memoria presentada por EVA TORRES SANGIAO para optar o grao de Doctor en Farmacia

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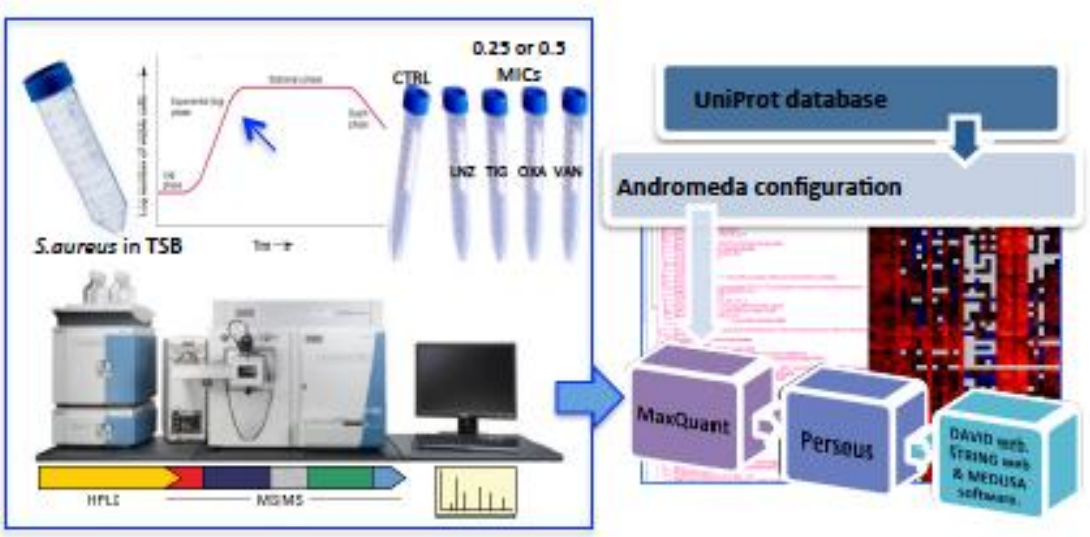
Sinopsis

Este estudio permite un conocimiento global de la adaptación de la cepa hipervirulenta de *Staphylococcus aureus* resistente a meticilina (SARM) aislado en la comunidad, USA300 clon, a la presión antibiótica. Además, marca una diferencia con los anteriores estudios de transcriptómica sobre el genoma, donde sólo es posible observar cambios en el ARNm, pero no a nivel de proteína, dónde realmente se reflejan de forma directa las adaptaciones fisiológicamente relevantes.

El estudio de los efectos de las concentraciones subinhibitorias de linezolid, tigeciclina, oxacilina y vancomicina, se ha centrado sólo en proteínas implicadas en la regulación de la patogénesis, virulencia, mecanismos de acción asociadas a cada antibiótico, incluyendo mecanismos de resistencia, y la respuesta al stress o presión antibiótica. Por lo tanto en este estudio proporcionamos como puede ser la adaptación *"in vitro"* del proteoma de USA300 clon, independiente y dependientemente del mecanismo de acción de cada antibiótico, y el posible efecto terapéutico de altas concentraciones subinhibitorias de varios antibióticos usados clínicamente.



Palabras clave: USA300, MRSA, patogénesis, proteoma, factores de virulencia.

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Introducción:

Staphlylococcus aureus es un patógeno humano, Gram-positivo, de gran importancia clínica, principalmente debido a su alta incidencia y propagación de la resistencia a los antibióticos. *S. aureus* resistente a meticilina (SARM), es una causa importante de infecciones nosocomiales y asociadas a la comunidad en todo el mundo. Las infecciones por SARM causan manifestaciones clínicas que van desde infecciones leves de la piel a infecciones graves como bacteriemia y neumonía necrotizante. En los últimos años la prevalencia de SARM se ha incrementado drásticamente y, SARM es ahora la principal causa de infecciones de piel y tejidos blandos.

La pérdida de la actividad de los antibióticos β-lactámicos frente a las cepas de *S. aureus* productores de PBP2a, *mec*A(+), ha obligado a la comunidad médica a usar numerosos antimicrobianos alternativos. Esto ha provocado un cambio en la terapia empírica de las infecciones estafilocócicas, de tal modo que antibióticos no β-lactámicos con actividad contra SARM son cada vez más prescritos. En consecuencia, las cepas de SARM que han sido típicamente susceptibles a los antibióticos no β-lactámicos (por ejemplo, clindamicina, tetraciclinas, trimetoprim-sulfametoxazol) se están volviendo resistentes a estos antibióticos.[278,279] Vancomicina, aunque ha conseguido mantenerse como el antibiótico de elección contra SARM, su uso clínico ha ejercido una presión selectiva continua desencadenando la aparición de *S. aureus* con susceptibilidad intermedia a vancomicina (VISA), o concentraciones mínimas inhibitorias (CMI) elevadas, un fenómeno conocido como vancomicina "creep CMI",[280-282] así como cepas hetero-resistentes (hVISA) e incluso resistentes a vancomicina (VRSA).[171,283-285] Estas cepas tienen una susceptibilidad reducida a los glucopéptidos, y están asociadas con fracaso[286] terapéutico a vancomicina.

S. aureus, además, produce una amplia gama de factores de virulencia que juegan un papel importante en la patogénesis de la infección estafilocócica, entre ellos α-hemolisina (Hla), leucocidinas y toxinas. Particularmente, las cepas de SARM adquiridas en la comunidad (SARM-AC) están mejor adaptadas a la evasión de la respuesta inmune innata, una característica que probablemente contribuye a su mayor virulencia.[102] Además, la infección por SARM-AC se asocia con necrosis intensa *in vivo*, que muy probablemente conduce a una peor difusión de los antibióticos, lo que consecuentemente resulta en bajas concentraciones en los sitios de infección. De acuerdo con las recientes directrices basadas en varias series de datos *in vitro*, se recomienda el uso de antibióticos que inhiban la expresión de los factores de virulencia para el tratamiento de infecciones graves causadas por *S. aureus,* incluidas las productores de la toxina Panton-Valentine

leucocidina (PVL).[287] Por otro lado, varios estudios han demostrado que las concentraciones subinhibitorias de antibióticos pueden modular la expresión de factores de virulencia en *S. aureus,* y por lo tanto, pueden afectar a la resolución de las infecciones estafilocócicas graves. Los resultados de estos estudios sugieren que niveles de sub-CMIs de los antibióticos, pueden mejorar la producción de algunos de los factores de virulencia mientras disminuyen la síntesis y liberación de los demás, en consecuencia, a través de esta `manipulación´ de los factores de virulencia, los antibióticos podrían empeorar o atenuar la enfermedad.[288] Por lo tanto, la eficacia del tratamiento para las infecciones por *S. aureus* puede depender no sólo de los efectos bacteriostáticos o bactericidas del antibiótico, sino también de su capacidad para evitar la liberación de los factores de virulencia.[178,245,289,290] También debe tenerse en cuenta que, la resistencia y tolerancia a antibióticos se cree que evoluciona en entornos con concentraciones subinhibitorias de antibióticos, como el entorno natural o cuerpo humano, además de la propia fluctuación de la concentración del antibiótico administrado en el tiempo y el espacio.

