# Analysis of the association between polymorphisms in intergenic regions of *Staphylococcus aureus* genes involved in biofilm formation and periprosthetic joint infections

Memoria presentada por

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para optar al grado de Doctor por la Universidad Pública de Navarra

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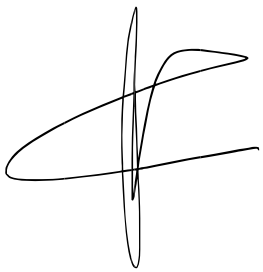

Fdo. Iñigo Lasa Uzcudun

Fdo. Cristina Solano Goñi

*A Mayoly*

*A mis padres*

*A mis hermanos*

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# Index

**Abbreviations**

| | |
|---|---|
| Ado: | adenosine |
| AIP: | autoinducing peptide |
| Amp: | ampicillin |
| AtlA: | autolysin A |
| Bap: | biofilm-associated surface protein |
| BIGSdb: | bacterial isolate genome sequence database |
| BSP: | bone sialoprotein |
| C1q: | complement protein |
| C4BP: | C4-binding protein |
| CC: | clonal complex |
| CCP20: | complement-control protein 20 |
| CFH: | complement factor H |
| CFI: | complement factor I |
| CFU: | colony-forming unit |
| Ck 10: | cytokeratin 10 |
| Cn1: | collagen 1 |
| Cna: | collagen-binding protein |
| CWA: | cell-wall-anchored |
| dAdo: | deoxyadenosine |
| Dsg1: | desmoglein 1 |
| Eap: | extracellular adhesion protein |
| Ebh: | extracellular matrix-binding protein homologue |
| ECM: | host extracellular matrix |
| eDNA: | extracellular DNA |
| Efb: | extracellular fibrinogen binding protein |
| Emp: | extracellular matrix binding protein |
| Ery: | erythromycin |
| Fg: | fibrinogen |
| Fg-α: | fibrinogen alpha chain |
| Fg-γ: | fibrinogen gamma chain |
| Fn: | fibronectin |
| FnBP: | fibronectin binding protein |
| GFP: | green fluorescent protein |
| Gp340: | glycoprotein 340 |
| Gp96: | glycoprotein 96 |
| GWAS: | genome-wide association studies |
| Hb: | hemoglobin |
| IGR: | intergenic region |
| Isd: | iron-regulated surface proteins |
| LPXTG: | Leu-Pro-X-Thr-Gly |
| mDa: | megadalton |
| MLST: | multi-locus sequence type system |
| MRSA: | methicillin-resistant *S. aureus* |

| | |
|---|---|
| MSCRAMMs: | microbial surface components recognizing adhesive matrix molecules |
| NCBI: | national center for biotechnology information |
| NEAT: | near iron transporter |
| NeuAc: | N-acetylneuraminic acid |
| ORF: | open reading frame |
| PIA/PNAG: | intercellular adhesin/poly-N-acetyl-glucosamine |
| PJI: | periprosthetic joint infection |
| PSMs: | phenol-soluble modulins |
| QS: | quorum sensing |
| Sas: | *Staphylococcus aureus* surface proteins |
| SigB: | sigma factor B |
| SNP: | single nucleotide polymorphism |
| SrtA: | sortase A |
| ST: | sequence type |
| TCS: | two-component system |
| TF: | transferrin |
| TNFR1: | tumor necrosis factor receptor 1 |
| TSB: | trypticase soy broth |
| TSB-gluc: | TSB supplemented with glucose 0.25% |
| TSB-NaCl: | TSB supplemented with NaCl 3% |
| UTR: | untranslated region |
| Vn: | vitronectin |
| vWbP: | von Willebrand factor binding protein |
| VWF: | von Willebrand factor |

# Summary

*Staphylococcus aureus* is a commensal bacterium that colonizes several body regions in healthy individuals. *S. aureus* is harmless in these locations, but it turns into an extremely threatening pathogen when it traverses the epithelial barrier and gains access to internal tissues. Due to its high capacity to sense and adapt to different environmental conditions, *S. aureus* has the ability to infect almost any organ and cause a broad spectrum of infections including abscesses, pneumonia, endocarditis, osteomyelitis, sepsis, and medical device-associated infections. A key success factor for *S. aureus* colonization of medical devices is its ability to attach to different surfaces and form a biofilm. The biofilm matrix provides the bacteria with the perfect environment to survive unharmed by the immune system and antibiotic treatment. Several elements in the *S. aureus* genome contribute to biofilm production. The polysaccharide PIA/PNAG, encoded by the *icaADBCR* locus, is a major compound of the biofilm matrix and promotes adhesion to the implant surface and cell-to-cell interactions. Surface adhesins can also promote adhesion of the bacteria to the naked surface of the medical device through non-specific interactions such as hydrophilic/hydrophobic forces. In addition, these adhesins play a relevant role once the prosthesis is implanted and coated with components of the host extracellular matrix (ECM), and plasma proteins. Interaction of bacterial surface adhesins with such components promotes *S. aureus* attachment to the implant surface.

