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# *Staphylococcus aureus* virulence factors and disease

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*Staphylococcus aureus* is a major cause of nosocomial infections worldwide, especially methicillin-resistant *S. aureus*. Patients subjected to broad-spectrum antibiotics and immunosuppressive therapies have higher risk of infection by this microorganism.

*S. aureus* infection are often extremely difficult to treat due to the large population heterogeneity, phenotypic switching, intra-strain diversity, hypermutability and most importantly the small colony variants.

It is very important to emphasise that host immune responses against persistent infections by *S. aureus* is insufficient resulting normally into chronic infections, which in turn can lead to life threatening situations.

So, throughout this chapter we will focus on the principal aspects of *S. aureus* virulence will be focused.

## 1. Microbiology

### 1.1. The *Staphylococcus* genus

The genus *Staphylococcus* is composed of Gram-positive bacteria with diameters of 0.5-1.5 µm, characterized by individual cocci that divide in more than one plane to form grape-like clusters [1]. These bacteria are non-motile, non-spore forming facultative anaerobes, featuring a complex nutritional requirement for growth [2-4], a low G+C content of DNA (in the range of 30-40 mol%) [5], a tolerance to high concentrations of salt [2] and resistance to heat [6].

The genus *Staphylococcus* is traditionally divided in two groups based on the bacteria ability to produce coagulase, an enzyme that causes blood clotting: the coagulase-positive staphylococci, which includes the most known species *Staphylococcus aureus*; and the coagulase-negative staphylococci (CoNS), which are common commensals of the skin [5,7].

### 1.2. *Staphylococcus aureus*

*S. aureus* is the most pathogenic specie of the genus *Staphylococcus*, being implicated in both community-acquired and nosocomial infections. It often asymptomatically colonizes the skin and mucous membranes of healthy individuals, in particular the anterior nares [8-10]. In effect, it has been estimated that about 20-30 % of the population are permanently colonized by this bacterium, while other 30 % are transient carriers [10,11]. This colonization represents an increased risk of infection by providing a reservoir from which bacteria are introduced when the host defense is compromised [12]. Due to the importance of *S. aureus* infections and the increasing prevalence of antibiotic-resistant strains, this bacterium has become the most studied staphylococcal species.

The name *aureus* refers to the fact that colonies formed on solid rich media have a golden color, caused by the presence of carotenoids, in opposition to the pale, translucent, white colonies formed by CoNS [13,14].

### 1.3. Expression of virulence determinants in *S. aureus*

*S. aureus* is known for its capacity to cause a broad range of important infections in humans. Such capacity is related to the expression of an array of factors that participate in pathogenesis of infection, allowing this bacterium to adhere to surfaces/tissues, avoid or invade the immune system, and cause harmful toxic effects to the host [15-17]. These factors are known as virulence determinants (Table 1), and can be divided into cell-surface-associated (adherence) and secreted (exotoxins) factors.

#### *Cell surface factors*

*S. aureus* expresses several cell surface factors that play a role in its virulence. These include microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), capsular polysaccharides, and staphyloxanthin (carotenoid pigment) [18].


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**Table 1** Virulence factors involved in the pathogenesis of *Staphylococcus aureus* and respective putative functions.