En los últimos años, la transcriptómica ha comenzado a ser aplicada al estudio de la resistencia antibiótica.[291-293] Estos estudios han demostrado que los antibióticos provocan respuestas transcripcionales específicas, involucrando muchas vías de transducción de señales metabólicas, que apoyan la idea de que la susceptibilidad a los antibióticos debe ser examinada desde un punto de vista global. El uso de métodos de espectrometría de masa (EM) con tecnología de última generación para perfilar los cambios en la expresión de las proteínas bacterianas ha sido un paso natural. Unos pocos estudios, proteo-transcriptómicos y de proteómica comparativas (iTRAQ combinan con 2-DE), han sido empleados para estudiar el mecanismo de resistencia en *S. aureus* a daptomicina y vancomicina, respectivamente.[294-296] En general, se observó una pobre correlación entre los datos de proteómica y transcriptómica. Los estudios de proteómica comparativa revelaron que proteínas asociadas a la pared celular y formación de biopelículas, se expresaban diferencialmente en la adaptación a daptomicina.[295] En el segundo estudio, rutas metabólicas como biosíntesis y metabolismo de la pared celular, se encontraban reguladas positivamente (+) en cepas con susceptibilidad intermedia a vancomicina.[296] El primer estudio que describe los cambios en todo el proteoma de SARM bajo concentraciones subinhibitorias de oxacilina, y utilizando cuantificación sin marcaje (LFQ), han sido publicado recientemente por Liu *et a*l.,[247] quienes investigaron los cambios globales en el perfil proteómico de SARM y *S. aureus* sensibles a meticilina, bajo condiciones de estrés a oxacilina, para entender los mecanismos de resistencia y de tolerancia desde un punto de vista sistemático.

Objetivos:

El objetivo de este estudio fue investigar la **respuesta celular** de la cepa de *S. aureus* USA300, bajo dos concentraciones subinhibitorias de cuatro antibióticos de uso clínico, linezolid, tigeciclina, oxacilina y vancomicina, y por lo tanto el **efecto** de los mismos en el perfil proteómico de USA300. Los resultados además nos proporcionaran una **visión global de la respuesta al estrés**, y/o **mecanismo de adaptación** de SARM-AC USA300 a estas concentraciones subinhibitorias, así como las proteínas involucradas.

Materiales y métodos.

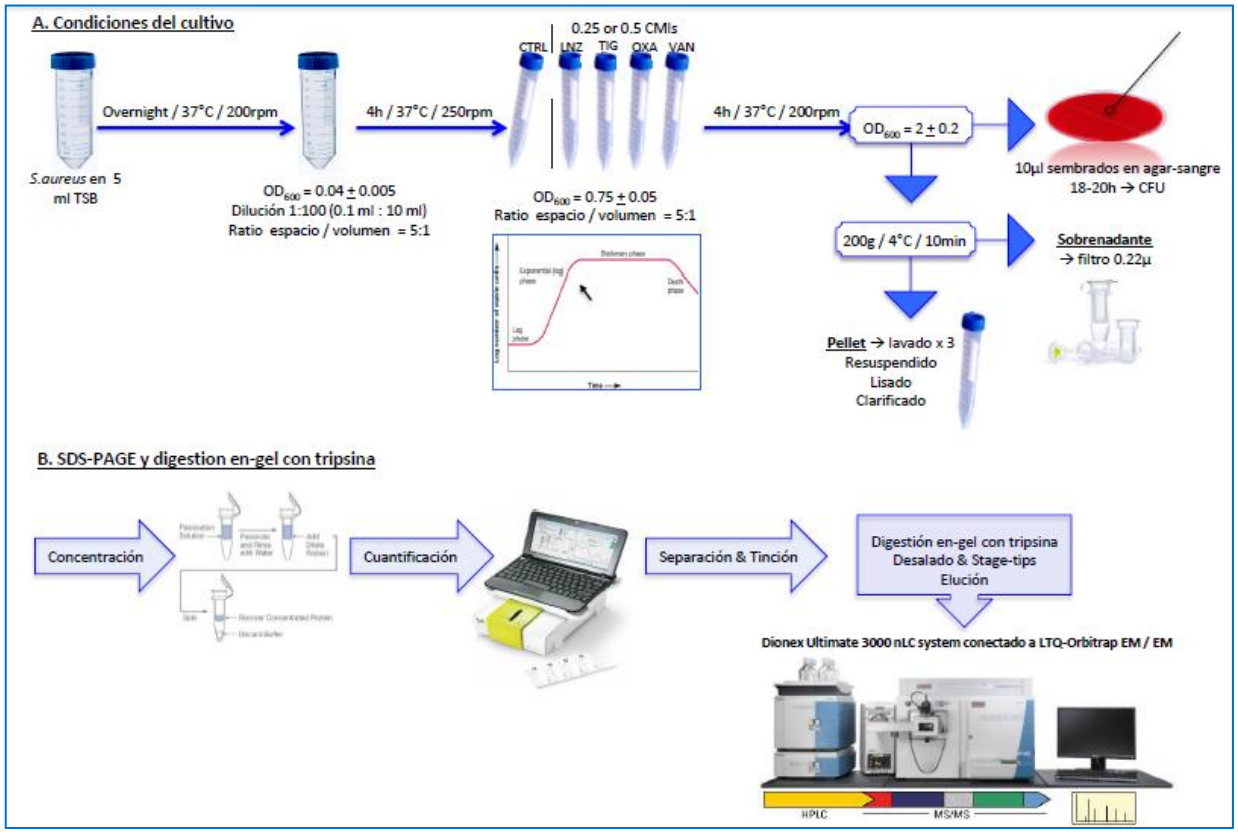
Cepas bacterianas y reactivos: USA300 clon[146,200] (*Staphylococcus aureus* resistente a meticilina adquirido en la comunidad, SARM-AC). Antibióticos: linezolid, tigeciclina, vancomicina y oxacilina.

Determinación de CMIs: Método de microdilución en caldo recomendado por el Clinical Laboratory Standards Institute (CLSI)[14] en medio Mueller-Hinton II y por E-Test ® siguiendo las instrucciones del fabricante (bioMérieux). Los experimentos se realizaron por triplicado.

Preparación de la muestra para el análisis proteómico: Condiciones de cultivo: Una única colonia de USA300 se inoculó en 5ml de TSB estéril toda la noche a 37°C/200 rpm. 100µl de este cultivo fueron inoculados en 10ml de TSB (**Figura 1**). El crecimiento se monitorizó espectrofotométricamente hasta fase estacionaria temprana. En este punto, se añadieron concentraciones de 0,25 y 0,5 CMIs de cada antibiótico. Las concentraciones utilizadas fueron: 0,25 y 0,5 µg/ml para linezolid, 0,12 y 0,25 µg/ml para la tigeciclina, 8 y 16 µg/ml para oxacilina, 0,5 y 1 µg/ml para la vancomicina, respectivamente. Los cultivos con o sin antibióticos se volvieron a incubar durante 4 horas/37°C/200 rpm hasta fase estacionaria. Posteriormente, las bacterias se sembraron en agar sangre para determinar UFC/ml. Los experimentos fueron realizados por triplicado. Las bacterias fueron recogidas por centrifugación a 2 000x g/10 min/4°C, se lavaron 3 veces con PBS a 1 000x g/5 min/4°C. El sedimento se resuspendió en Tris-HCl-SDS. Las paredes de las células se lisaron mecánicamente en un Ribolyser. Lisado y fracción soluble fueron clarificados, filtrados y concentrados, y el rendimiento se evaluó con un DetectTM Espectrómetro directo. SDS-PAGE y en gel de digestión con tripsina: 20µgr de extracto de proteína total fueron posteriormente fraccionados por SDS-PAGE usando un 4-12% Bis-Tris gradiente. Después de la tinción, cada carril de gel fue dividido en 4 fracciones (2.5x10mm), y cada fracción se sometió a reducción en gel- alquilación-digestión con

tripsina de acuerdo con el protocolo utilizado en la Unidad de Proteómica de la Universidad de Bergen (PROBE). La mezcla de péptidos resultante se desaló en fase inversa C18 y se diluyó en 0,1% de Tri-fluor-acético[234] antes del análisis nano-LC-ESI-QTOF-EM/EM. (**Figura 1**)

Figura 1. Preparación de las muestras (**A**) cultivo y (**B**) digestión. LNZ= linezolid; TIG= tigeciclina; OXA= oxacilina; VAN= vancomicina.