It has been long suspected that some strains of *S. aureus* have a better ability to colonize medical implants. However, despite significant research efforts made by many laboratories, the molecular determinants that confer a

greater ability to colonize medical devices and cause infections on specific lineages remain unknown. Several tools for searching the connection between genetic features and phenotypes have been developed. Genome-wide association studies (GWAS) help to identify genes associated with a particular trait. When applied to bacteria, this method studies the entire set of DNA (the genome) of a large group of bacterial genomes, searching for small variations, called single nucleotide polymorphisms or SNPs. Despite the availability of complete genomes, most GWAS studies have been mainly centered on polymorphisms within coding regions whereas those variations that occur within non-coding sequences have remained mainly disregarded. Hence, the contribution of SNPs in the intergenic regions (IGRs) adjacent to genes important for biofilm development to the differences in the ability of clinical *S. aureus* isolates to colonize medical implants has not been explored. At least three reasons may explain the lack of these studies: i) the belief that mutations in IGRs are mostly neutral; ii) the presence of numerous SNPs in some IGRs which complicates the analysis of the contribution of each SNP to the phenotype, and iii) the requirement of experimental validation in order to demonstrate that specific SNPs in IGRs are associated with the implant colonization phenotype.

In this thesis, we have focused on studying variants found in IGRs adjacent to the most important genes involved in *S. aureus* biofilm formation; the *icaADBCR* locus, and the genes encoding the family of surface adhesins. For this purpose, we sequenced the whole genome of a collection of 71 *S. aureus* isolates from periprosthetic joint infections (PJI) and wound infections stored at

the Clinical Bacteriological Laboratory of the Sahlgrenska University Hospital and at the Culture Collection University of Gothenburg (CCUG), respectively.

In the **first chapter**, we explored the regulatory regions of the *icaADBCR* locus to identify patterns that might be associated with an increased capacity of the isolates to produce PIA/PNAG and form a biofilm. This study compared the regulatory regions of the *icaADBCR* locus in the genomes of PJI and wound isolates with those in the genome of the reference strain MW2. From these analyses, strains were grouped based on the SNPs found in the IGRs of the operon and also within the coding region of the transcriptional regulator IcaR. These regions showed high conservation rates, and no pattern associated with the origin of the isolates, either PJI or wounds, was detected. On the other hand, using transcriptional fusions between the regulatory region of the *icaADBCR* locus and the green fluorescent protein gene (*gfp*), we demonstrated that the expression of *icaADBC* genes was not affected by the presence of variations in IGRs. Notably, a SNP within the coding region of *icaR*, which results in an amino acid change in the transcriptional repressor IcaR V176E, led to a significant increase in the transcription of the *icaADBC* operon and the production of PIA/PNAG. Using a *Galleria mellonella* infection model, we were able to demonstrate a significant reduction in *S. aureus* virulence associated with the increase in PIA/PNAG production.

In the **second chapter**, we focused on analyzing the association between SNPs in the promoter regions of genes encoding adhesion-related proteins with adhesins expression levels and therefore, the ability of the strain to adhere to medical devices. Genome analyses of PJI and wound isolates showed different

profiles in the content of adhesin-encoding genes. Some of these, such as *sasG* and *cna*, were lineage-associated, and fifteen genes were present in the whole collection of strains. When the variability in the SNPs contained in regulatory regions that control the expression of each adhesin was investigated, different variation rates were found among the isolates. Following the same approach as in chapter I, based on transcriptional fusions between regulatory regions and the *gfp* gene, results showed that each genetic lineage contained a specific profile of adhesins expression under the same environmental condition. Moreover, we developed a biomaterial-associated murine infection model together with a metagenomic analysis to simultaneously compare the capacity of different *S. aureus* isolates to colonize medical implants.