| VIRULENCE FACTOR | PUTATIVE FUNCTION |
|---|---|
| CELL SURFACE FACTORS | |
| *Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)* | |
| Staphylococcal protein A (SpA) | Bind to IgG, interfering with opsinization and phagocytosis |
| Fibronectin-binding proteins (FnbpA and FnbpB) | Attachment to fibronectin and plasma clot |
| Collagen-binding protein | Adherence to collagenous tissues and cartilage |
| Clumping factor proteins (ClfA and ClfB) | Mediate clumping and adherence to fibrinogen in the presence of fibronectin |
| *Capsular polysaccharides* | Reduce phagocytosis by neutrophils; enhance bacterial colonization and persistence on mucosal surfaces |
| *Staphyloxanthin* | Resistance to neutrophil reactive oxidant-based phagocytosis |
| SECRETED FACTORS | |
| *Superantigens* | |
| Staphylococcal enterotoxins (SEA, B, C, D, E, G and Q) | Massive activation of T cells and antibody presenting cells |
| Toxic shock syndrome toxin-1 (TSST-1) | Massive activation of T cells and antibody presenting cells |
| *Cytolytic toxins* | |
| Cytolysins | |
| α-hemolysin | Induce lysis on a wide spectrum of cells, mainly platelets and monocytes |
| β-hemolysin | Hydrolysis of sphingomyelin of the plasmatic membrane of monocytes, erythrocytes, neutrophils and lymphocytes; make cells susceptible to other lytic agents |
| γ-hemolysin | Induce lysis on erythrocytes and leukocytes |
| Leukocidin family | |
| Leukocidins E/D and M/F-PV | Induce lysis on leukocytes |
| Panton-Valentine leukocidin (PVL) | Induce lysis on leukocytes |
| *Various exoenzymes* | |
| Lipases | Inactivate fatty acids |
| Nucleases | Cleave nucleic acids |
| Proteases | |
| Serine (e.g. exfoliative toxins ETA and ETB) | Inactivate neutrophil activity; activate T cells (only ETA and ETB) |
| Cysteine (e.g. staphopain) | Block neutrophil activation and chemotaxis |
| Aureolysin | Inactivate antimicrobial peptides |
| Hyaluronidase | Degrade hyaluronic acid |
| Staphylokinase (SAK) | Activate plasminogen; inactivate antimicrobial peptides |
| *Miscellaneous proteins* | |
| Staphylococcal complement inhibitor (SCIN) | Inhibit complement activation |
| Extracellular fibrinogen binding protein (Efb) | Inhibit complement activation |
| Chemotaxis inhibitory protein of *S. aureus* (CHIPS) | Inhibit chemotaxis and activation of neutrophils |
| Formyl peptide receptor-like 1 inhibitory protein (FLIPr) | Inhibit chemotaxis of neutrophils |
| Extracellular adherence protein (Eap) | Inhibit neutrophil migration |

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*Secreted factors (exotoxins)*

One important feature of *S. aureus* is the ability to secrete toxins that, in contrast to the protective and passive role of the cell-wall associated virulence factors mentioned above, play active roles in disarming host immunity. Indeed, they disrupt host cells and tissues and interfere with the host immune system to release nutrients and facilitate bacteria dissemination [18,19]. These secreted factors can be divided into four categories: superantigens, cytolytic (pore-forming) toxins, various exoenzymes and miscellaneous proteins [18].

*Superantigens*

Superantigens are a group of powerful secreted immune-stimulatory proteins capable of inducing a variety of human diseases, including toxic shock syndrome (TSS).

*Cytolytic (pore-forming) toxins*

*S. aureus* secretes a large number of cytolytic toxins that, although structurally diverse and with different target specificity, share a similar function on host cells. These toxins form β-barrel pores in the cytoplasmic membranes of target cells and cause leakage of the cell's content (when at low doses) and cell lysis (at high doses) [18,19].

*Various exoenzymes*

Nearly all strains of *S. aureus* secrete several extracellular enzymes whose function is thought to be the disruption of host tissues and/or inactivation of host antimicrobial mechanisms (e.g. lipids, defensins, antibodies and complement mediators) to acquire nutrients for bacterial growth and facilitate bacterial dissemination [17,18]. These exoenzymes include lipases, nucleases, proteases (serine, cysteine (e.g. staphopain), aureolysin), hyaluronidase, and staphylokinase (SAK) [17,18,20].

*Miscellaneous proteins*

*S. aureus* has also other specific proteins that can have a profound impact on the innate and adaptative immune system. These proteins include staphylococcal complement inhibitor (SCIN) [21], extracellular fibrinogen binding protein (Efb) [22,23], chemotaxis inhibitory protein of *S. aureus* (CHIPS) [19], formyl peptide receptor-like-1 inhibitory protein (FLIPr) [24,25], and extracellular adherence protein (Eap) [26].

### 1.4. Regulatory mechanisms of virulence determinants in *S. aureus*

The diverse array of cell wall and extracellular components involved in *S. aureus* virulence implies that the pathogenicity of this bacterium is a complex process requiring the tightly coordinated expression of these factors during different stages of infection (i.e. colonization, avoidance of host defense, growth and cell division, and bacterial spread) [27,28].

Indeed, the regulation of the virulence genes in *S. aureus* appears to follow a strategy that begins with the establishment of the bacterium in the host, followed by the attack of its defenses. For this, *S. aureus* begins by up-regulating the expression of genes coding for surface proteins involved in adhesion and defense against the host immune system; and only late in infection it starts to up-regulate the production of toxins that facilitate tissue spread [29-31].

To control the production of the virulence determinants during infection, *S. aureus* has several regulatory systems that respond to bacterial cell density (quorum-sensing) and environmental cues (e.g. nutrient availability, temperature, pH, osmolarity, and oxygen tension) [28,32-34]. These systems can be divided into two broad categories: two-component signal transduction systems and global transcriptional regulators [31,35].