Espectrometría de masas (EM): Todos los experimentos se realizaron en un sistema de nano-3000 LC Dionex final) conectado a un LTQ-Orbitrap espectrómetro de masas. El disolvente A fue acetonitrilo acuoso al 2% en ácido fórmico al 0,1%, y el disolvente B fue acetonitrilo acuoso al 90% en ácido fórmico al 0,1%.[297]

Secuencia de búsqueda de base de datos: Todos los datos obtenidos fueron procesados y analizados utilizando MaxQuant (versión 1.4.1.2)[235] software desarrollado específicamente para datos adquiridos por instrumentación de alta resolución. La identificación de proteínas se realizó mediante la búsqueda de los datos por separado frente a las proteínas de USA300 desde la base de datos descargada de UniProt. La identificación de proteínas y su validación fue realizada con el uso de Identify.exe y los siguientes parámetros: FDR:[298] 0,01 (1%), longitud mínima de péptido fue de 7

aminoácidos, y para garantizar una tasa de identificación de alta confianza, y el número mínimo de péptidos únicos por proteínas: 1. Entonces las proteínas fueron validadas estadísticamente en base a la puntuación de sus péptidos individuales. Utilizando estos criterios, todas las identificaciones EM/EM de péptidos presentes en las entradas con secuencias invertidas, sólo identificados por sitio y los contaminantes no fueron validadas. Proteínas identificadas con confianza en al menos dos de cada tres réplicas biológicas para los ensayos de antibióticos y tres de las seis réplicas biológicas para el control no tratado se incluyeron en el análisis posterior.

Cuantificación sin marcaje: La cuantificación basada en el área del pico se realizó en MaxQuant. El análisis estadístico y visualización fue realizado usando Perseus (v.1.4.1.3).[237] Las proteínas expresadas diferencialmente se filtraron por el siguiente corte de valor de *p* para la prueba t < 0.05. El *fold change* fue calculado como t-test diferencia (antibiótico -control) para los datos transformados por log2 y como el ratio (antibiótico / control) de los datos sin transformar.

Análisis Bioinformático: Para determinar la localización subcelular de las proteínas, así como su proceso biológico (BP), la función molecular, y otras anotaciones, se utilizó UniProt-database. Todas las proteínas identificadas se analizaron mediante el uso de la web DAVID[239] a través de la cual se calculó el algoritmo para evaluar el enriquecimiento de las vías metabólicas. Para mostrar estas proteínas en el contexto de su red interactuante, se utilizó STRING 9.1[240] para predecir las interacciones proteína-proteína y Medusa 1.5[241] para mostrar el análisis gráfico.

Resultados y discusión

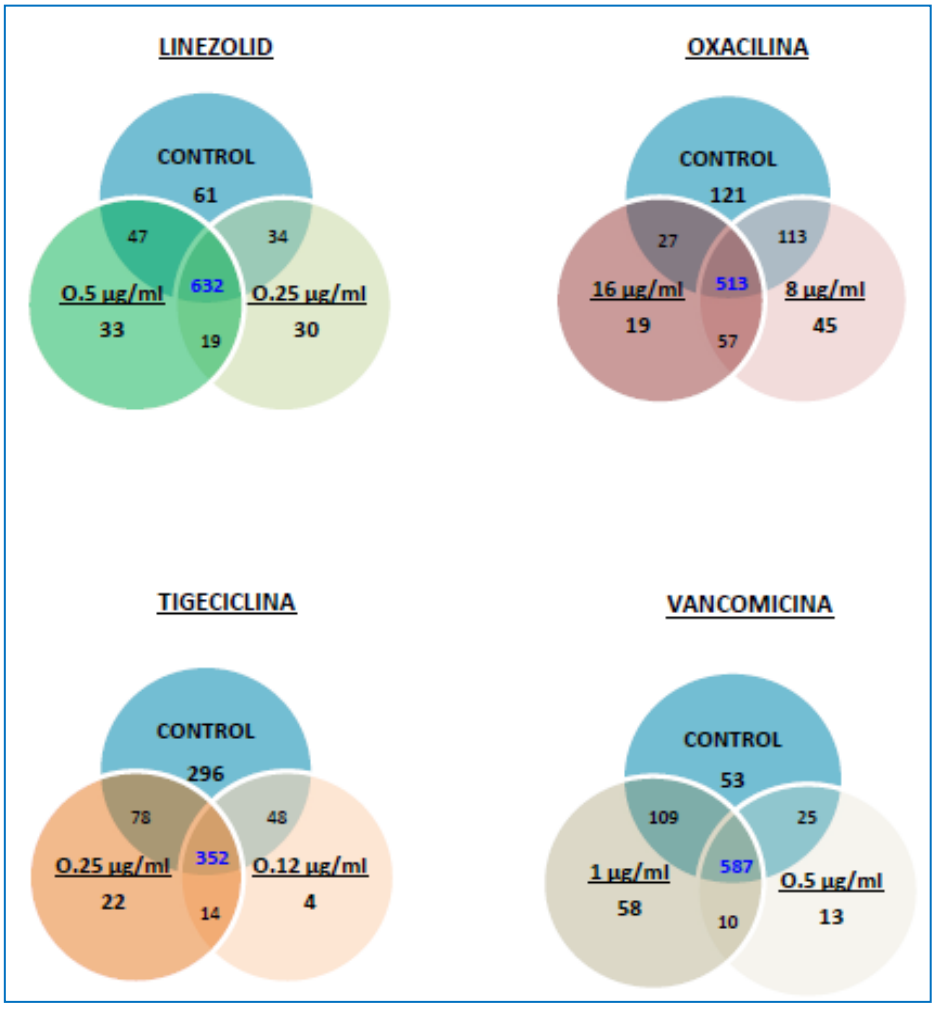
Identificación global de las proteínas de USA300: Se identificaron un total de 1 284 proteínas, de las cuales 980 estaban presentes en al menos dos réplicas biológicas y por lo tanto fueron considerados como proteínas válidas para su posterior análisis. Esto representó aproximadamente el 40% de las aproximadamente 2 600 proteínas codificadas en el genoma de USA300 anotado por Diep *et al.*[146]

La **Figura 2** muestra los correspondientes diagramas de Venn que describen las proteínas comunes e identificadas de forma selectiva para cada antibiótico y condición.

Análisis cualitativo. Distribución de las proteínas identificadas con respecto a la localización celular, función biológica y ruta metabólica: Para comparar los cambios entre las diferentes condiciones de antibióticos, primero analizamos la localización celular de las proteínas identificadas. Linezolid y vancomicina no mostraron cambios significativos

con respecto a la distribución de la localización celular. Sin embargo, en las muestras tratadas con 0.5sub-CMI de oxacilina y tigeciclina, las proteínas de membrana, especialmente proteínas implicadas en procesos de transporte, fueron reducidas en número en más de un 30%. Por otra parte, el número de proteínas extracelulares aumentó más del 40% para estas mismas condiciones de 0.5sub-CMI de tigeciclina y oxacilina. Esta pérdida de proteínas transportadoras de membrana podría estar asociado a una pérdida de la viabilidad de la función de la pared celular, y el gran número de proteínas extracelulares podría estar asociado con un incremento en los factores de virulencia y / o respuesta al estrés, para estas sub-concentraciones.