In summary, our results evidenced that SNPs in the IGRs flanking the genes encoding factors important for biofilm development may contribute to the generation of variability in the capacity of *S. aureus* to colonize medical implants. In particular, our results revealed that IGRs controlling the expression of the *icaADBC* locus and production of the PIA/PNAG exopolysaccharide are highly conserved and that very few silent SNPs can be detected between strains. On the contrary, SNPs in the IGRs of genes encoding surface adhesins provide a profile of proteins expression that is specific for each *S. aureus* clonal complex (CC). Altogether, these studies emphasize the importance of investigating the potential impact of SNPs inside IGRs on gene expression and specific bacterial traits, such as pathogen colonization success.

# *Resumen*

*Staphylococcus aureus* es una bacteria comensal que se encuentra colonizando diferentes zonas del cuerpo en individuos sanos. La presencia de *S. aureus* en estas localizaciones es inofensiva, sin embargo, puede convertirse en un patógeno peligroso cuando atraviesa la barrera epitelial y accede a los tejidos internos. Debido a su gran capacidad para percibir y adaptarse a diferentes condiciones ambientales, *S. aureus* tiene la habilidad de infectar casi cualquier órgano y causar un amplio rango de infecciones, incluyendo abscesos, neumonía, endocarditis, osteomielitis, sepsis e infecciones asociadas a dispositivos médicos. Un factor clave para la colonización de dispositivos médicos por parte de *S. aureus* es su capacidad para adherirse a diferentes superficies y formar un biofilm. La matriz del biofilm proporciona a la bacteria el entorno perfecto para sobrevivir sin ser dañada por el sistema inmune y el tratamiento con antibióticos. Varios elementos en el genoma de *S. aureus* contribuyen a la producción y regulación del biofilm. En este sentido, el polisacárido PIA/PNAG, codificado por el locus *icaADBCR*, es uno de los principales componentes de la matriz del biofilm; promueve la adhesión a la superficie del implante y la interacción intercelular. Las adhesinas de superficie también pueden promover la adhesión de las bacterias a la superficie desnuda del dispositivo médico, a través de interacciones no específicas, como las fuerzas hidrofílicas/hidrofóbicas; además, estas adhesinas desempeñan un papel relevante una vez que la prótesis se implanta y se recubre con componentes de la matriz extracelular del huésped y proteínas presentes en el plasma. La interacción de las adhesinas de superficie bacterianas con dichos componentes favorece la adhesión de *S. aureus* a la superficie del implante.

Existe la sospecha de que ciertas cepas de *S. aureus* tienen una mayor capacidad para colonizar dispositivos médicos; sin embargo, a pesar de los importantes esfuerzos de investigación realizados por muchos laboratorios, siguen sin conocerse los determinantes moleculares que confieren a linajes específicos una mayor capacidad para colonizar dispositivos médicos y causar infecciones. Se han desarrollado diferentes herramientas para establecer la conexión entre los determinantes genéticos y el fenotipo. Los estudios de asociación de genoma completo (GWAS) permiten identificar genes asociados a una característica concreta. Cuando se aplica a las bacterias, este método estudia el conjunto de ADN (el genoma) de un gran grupo de genomas bacterianos, buscando pequeñas variaciones, llamadas polimorfismos de un solo nucleótido o SNPs. A pesar de la disponibilidad de genomas completos, la mayoría de los estudios de GWAS se centran en los polimorfismos dentro de las regiones codificantes, mientras que las variaciones que se producen dentro de las secuencias no codificantes son mayoritariamente ignoradas. Por lo tanto, la contribución de los SNPs en las regiones intergénicas (IGRs), adyacentes a genes importantes para el desarrollo del biofilm, a las diferencias en la capacidad de los aislados clínicos de *S. aureus* para colonizar los implantes médicos no ha sido explorada. Al menos tres razones podrían explicar la ausencia de estos estudios: i) la creencia de que las mutaciones en las IGRs son mayoritariamente neutras; ii) la presencia de numerosos SNPs en algunas IGRs que dificulta el análisis de la contribución de cada SNP al fenotipo, y iii) la necesidad de realizar una validación experimental para demostrar que SNPs específicos en las IGRs están asociados con el fenotipo de colonización de implantes.