*Two-component regulatory systems*

The two-component regulatory systems in *S. aureus* include the accessory gene regulator (*agr*) [36] and the staphylococcal accessory element (*sae*) [37].

The *agr* locus regulates more than 70 genes, of which 23 are related to virulence [38]. It is responsible for up-regulating the expression of many exoproteins (e.g. α-hemolysin, serine proteinase, TSST-1, enterotoxins, and proteases), and down-regulating the synthesis of cell wall-associated proteins (e.g. FnbpA, FnbpB, and SpA) [30,33,39,40].

The *sae* locus codes for another two-component system that regulates the expression of many virulence factors involved in bacterial adhesion, toxicity and immune evasion [41]. This includes the up-regulation of α-, β- and γ-hemolysins [42,43] and the down-regulation of SpA [44].

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*Global regulatory systems*

Several global regulatory systems have been identified in *S. aureus*, including the staphylococcal accessory regulator A (*sarA*) [45,46] and its several homologues [47,48].

*sarA* up-regulates the expression of some virulence factors (e.g. Fnbps, α- and β-hemolysins) and down-regulates others (e.g. SpA and proteases) [49,50]. Additional genes with homology to *sarA* have been described, including *sarS* [51] and *sarT* [52].

The regulation of virulence determinants may also involve sigma factors (σ), which are proteins that bind to the core RNA polymerase to form the holoenzyme that binds to specific promoters [52,53]. *S. aureus* have two sigma factors: the primary sigma factor, $\sigma^A$, which is responsible for the expression of housekeeping genes essential for growth [54]; and the alternative sigma factor $\sigma^B$, which regulates the expression of different genes involved in cellular functions (e.g. stress response) [55] and at least 30 virulence genes [56,57]. It up-regulates capsule, FnbpA and coagulase, and down-regulates hemolysins and serine protease A [38,58,59].

All the above mentioned regulators do not exert their influence singly; instead they form an interactive regulatory network to ensure that specific virulence genes are expressed only when required.

## 2. Population diversity/heterogeneity

Bacteria within the human host undergo genetic, morphological and physiological changes to survive for long periods of time under the challenging selective pressure imposed by the immune system and antibiotic treatments. As abovementioned, *S. aureus* is a successful human pathogen as it carries virulence machinery that turns it able to colonize host tissues and evade immune responses. *S. aureus* can survive and adapt to human environment trigging predetermined sensor-effect regulatory circuits as the classical response to stress by the regulation of gene expression through sigma factors ($\sigma^A$ and $\sigma^B$) [60]. However, this gene regulation is insufficient to face unpredictable stresses. Therefore, bacteria use alternative mechanisms, such as generation of microbial heterogeneity in a population.

The production of microbial diversity generates several variants that some of them are “fitter” and thus better adapted to a new environment than the other members of the population [61-64]. By this way, bacteria ensure the survival of the population, maintaining or enhancing their functioning against environmental fluctuations and, consequently, the infection persistence.

Clinically, chronic and exacerbations of staphylococcal infections have been associated with altered phenotypes. *S. aureus* might create phenotypic variants through mutations. The occurrence of mutations is frequently associated with antibiotic resistance. However, irreversible mutations represent a fitness cost to bacteria in the absence of the antibiotic. The evolution of fitness-compensatory mechanisms favoured the selection of reversible stress-resistance mechanisms such as phenotypic switching.

*Phenotypic Switching*

Phenotypic switching consists in a reversible conversion of phenotypic states according environmental changes, analogue to a mechanism ON/OFF. Although bacteria exhibit one of the phenotypic states, they retain the possibility to switch again, if advantageous, when new environmental stimuli occur, or switch back to the previous phenotype state when the external stressor, that had provoked the switching, vanishes [65]. Stress-inducible mechanisms as phenotypic switching can greatly accelerate the adaptive evolution of bacteria and are of serious concern. In contrast to DNA replication or transcription, a general stress-inducible mechanism does not exist, but just similarities and differences among a series of this kind of mechanisms. This means that there are not available specific target molecules to antimicrobial agents block these stress-inducible mechanisms but just some probable active components since the activation of those mechanisms is highly dependent of environment stresses. In addition, those processes might have impact on antibiotic susceptibility and virulence factors expression [65].