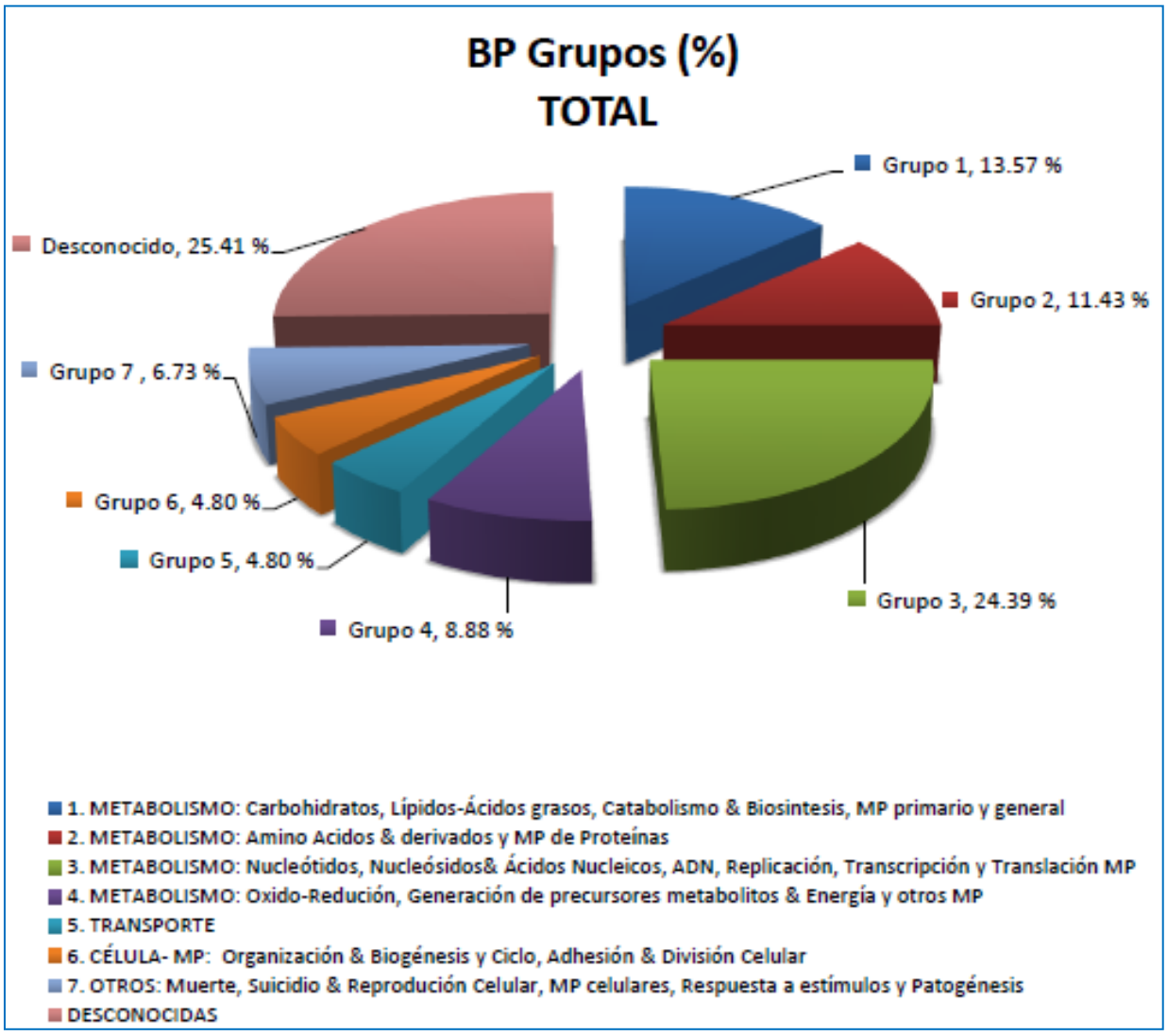
Figura 2. Diagrama de Venn. Diagrama de Venn para la comparación del proteoma entre el control-no tratado y las concentraciones subinhibitorias (0.25 MIC y 0.5 MIC) de linezolid, tigeciclina, oxacilina y vancomicina.



Para seguir con este análisis cualitativo, utilizamos el programa disponible en la web DAVID. Este análisis nos permitió conocer la distribución de estas 980 proteínas respecto

a los procesos biológicos (BP) anotados, y a las rutas metabólicas anotadas, con sus respectivos "escores de enriquecimiento", para cada condición de antibiótico respecto del control. Las proteínas anotadas por estar implicadas en el metabolismo de carbohidratos (grupo 1), aminoácidos y derivados de los procesos metabólicos y metabolismo de las proteínas (grupo 2), y procesos metabólico de los ácidos nucleicos, nucleótidos y nucleósidos (grupo 3), constituyeron el 50% sobre el total (**Figura 3**) para todas las condiciones.

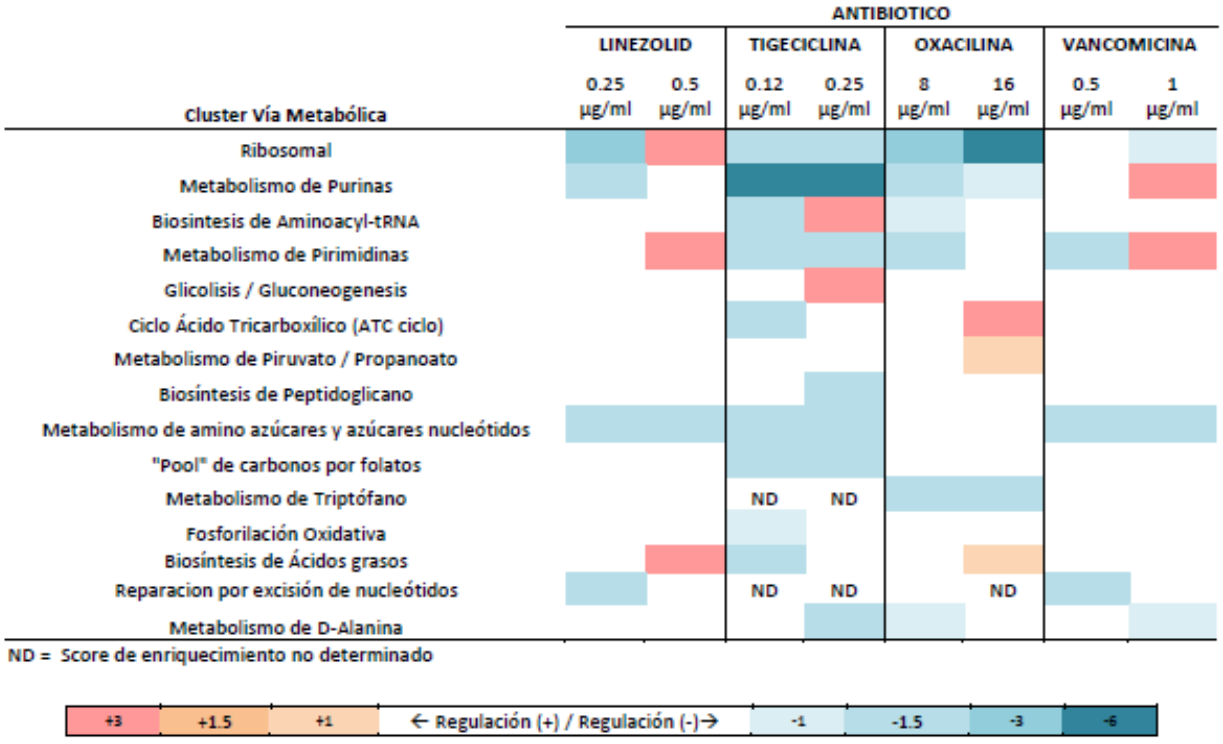
Figura 3. Distribución de los Procesos Biológicos de las proteínas diferencialmente expresadas agrupadas en diferentes grupos.



El análisis de los "scores de enriquecimiento" con respecto a las rutas metabólicas (**Figura 4**) difirieron enormemente entre los cuatro antibióticos, *por ejemplo*, para la 0.25sub-CMI de linezolid se observó una regulación negativa (-) de la vía ribosomal, sin embargo con la concentración de 0.5sub-CMI esta misma ruta metabólica estaba regulada

positivamente (+). Ahora bien, todas las sub-CMIs consiguieron modificar la regulación de la ruta del metabolismo ribosomal o metabolismo de purinas/pirimidinas. Resumiendo, las concentraciones más altas, pero todavía por debajo de la concentración que se considera terapéutica, las bacterias responder de una manera completamente diferente para contrarrestar el efecto de la presión antibiótica. Suponemos así, que bajo 0.25sub-CMIs, USA300 adapta su metabolismo a través de la regulación negativa de las principales vías metabólicas. Sin embargo, a dosis más altas y próximas a la CMI de los antibióticos, la bacteria parece redireccionar su metabolismo a través de la regulación del ciclo acido tricarboxílico, biosíntesis de ácidos grasos o de las vías del metabolismo de purinas/pirimidinas, para de este modo poder superar la presión antibiótica, es decir, se aumenta la respuesta al estrés.