Esta tesis se ha centrado en el estudio de las variantes encontradas en las IGRs adyacentes a los genes más importantes implicados en la formación de biofilms de *S. aureus*; el locus *icaADBCR*, y los genes que codifican adhesinas de superficie. Para ello, se ha secuenciado el genoma completo de una colección de 71 aislados de *S. aureus* procedentes de infecciones periprotésicas (IPP) e infecciones de heridas, almacenadas en el Laboratorio de Bacteriología Clínica del Hospital Universitario Sahlgrenska y en la Colección de Cultivos de la Universidad de Gotemburgo, respectivamente.

En el **primer capítulo** exploramos las regiones reguladoras del locus *icaADBCR* con el fin de identificar patrones que pudiesen estar asociados con una mayor capacidad de los aislados para producir PIA/PNAG y formar biofilm. Este estudio comparó las regiones reguladoras del locus *icaADBCR* en los genomas de los aislados de IPP y heridas con las del genoma de la cepa de referencia MW2. A partir de estos análisis, las cepas se agruparon en función de los SNPs encontrados en las IGRs del operón y también dentro de la región codificante del regulador transcripcional IcaR. Estas regiones mostraron altos índices de conservación y no se detectó ningún patrón asociado al origen de los aislados (IPP o heridas). Por otro lado, utilizando fusiones transcripcionales entre la región reguladora del locus *icaADBCR* y el gen de la proteína verde fluorescente (*gfp*), demostramos que la expresión de los genes *icaADBC* no se ve afectada por la presencia de variaciones en las IGRs. Por otra parte, un SNP dentro de la región codificante de *icaR*, que da lugar a un cambio de aminoácido en el represor transcripcional IcaR V176E, condujo a un aumento significativo de la transcripción del operón *icaADBC* y de la producción de PIA/PNAG.

Utilizando un modelo de infección de *Galleria mellonella*, pudimos demostrar una reducción significativa de la virulencia de *S. aureus* asociada al aumento de la producción de PIA/PNAG.

En el **segundo capítulo**, nos centramos en analizar la asociación entre los SNPs en las regiones promotoras de los genes que codifican proteínas relacionadas con la adhesión con los niveles de expresión de estas adhesinas y, por tanto, con la capacidad de la cepa para adherirse a dispositivos médicos. Los análisis del genoma de los aislados de IPP y de heridas mostraron diferentes perfiles en el contenido de genes que codifican las adhesinas. Algunos de ellos, como *sasG* y *cna*, se encontraron asociados a linajes específicos, mientras que quince genes estaban presentes en toda la colección de cepas. Asimismo, cuando se investigó la variabilidad de los SNPs contenidos en las regiones reguladoras que controlan la expresión de cada adhesina, se encontraron diferentes tasas de variación entre los aislados. Siguiendo el mismo enfoque del primer capítulo, basado en fusiones transcripcionales entre las regiones reguladoras y el gen *gfp*, los resultados demostraron que cada linaje genético contenía un perfil específico de expresión de adhesinas bajo la misma condición ambiental evaluada. Además, se desarrolló un modelo de infección murino asociado a biomateriales junto con un análisis metagenómico para comparar simultáneamente la capacidad de diferentes aislados de *S. aureus* para colonizar implantes médicos.

En resumen, nuestros resultados han demostrado que los SNPs en las IGRs que flanquean los genes que codifican factores importantes para el desarrollo del biofilm, pueden contribuir a generar variabilidad en la capacidad

de *S. aureus* para colonizar implantes médicos. En particular, nuestros resultados evidencian que las IGRs que controlan la expresión del locus *icaADBC* y la producción del exopolisacárido PIA/PNAG están muy conservadas y que se pueden detectar muy pocos SNPs silenciosos al comparar las distintas cepas. Por el contrario, los SNPs en las IGRs de los genes que codifican adhesinas de superficie proporcionan un perfil de expresión de proteínas que es específico para cada complejo clonal (CC) de *S. aureus*. En conjunto, estos estudios destacan la importancia de investigar el impacto potencial de los SNPs dentro de las IGRs en la expresión de genes y fenotipos bacterianos específicos, como puede ser la capacidad de colonización del patógeno.