*Small Colony Variant*

The heterogeneity of *S. aureus* population is frequently analysed regarding antibiotic resistance, in particular, the detection of small colony variant (SCV). SCV are frequently isolated from patients with cystic fibrosis, chronic infections and device-associated infections [66,67]. SCV designation comes from their small-colony size, typically 10 times smaller than the usual size of *S. aureus* colonies, after 24-48 h of growth on agar media [66,67]. These colony variants are normally hyperpiliated, hyperadherent, mostly auxotrophic for either menadione or hemin, excellent biofilm formers and exhibit autoaggregative behaviour [66]. In addition, SCV display augmented resistance to several classes of antibiotics [66,68], as well as changed virulence factor expression [69], contributing to their persistence in the human body. Its phenotypic abnormality arises from deficiency in electron transport due to their single or multiple auxotrophism. The inability to synthesize hemin, thiamine, menadione or thymidine affects the electron transport and, consequently, their growth [66,70]. The electron transport chain produces ATP that is important for many metabolic processes in bacteria, including cell wall biosynthesis, amino acid transport and protein synthesis. Its disruption causes

impaired ability to grow and therefore colonies are of small size. In contrast to the majority of in vitro SCV generated, SCV recovered from clinical specimens are frequently instable and reverse to normal phenotype under nutritious growth conditions.

SCV represents the major challenge concerning disease management. The intracellular uptake of *S. aureus* by non-professional phagocytes, such as endothelial, epithelial cells, fibroblasts and osteoblasts confers protection from antibiotics and host immune defences. The intracellular location can trigger the conversion to SCV and is usually associated with the persistence of *S. aureus* infections [66,71]. This feature has been challenged the microbial diagnosis and therapy design to control or eradicate *S. aureus* infections.

## 3. Antimicrobial Resistance and Molecular Epidemiological Aspects

To all the virulence factors described earlier it is important to mention that a key factor for the success of *S. aureus* as a pathogen is its remarkable capacity to acquire antibiotic resistance [72,73]. Therefore, from a clinical point of view, the major problem that physicians have to face when treating *S. aureus* infections is antibiotic resistance, due to the likelihood of therapeutic failure and consequently poor prognostic [73].

### *Antimicrobial Resistance Overview*

The resistance to the first antibiotic, penicillin, emerged in 1942, only a few years after its introduction into the clinical practice [73-75]. Penicillin-resistant strains soon began to cause community infections, and by the early 1950s, they had become pandemic [76,77]. Since 1960, around 80% of all *S. aureus* strains were resistant to penicillin [78]. These strains produce a plasmid-encoded penicillinase, which hydrolyses the β-lactam ring of penicillin deactivating the molecule's antibacterial properties [76].

To treat infections caused by penicillin-resistant *S. aureus*, a semi-synthetic antibiotic methicillin, which is derived from penicillin, but resistant to β-lactamase inactivation, was introduced in 1959 [78]. However, in 1961 there were reports from the United Kingdom that *S. aureus* isolates had acquired resistance to methicillin (MRSA, methicillin-resistant *S. aureus*) and MRSA isolates were soon recovered from other European countries, and later from Japan, Australia, United States, and now, it is endemic in various hospitals worldwide, mainly in developing countries [76,79-81].

### *Molecular Epidemiological Aspects*

Methicillin resistance is associated with acquisition of a large transmissible genetic element known as staphylococcal cassette chromosome *mec* (SCC*mec*). The SCC*mec* contains two essential components: the *mec* gene complex and the *ccr* gene complex. The *mec* gene complex consists of *mec*A, the regulatory genes and associated insertion sequences, and it is classified into six different classes: A, B, C1, C2, D and E. The *mec*A gene encodes a penicillin binding protein PBP2a, a transpeptidase with low affinity for β-lactams that replaces the wildtype penicillin binding protein and is directly responsible for resistance to methicillin and all other β-lactam antibiotics. The *ccr* gene complex consists of cassette chromosome recombinase (*ccr*) genes (*ccrC* or the pair of *ccrA* and *ccrB*) encoding recombinases mediating integration and excision of SCC*mec* into and from the chromosome and surrounding genes [82-84]. In addition to *ccr* and *mec* gene complexes, SCC*mec* contains some other genes and various other mobile genetic elements, i.e., insertion sequences (e.g. IS431), transposons (e.g. Tn554, ΨTn554 and Tn4001) and plasmids (e.g. pUB110, pI258 and pT181) that encode multiple resistance to different classes of antibiotics [73,76,85].

To date, eleven types of SCC*mec* have been assigned for *Staphylococcus aureus* based on the classes of the *mec* gene complex and the *ccr* gene types (I to XI) [85].