Figure 4. Estudio del "score de enriquecimiento" de las rutas metabólicas por DAVID software con una cobertura para las anotaciones del 45%. La regulación positiva (up), o negativa (down) de las rutas metabólicas se definió como la substracción entre (*score*-puntuación del enriquecimiento para cada condición de antibiótico - *score* -puntuación del enriquecimiento para el control). Las diferencias por encima de ± 1 fueron consideradas significativas.



Como conclusión, nuestros resultados fueron consistentes con la literatura reciente, la cual sugiere que los cambios en el metabolismo energético, ciclo acido tricarboxílico y metabolismo de purinas/pirimidinas, son respuestas bacterianas comunes frente a la

presión antibiotica,[244] al mismo tiempo que la respuesta al estrés y la formación de biopelículas se incrementan.[267]

Análisis cuantitativo del perfil del proteoma de USA300 en respuesta a la concentración subinhibitoria de 0.5CMI: Previamente al análisis estadístico y comparativo, se comprobaron las correlaciones entre las réplicas biológicas. Estas correlaciones representadas como coeficiente de correlación Pearson R, fueron entre 0.8–0.99 para todos los extractos de las células (*pellet*), excepto para las tratados con tigeciclina. Sin embargo, para los sobrenadantes del cultivo, este coeficiente R fue mucho menor. Los bajos coeficientes de correlación R para tigeciclina podrían ser explicados por una mayor actividad de este antibiótico, y la mayor presencia de proteínas intracelulares en los sobrenadantes tratados con oxacilina podría ser debido a una extensiva lisis bacteriana.[249](Datos no mostrados).

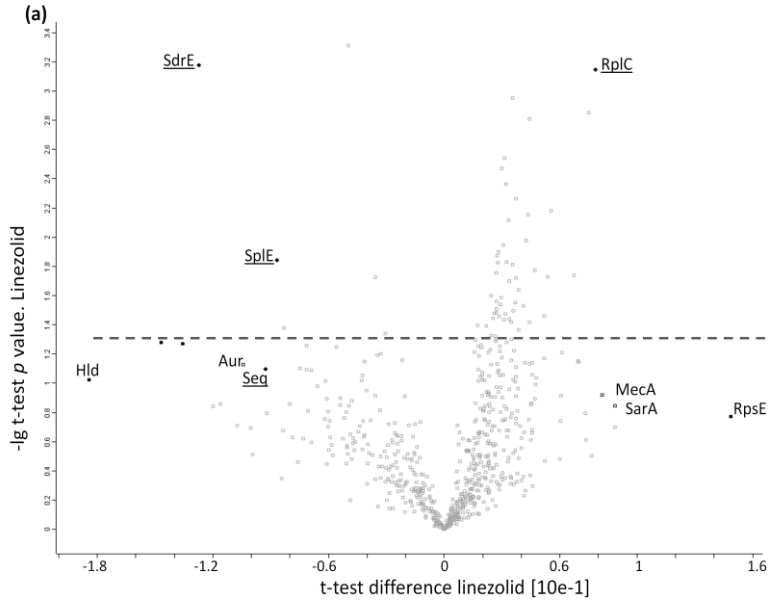
Para este análisis comparativo de la respuesta específica a cada antibiótico, sólo se consideraron las proteínas expresadas diferencialmente en las condiciones de 0.5sub-MICs. Para analizar estas proteínas expresadas diferencialmente, consideramos el diseño del experimento y las limitaciones de detección. Por lo tanto, las proteínas expresadas diferencialmente pero no identificados bajo alguna condición, antibióticos o control, y con unas intensidades normalizadas LFQ (*N*–LFQ) por debajo de 0.20 (equivalente a LFQ $< 10^6$), no fueron consideradas. Entre las proteínas expresadas diferencialmente en el control, 26, 205, 117 y 21 no fueron observadas con linezolid, tigeciclina, oxacilina y vancomicina, respectivamente. También se determinaron 19, 32, 39 y 17 proteínas expresadas diferencialmente con linezolid, tigeciclina, oxacilina y vancomicina, respectivamente, las cuales no fueron observadas en el control. El análisis estadístico, para las proteínas expresadas diferencialmente en ambas condiciones, antibióticos y control, reveló 4, 37, 24 y 3 proteínas estadísticamente significativas para linezolid, tigeciclina, oxacilina y vancomicina, respectivamente (**Figura 3**). Entre estas proteínas diferencialmente expresadas observamos, como era de esperar, proteínas asignadas por estar implicadas en, i) *mecanismo de resistencia* a, quinolonas (GyrA, GyrB, Parc ParE), vancomicina (RpoB, RpoC),[252] β-lactámicos (BlaZ, FemA y PBP2`a/MecA) y aminoglucósidos (AphA(3')-III); ii) *sistemas de regulación*, *p.ej.*, de resistencia antibiótica (MarR), virulencia, como el represor CodY cuya delección ha sido relacionado con un aumento de la virulencia de USA300,[250] o ambos, como las proteasas-dependiente de ATP Clp asociadas a la resistencia a β-lactámicos y virulencia de USA300;[263] iii) *respuesta al estrés,* como superóxido-dismutasa SodA/D; y *iv) patogenia y virulencia*, como inhibidores de la vía del complemente (Sbi, EfB, Chp),[299] enterotoxinas (SEC), y las

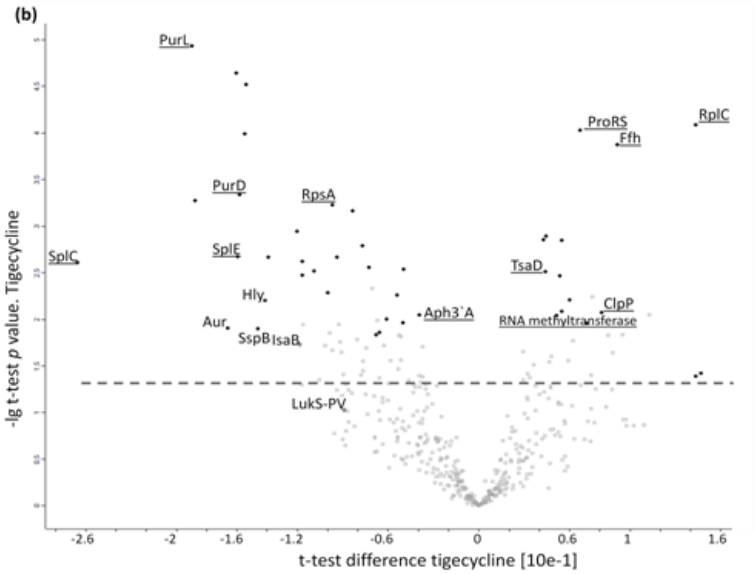
principales toxinas características del hipervirulento SARM-AC, clon USA300: fenol-soluble-modulinas (PSM-A4), Hla/Hly y PVL (LukF-PV y LukS-PV).[300] Las proteínas con una mayor expresión diferencial en condiciones de antibióticos se recogen en la **Tabla 1**, y las proteínas expresadas diferencialmente en el control pero no observados con antibióticos se resumen en la **Tabla 2**.