# Introduction

## 1. *Staphylococcus aureus*

*Staphylococcus aureus* is a Gram-positive bacterium, a member of the class Bacilli and the family Micrococcaceae, observed under the microscope forming grape clusters, short chains, or tetrads (Cohen, 1986; Thiemann *et al.*, 2016). *S. aureus* colonizes the nasopharynx of approximately 30% of the healthy human population (Fitzgerald, 2014). If *S. aureus* passes through the epithelial barrier and reaches internal organs, it can cause a variety of diseases, ranging from minor skin infections, such as furuncles or boils to severe infections, such as bacteremia, pneumonia, osteomyelitis, or endocarditis (Cheung *et al.*, 2021; Thiemann *et al.*, 2016). Despite the progress achieved with the use of antibiotics in the treatment of bacterial infections over the last two decades, the number of infections due to this pathogen has increased (Gordon & Lowy, 2008; Wertheim *et al.*, 2005). In addition to its immense capacity to adapt to grow in different tissues under different environmental conditions, *S. aureus* is one of the bacteria that more often cause infections associated with medical implants. In this regard, the capacity of *S. aureus* to adhere to surfaces and form a biofilm is associated with the progress and origin of many primary infections (Donlan, 2001). A wide variety of virulence factors and biofilm-associated genes allow *S. aureus* to colonize, adhere to medical surfaces, evade the immune system, develop resistance to antimicrobials, and cause toxicity (Ahmadrajabi *et al.*, 2017). The regulation of the expression of these biofilm-associated genes is important for bacterial pathogenicity and leads to important challenges in the treatment and eradication of *S. aureus* infections (Bjarnsholt, 2013).

## 2. Bacterial biofilms

### 2.1 General definition

A microbial biofilm is a community of microorganisms embedded within an extracellular matrix, which grows attached to a biological or inert surface (Costerton *et al.*, 1995). Biofilms represent a common form of growth of microorganisms in nature, other than planktonic, which allows them to adapt to and resist hostile environmental conditions such as desiccation, ultraviolet radiation, and extreme temperatures (Yin *et al.*, 2019). Moreover, the physiological states inside the biofilm, which are induced by high cell density, accumulation of metabolic wastes, and lack of nutrients allow the bacteria to reduce their metabolic rate and growth in order to survive for long periods under environmental stress conditions (Hu & Coates, 2012).

### 2.2 Steps in biofilm formation

Biofilm formation is a multifactorial process that requires the participation of specific sensory systems and the transcription of gene clusters distinct from those expressed in the planktonic state of the same microorganism (Jamal *et al.*, 2018). In nature, biofilms are usually poly-microbial, and their formation process requires four major steps: first, i) initial adhesion of the bacteria to an alive or inert surface occurs. In this phase, planktonic bacteria bind to the surface through non-specific interactions. This process differs between Gram-negative and Gram-positive bacteria; in the former case, it involves the existence of appendages such as *pili* or flagella, while the latter mainly involves proteins with adhesin function found on the bacterium's

surface. Following the initial adhesion, physiological changes occur in the cells that lead to the ii) synthesis of the biofilm matrix. In this phase, adhesion becomes stable and irreversible. From this moment, iii) the biofilm maturation process begins whereby the cells continue dividing and producing the matrix, composed mainly of exopolysaccharides, proteins, and extracellular DNA (eDNA). The final composition of the biofilm matrix may vary depending on the strain, or environmental conditions. Finally, once there is a limitation of nutrients, iv) the separation and dissemination phase occurs, and cells disaggregate and disseminate to new sites in search of fresh nutrients (Costerton *et al.*, 1995; Flemming & Wingender, 2010; Otto, 2018). Figure 1 graphically depicts the stages of biofilm formation.