The first MRSA isolated, called as the archaic clone, harbored the staphylococcal chromosome cassette *mec* I (SCC*mec*I). This strain circulated in hospitals throughout Europe, but the rest of the world was almost unaffected and, in 1980s, for reasons that remain unclear, the archaic MRSA clone largely disappeared from European hospitals [76]. In the mid to late 1970s, new MRSA strains that contained the new SCC*mec* allotypes, SCC*mec*II and SCC*mec*III emerged, leading to a worldwide pandemic of MRSA [76,78,86,87]. For a long time MRSA infections were limited to hospitalized patients, but during the 1990s reports of community-associated MRSA (CA-MRSA) infections among healthy individuals, without well-established MRSA risk factors, began to be described and were soon shown to be associated with genetically distinct lineages of MRSA, apparently unrelated to existing healthcare-associated MRSA (HA-MRSA) strains [88].

Indeed, genetic differences are observed with respect to SCC*mec* type between hospital and community strains. SCC*mec* types I, II and III are characteristic HA-MRSA strains and cause resistance to multiple classes of antibiotics due to the additional drug resistance genes integrated into SCC*mec.* SCC*mec* types IV, V and VI are generally associated with CA-MRSA, which in most cases, does not contain additional antimicrobial resistance genes, and therefore, cause only β-lactam antibiotic resistance [78,88].

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In addition to genotypic differences between HA-MRSA and CA-MRSA, the strains affect distinct population and cause different clinical syndromes. CA-MRSA infections tend to occur in previously healthy children and young adults and have been linked to skin and soft-tissue infections and severe invasive infections, including necrotizing fasciitis, necrotizing pneumonia and sepsis. In contrast, HA-MRSA strains are isolated largely from older adults and people with weakened immune systems; residing in a long-term care facility; under antibiotic treatment; with invasive medical devices; with one or more comorbid conditions. HA-MRSA strains are common cause of pneumonia, bacteremia, and invasive infections [89].

Despite this epidemiological data, the increase of antimicrobial resistance in CA-MRSA strains and its spread to the hospital settings replacing traditional HA-MRSA strains make unfeasible the distinctions between HA-MRSA and CA-MRSA based only on clinical epidemiology and susceptibility becoming necessary the use of molecular tools such as PCR and sequence-based molecular methods to study and understanding about the epidemiology of this pathogen [88-91].

The evolution of sequence-based molecular methods for genotyping strains, particularly, the multilocus sequence typing (MLST) technology, has made possible to know the molecular epidemiology of *S. aureus.* The MLST is based on sequencing of well conserved evolutionarily genes (*housekeeping genes*) that allows the grouping of strains into clonal complexes. Almost nosocomial MRSA strains detected worldwide belong to five clonal complexes (CCs): 5, 8, 22, 30 and 45 [76,92].

The global distribution and impact of HA-MRSA infections led to the increased use of vancomycin, the last remaining antibiotic to which MRSA strains were reliably susceptible. This intensive selective pressure resulted in the emergence of vancomycin-intermediate *S. aureus* (VISA) strains, which are not inhibited *in vitro* at vancomycin concentrations below 4–8 μg/ml and vancomycin-resistant *S. aureus* (VRSA) strains, which are inhibited only at concentrations of 16 μg/ml or more [76,93-95].

The glycopeptides vancomycin and teicoplanin exert their antimicrobial effects by binding irreversibly to the terminal D-alanyl-D-alanine (D-Ala-D-Ala) of bacterial cell wall precursors, inhibiting the synthesis of the *S. aureus* cell wall. The reduced susceptibility to vancomycin in VISA strains is due to the synthesis of an unusually thickened cell wall containing dipeptides (D-Ala-D-Ala) capable of binding vancomycin, sequestering them effectively, thereby reducing availability of the drug for intracellular target molecules. The genetic basis for these cell wall alterations has not yet been determined. On the other hand, the vancomycin resistance in VRSA is due to the plasmid-mediated transfer of the *van*A gene cluster (*van*R, *van*S, *van*H, *van*A and *van*X) carried by the mobile genetic element Tn1546 from vancomycin-resistant enterococci (VRE). The *van*A cluster confers vancomycin resistance due to the synthesis of an alternative cell wall terminal peptide (D-Ala-D-Lac) with a reduced affinity for vancomycin that replaces the normal dipeptide D-Ala-D-Ala in peptidoglycan synthesis. Fortunately, these strains did not spread substantially, possibly due to increased fitness cost associated with high-level resistance to vancomycin [76,93].

## 4. Final Remarks

The epidemiology of *Staphylococcus aureus* is dynamic and has changed significantly over the years. The proven ability of *Staphylococcus aureus* to acquire resistance genes is a concern among physicians worldwide. The search for new therapeutic alternatives associated with policies to control antibiotic use and hospital-acquired infections guided by epidemiological surveillance studies should be constant habits among health professionals and hospitals as an alternative to minimize the problem.

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