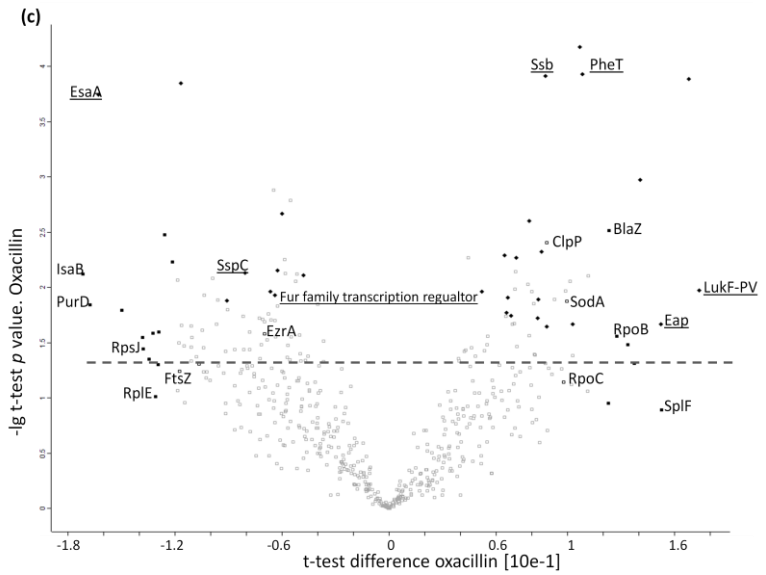
Figura 3. **Volcano Plots**. Volcano Plots donde se muestran las diferencias más significativas de las proteínas expresadas diferencialmente bajo 0.5MIC de, (**a**) linezolid, (**b**) tigeciclina, (**c**) oxacilina, y (**d**) vancomicina, con respeto al no tratado-control-USA300, relacionadas con el mecanismo de acción y patogénesis.

p-valor estadístico < 0,05 (eje *y*), y `*fold-change´* antibióticos *versus* control (eje *x*). La línea discontinua muestra donde *p*-valor = 0,05 expresados como log de t-test *p*-valor (log 10 *p*-valor) = 1,30103. Puntos por encima de la línea se corresponden con p < 0,05 y por debajo de la línea se corresponden con *p* > 0,05. El ■ se corresponde con los puntos que tienen un `*fold change´* más de 2x (2 <x <0.5), expresado como el ratio, antibióticos / control, y de acuerdo a la diferencia de la prueba t [antibiótico - control]. El ◆ se corresponde con los puntos que son estadísticamente significativos.

Aph3'A: aminoglucósido 3'-fosftransferasa; **Aur:** zinc metaloproteinasa aureolisina; **BlaZ**: beta-lactamasa; **ClpP**: ATP-dependiente Clp proteasa subunidad proteolítica; **Ffh:** señal de reconocimiento de partículas; **FtsZ**: proteína de la división celular; **HemG**: protoporfirinógeno oxidasa; **Hld**: delta-haemolisina; **Hly:** alfa-haemolisina; **IsaB**: antígeno inmunodominante B; **RpsA**: proteína ribosomal 30S S1; **LukF-PV**: Panton-Valentine leucocidina subunidad F; **LukS-PV**: Panton-Valentine leucocidina subunidad S; **MecA**: proteína de unión a penicilina 2a (PBP 2a); **PheT**: fenilalanina - ARNt ligasa subunidad beta; **ProRS**: Proline-tARN ligasa; **PurD**: fosforribosil-amina-glicina ligasa; **PurI:** fosforibosil-formil-glicin-amidina sintasa 2; **RplC**: proteína ribosomal 50S L3; **RplE**: proteína ribosomal 50S L5; **RpoB**: ARN polimerasa DNA-dirigida subunidad beta; **RpoC**: ARN polimerasa DNA-dirigida subunidad beta; **RpsE**: proteína ribosomal 30S S5; **RpsJ**: proteína ribosomal 30S S10; **SarA**: regulador accesorio estafilocócica A; **SdrE:** serina-aspartato proteína E; **Seq**: enterotoxina Q; SodA: superóxido dismutasa A; **SplC**: serinproteasa C; **SplE**: serinproteasa E; **SplF**: serinproteasa F; **Ssb**: proteína de unión al ADN monocatenario; **SspB**: C47 estafopaína familia B; **SspC**: Familia I57 estafostatina B; **TsaD**: N6-adenosina ARNt treonil-carbamoil-transferasa.




(b)
PurL
ProRS
RplC
Ffh
PurD
RpsA
SplE
SplC
TsaD
Hly
ClpP
Aph3`A
Aur
RNA methyltransferase
SspB
IsaB
LukS-PV
-lg t-test p value. Tigecycline
t-test difference tigecycline [10e-1]


(c)
Ssb
PheT
EsaA
BlaZ
ClpP
IsaB
SspC
LukF-PV
PurD
Fur family transcription regualtor
SodA
EzrA
RpoB
Eap
RpsJ
FtsZ
RpoC
RplE
SplF
-lg t-test p value. Oxacillin
t-test difference oxacillin [10e-1]

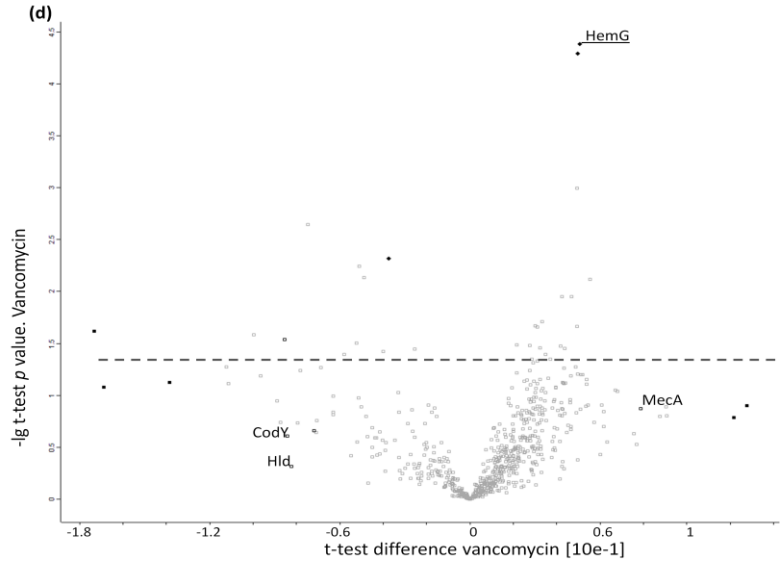

(d)
HemG
MecA
CodY
Hld
-lg t-test p value. Vancomycin
t-test difference vancomycin [10e-1]

Tabla 1. Proteínas altamente expresadas sólo bajo 0.5MICs de cada antibiótico. LFQ intensidades normalizadas no transformadas por log_2, representadas como la media entre las tres réplicas biológicas, y mayores de 0.75.

Num Ids	Protein Name	ANTIBIOTIO				Grupo Biológico
		Linezolid 0.5 µg/ml	**Tigeciclina** 0.25 µg/ml	**Oxacilina** 16 µg/ml	**Vancomicina** 1 µg/ml	
472	CHAP dominio-familia. Posible LysM dominio	**1.45**	0.38			1
527	Proteína relacionada con la familia de chitinasas (EC 3.2.1.14)		**3.29**			1
1164	Ornitina carbamoiltransferasa (OTCasa) (EC 2.1.3.3) **ArcB ArgF**		**1.83**			2
123	Proteína fijadora de fibronectina B **FnbB**		**3.93**			6
124	Proteína fijadora de fibronectina B **FnbA**	**0.79**	**1.46**			6
488	Factor de agregación A **ClfA**		**1.59**	0.13		6
503	Enterotoxina K **Sek**		**0.91**	0.72		7
741	inhibidor estafilococal del complemento (SCIN) **Scn**			**2.71**		7
379	Estafilocoagulasa (EC 3.4.23.48) **Coa**		**0.85**			
413	Proteína putativa de superficie		**6.80**			
421	Fenol-soluble-modulina péptido alfa 1 **PsmA1**			**8.33**		
604	Proteína UPF0154 SAUSA300_1240, USA300HOU_1280 (1)	**0.79**			**0.85**	
808	D-lactato dehidrogenasa (EC 1.1.1.28) **Ddh**		**2.58**	0.58	0.18	4
919	Proteína fijadora de fosfato **PstS**	0.64	**2.51**	0.29		5
303	Transferrina, receptor de hierro (Fe^{+3})	0.10	0.29	0.26		5
1163	Factor de agragación B **ClfB**	**1.20**	**3.50**	**0.43**		6
560	Proteína estafilococcal fijadora de transferrina, proteina A **IsdA**	**0.57**	**0.99**	**1.82**		7
1173	PXTG familia, proteína de anclaje de la pared celular **SasF**	**1.63**	**1.25**	**0.54**		
743	Estafiloquinasa **Sak**	**3.66**	**4.74**	**1.59**		
1182	Proteína no caracterizada (2)	**2.62**	**2.02**	**1.02**		

(1) Putativa proteína exportadora. Membrana Citoplasmática. (Probabilidad = 0.955). Sólo identificada en sobrenadantes.
(2) Putativa proteína de inmuniidad/bacteriocina. Sólo identificada en los sobrenadantes.

Tabla 2. Proteínas diferencialmente expresadas en el control sin ser observadas usando 0.5MICs de cada antibiótico. Proteínas asociadas con el mecanismo de acción de los antibióticos o implicadas en la patogénesis. LFQ intensidades normalizadas no transformadas por log_2, representadas como la media entre las seis (control) o las tres (antibióticos) réplicas biológicas, y mayores de 0.20.

Num id	Nombre de la proteína	Control	ANTIBIÓTICO				Grupo Biológico
			Linezolid 0.5µg/ml	Tigecidina 0.25µg/ml	Oxacilina 16µg/ml	Vancomicina 1µg/ml	
1076	Serinproteasa **SplC** (EC 3.4.21.-)	**20.33**		0.16	1.29	15.40	2
1078	Serinproteasa **SplA** (EC 3.4.21.-)	**3.15**	0.29		1.07	2.15	2
1103	Regulador Factor Sigma B **RsbU**	0.17	0.27			0.35	2
61	Probable ARN polimerasa dirigida por ADN subunidad delta (RNAP factor delta) **RpoE**	0.33	0.33		0.30	0.25	3
313	Sistema UvrABC, proteína B (Proteína **UvrB**) (Excinucleasa ABC subunidad B)	0.22	0.23			0.30	3
314	Sistema UvrABC, proteína A (**UvrA** proteína) (Excinucleasa ABC subunidad A)	0.36	0.36			0.42	3
796	Regulador transcripcional **SarR** (Accesorio Staphylococcal reguladorR)	0.92	0.97		1.25	0.79	3
1000	ARN polimerasa Factor Sigma SigA **RpoD**	0.51	0.62		0.56	0.67	3
1101	Serin-proteína-quinase **RsbW** (EC 2.7.11.1) (Factor Anti-sigma-B) (efector negativo Sigma-B RsbW)	1.01	0.91		1.28	1.09	3
1175	Operón de adhesión intercelular (Ica operón), regulador transcripcional, **TetR** familia	0.13	0.09			0.11	3
1195	Sensor-redox, represor transcripcional **Rex**	0.59	0.69		0.39	0.74	3
873	Probable dual RNA metiltransferasa **RlmN** (EC 2.1.1.192)	**1.71**	1.48			1.30	3
1267	Canal de larga-conductancia mecano-sensible **MscL**	0.68	0.89		0.15	0.98	5
520	D-alanina—poli(fosforibitol) ligasa subunidad 2 (EC 6.1.1.13) (proteína transportadora de D-alanil) **DltC**	0.35	0.31			0.55	6
619	Factor de resistencia a meticilina **FemB**	0.31	0.31	0.25		0.77	6
666	Proteína determinante de la forma celular **MreC**	0.12	0.15			0.14	6
781	Proteína de biosíntesis del interpéptido de pentaglicina **FemB** (EC 2.3.2.16)	0.70	0.56		0.64	0.86	6
953	Proteína del cido celular **GpsB** (proteína *Guiding* PBP1-*shuttling*)	0.13	0.13			0.11	6
1185	Proteína reguladora de beta-lactamasas (Fragmento) **BlaR**	0.28	1.73		0.43	0.40	6
69	Proteína de stress general 20U (EC 1.16.3.1), familia **Dps**	0.52	0.39		1.64	0.54	7
145	Proteína de regulación transcripcional **WalR**	0.32	0.23		0.14	0.34	7
363	I57 estafostatina B **SspC**	0.23			0.04	0.22	7
629	Proteína estafilocócica de respuesta respiratoria, **SrrA**	0.25	0.21		0.24	0.27	7
887	Proteína cromosómica de división **Smc**	0.23	0.22	0.19		0.19	7
1088	Posible leucocidina, subunidad	0.37	0.34	0.78		0.63	7
1397	Delta-haemolisina **Hld**	**47.83**	7.07		36.95	31.06	7
14	Proteína UPF0342 SAUSA300_1795. Proteína UPF0342 USA300HOU_1838 (1)	**1.17**			0.87	0.63	
402	Proteína de virulencia **EsaA**	**8.27**	6.02		0.70	5.43	
509	Proteína **Ear**	**1.26**			1.19	3.05	
521	D-alanina-activador enzima/D-alanina-D-alanil, proteína dltD. Transferidora de D-alanina **DltD**	0.48	0.41			0.52	
571	Proteína fijadora de fibrinógeno	0.34	1.27	0.68		0.36	
751	Proteína(2) no caracterizada	**2.12**	6.79		0.30		
865	Proteína fijadora de fibrinógeno **FbpA**	0.24	0.15		0.42	0.17	
1004	Endoribonucleasa **YbeY** (EC 3.1.-.-)	0.49	0.08			0.07	
1079	Proteína no caracterizada. Probable Beta-lactamasa	**15.36**	0.61		4.51	12.50	

(1) Probablemente una proteína reguladora involucrada en el desarrollo de competencia y esporulación. [Replicación, recombinación y reparación, y mecanismo de traducción de señales] Citoplasmática (Probabilidad = 0.75).
(2) Putativa proteína de transporte o de superficie, o toxina con beta-dominio. La familia del beta-dominio ha sido relacionada con un amplio rango de proteínas de matriz extracelular truncadas análogas a MHC-II.

Linezolid y tigeciclina mostraron un efecto sobre la síntesis de proteínas, observado por el aumento estadísticamente significativo de la expresión de RplC (50S L3) (**Figura 3a – b**). Oxacilina mostró un efecto sobre la síntesis de la pared celular, ya que la expresión de FtsZ y su regulador negativo EzrA (**Figura 3c**) estaba disminuida, y proteínas como DltD o MurF (**Tabla 2**) no fueron observadas.

Los inhibidores de la síntesis de proteínas, linezolid y tigeciclina, fueron mejores inhibidores de la expresión de proteínas implicadas en la virulencia, y los únicos antibióticos con efecto sobre las serinproteasas, toxinas y hemolisinas (**Figura 3**, **Tabla 2**), las cuales desempeñan un importante papel en la patogénesis de *S. aureus*. Tigeciclina además disminuyó la expresión del principal factor de virulencia de *S. aureus* Hla/Hly y la controvertida toxina PVL[255], por disminución de la expresión de la subunidad LukS-PV (**Figura 3**). Por el contrario, oxacilina aumentó significativamente la expresión del principal factor de virulencia de USA300, LukF-PV (PVL)[291] (**Figura 3c**), y de forma inesperada, PsmA1, otro factor de virulencia implicado además en la formación de biopelículas (**Tabla 1**). Por lo tanto, hubo un incremento en la expresión diferencial de proteínas implicadas en la respuesta al estrés incluyendo la formación de biopelículas, la cual fue superior con tigeciclina y oxacilina, probablemente debido al aumento de la presión llevado a cabo por estos antibióticos (**Tabla 1**).

Linezolid incrementó la expresión de MecA (**Figure 3a**) y PBP3, ambas proteínas están implicadas en la resistencia a todos los β-lactámicos, por lo que esto podría indicar que linezolid confiere una mayor resistencia a esta familia de antibióticos, al menos con esta sub-concentración. Oxacilina incrementó la expresión de RpoB[256] (**Figura 3c**), por lo tanto la resistencia a glucopéptidos. Tigeciclina disminuyó la resistencia a los aminoglucósidos por la significativa menor expresión de AphA (3 ‘) III (**Figura 3b**).

Perfil proteómico de USA300 bajo cada concentración subinhibitorias:

Linezolid: El efecto de linezolid fue dosis-dependiente, probablemente en relación a las condiciones de estrés y densidad celular que actuaron de manera diferente a través de proteínas reguladoras como AgrC (*agr*-locus). La 0.25sub-CMI fue pobremente eficaz, sin embargo cuando se utilizó la 0.5sub-CMI la síntesis de proteínas fue inhibida, y la expresión de los principales factores de virulencia como PVL y Hla[255] fue menor, respecto al control y 0.25sub-CMI. La 0.5sub-CMI disminuyó la expresión de otros factores de virulencia implicados en la patogénesis, como enterotoxinas, serinproteasas y hemolisinas.

Tigeciclina: Ambas sub-concentraciones de tigeciclina mostraron ser efectivas, e inhibir la síntesis de proteínas. La fuerte reducción del proteoma de USA300 afectó

principalmente a proteínas ribosomales o sistemas reguladores como AgrA/C, entre otros. Como consecuencia, los principales factores de virulencia implicados en la patogénesis de USA300, Hla y PVL,[255] estaban menos expresados. Además, consideramos que tigeciclina podría tener un efecto bactericida[246] debido a, i) la disminución en 2 log10 del recuento de colonias (UFC/ml), ii) la menor expresión de proteínas implicadas en la biosíntesis de la pared celular y homeostasis, iii) la mayor expresión de proteínas implicadas en la formación de biopelículas, lo que ayuda a la bacteria a escapar de la muerte inducida por antibióticos,[260] y iv) la menor expresión de sistemas de regulación como WalK/R-TCS, o vi) la menor expresión de sistemas de reparación del ADN (sistema UvrA/B SOS).

Oxacilina: Ambas sub-concentraciones provocaron una lisis de la pared celular, lo que fue interpretado como sub-concentraciones efectivas frente a USA300. Este efecto fue dosis-dependiente. El efecto de 0.5sub-CMI fue superior, incluyendo el aumento significativo de la expresión de PVL[291] y PSMs, principales factores de virulencia de USA300. Sin embargo, no hubo un aumento en la resistencia a β-lactámicos. Esto no quiere decir que no pueda existir una adaptación y selección de alto nivel o resistencia homotípica (HoR-SARM),[261,262] lo que significaría una total no efectividad de la oxacilina, puesto que si hubo una mayor actividad del ciclo tricorbaxílico o producción de acetil-CoA,[261,262] ambos hechos vinculados con la supervivencia en presencia de antibióticos β-lactámicos.[261,262]

Vancomicina: Las sub-CIMs de vancomicina fueron poco efectivas. La diana terapéutica de la vancomicina mostró pocos cambios, y sólo la dosis más alta de 0.5sub-CMI, mostró algún efecto sobre la división celular, aumentando la expresión de SepF. Las sub-CMI de vancomicina no tuvieron efecto en los factores de virulencia, ni sobre la respuesta al estrés.

Conclusión

S. aureus tiene una notable habilidad para adaptarse a diferentes condiciones, ambientes, hospedadores, medios de cultivo, etc., con la finalidad de mejorar su capacidad de supervivencia. Esta adaptación se realiza mediante la alteración o redireccionamiento de su metabolismo central, de acuerdo a las condiciones del medio e incluso fase de crecimiento. Esta adaptación es controlada y dirigida por sistemas regulatorios de dos componentes, como el "quórum sensing", o también mediante la carga de la superficie bacteriana. Todo ello contribuye a esa respuesta de supervivencia frente al estres oxidativo y presión antibiótica. Nuestras observaciones, al igual que otros estudios

recientes, apoyarían que las proteínas Clp puedan contribuir a esta adaptación a la presión antibiótica. El mejor conocimiento de esta interesante maquinaria proteolítica haría un mejor entendimiento sobre la patogenicidad de USA300 y mejoraría el tratamiento de las infecciones estafilocócicas.

Esta respuesta a la presión antibiótica se caracterizó por una mayor expresión de proteínas involucradas en la formación de biopelículas, especialmente con tigeciclina y oxacilina. Las sub-concentraciones más altas (0.5sub-MICs) fueron más efectivas, pero sólo los inhibidores de la síntesis proteica, linezolid y tigeciclina inhibieron la virulencia. Linezolid fue efectivo, en una manera dosis-dependiente, contra al clon USA300, y aunque tuvo pocos efectos sobre los principales factores de virulencia, PVL o Hla/Hly, inhibió otros factores de virulencia implicados en la patogénesis, especialmente la 0.5sub-CMI la cual podría ser eficaz en infecciones sistémicas. La tigeciclina fue efectiva utilizando ambas concentraciones subinhibitorias, y los principales factores de virulencia implicados en la patogénesis, Hla y PVL, estuvieron menos expresados, así como la resistencia a aminoglucósidos. Las sub-CIMs de oxacilina fueron efectivas contra USA300 en una forma dosis-dependiente, sin embargo, la mayor expresión de los principales factores de virulencia implicados en la patogénesis, como PSM y Luk-F (PVL), más que la posible selección de subpoblaciones homo-resistentes, desaconsejaría su uso clínico. La vancomicina no tuvo efecto sobre los factores de virulencia y en general fue menos efectiva. Este estudio principalmente proporciona un mejor conocimiento de la respuesta celular de USA300 a diferentes sub-concentraciones de antibióticos de uso clínico.

Abreviaturas:

-AC: Adquirido en la Comunidad
CMI: Concentración Mínima Inhibitoria
ES /MS Espectrofotometría de Masas
Hla / Hly: α-haemolisina
LFQ: "Label Free Quantification": Cuantificación sin marcaje
N- : Normalizado
PBP: Proteínas Fijadoras de Penicilina
PBS: Buffer de Fosfato Salino
PVL: Panton Valentine Leucocidina
SARM: *Staphylococcus aureus* Resistente a Meticilina
SDS: Sodio-Dodecil-Sulfato
TCS: "Two-Component regulatory System": Sistemas regulatorios de dos componentes
TSB: Tripticase Soja caldo de cultivo
UFC: Unidades Formadoras de Colonia

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ABSTRACTS

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«Lo peor no es cometer un error, sino tratar de justificarlo, en vez de aprovecharlo como aviso providencial de nuestra ligereza o ignorancia"

«Se ha dicho hartas veces que el problema de España es un problema de cultura. Urge, en efecto, si queremos incorporarnos a los pueblos civilizados, cultivar intensamente los yermos de nuestra tierra y de nuestro cerebro, salvando para la prosperidad y enaltecimiento patrios, todos los ríos que se pierden en el mar y todos los talentos que se pierden en la ignorancia».

«Me reservo el derecho a pensar de acuerdo con mis ideas actuales».

"The worst is not making a mistake, is to try justifying it, rather than use it as providential notice of our levity or ignorance"

"It has been said a lot of time that the problem of Spain is a cultural problem. Urge, indeed, if we incorporate us to civilized peoples, cultivate intensively the wilderness of our land and of our brain, saving for prosperity and patriotic exaltation, all rivers are lost at sea and all the talents that are lost in the ignorance. "

"I reserve the right to think according to my current ideas."

Santiago Ramón y Cajal

Nobel Prize of Medicine 1906

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