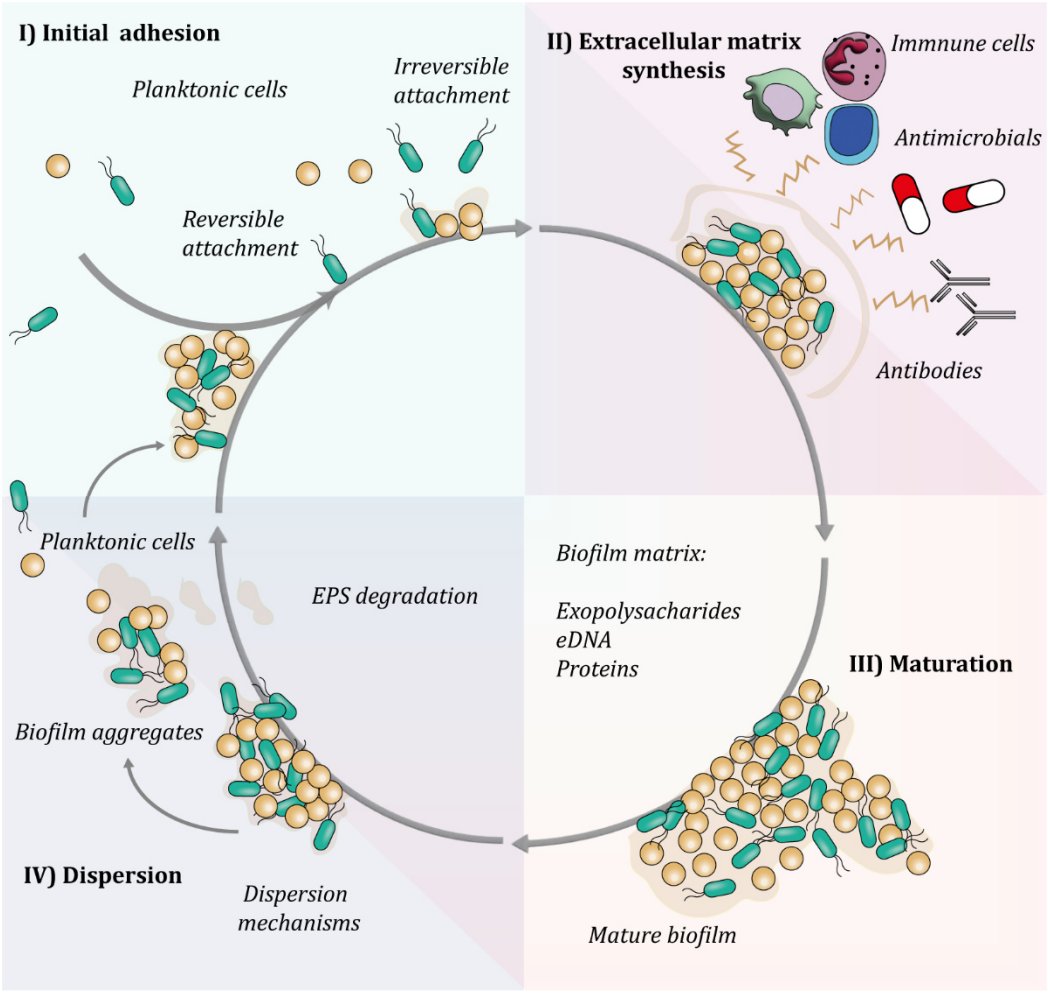



**Figure 1. Schematic representation of the stages during the biofilm formation cycle. (I)** Initial adhesion: planktonic state bacteria attach to the surface by either specific or unspecific interactions between bacterial structures and the naked surface, or the surface coated by the host extracellular matrix (ECM) and proteins present in the plasma. **(II)** Extracellular matrix synthesis: once the adhesion becomes irreversible, bacteria trigger matrix production, composed of exopolysaccharides, eDNA and proteins. **(III)** Maturation: aggregates keep forming and the biofilm matrix grows. **(IV)** Dispersion/detachment: once there is a limitation on nutrients, bacteria produce dispersion factors; the bacterial aggregates and planktonic cells move to other locations where the cycle continues. Figure adapted from (Koo *et al.*, 2018).

### 2.3 Clinical relevance of microbial biofilms

It is estimated that 65% of all infections caused by bacteria are related to biofilm formation (Jamal *et al.*, 2018). These can be primary, or secondary infections associated with the implantation of medical devices. The degree of severity of *S. aureus* biofilm-associated infections is determined by the location, the toxicity of the particular strain, differences in the composition of the biofilm matrix, and the immune response (Mohamed & Huang, 2007; Paharik & Horswill, 2016; Schinner *et al.*, 2020; Speziale & Geoghegan, 2015). Primary infections include oral infections, endocarditis, cystic fibrosis, osteomyelitis, wound infections, otitis media, and vaginitis (Chenicheri *et al.*, 2017; Machado & Cerca, 2015; Vestby *et al.*, 2020). Regarding medical device-associated infections, these account for 70% of nosocomial infections, and the formation of the biofilm on the surface of the implant represents the leading cause of implant failure (Bryers, 2008; Veerachamy *et al.*, 2014).

Biofilms can be formed on virtually any medical device, including catheters, heart valves, contact lenses, and orthopedic prostheses, among others (Bryers, 2008; Del Pozo, 2018; Elder *et al.*, 1995; Elgharably *et al.*, 2016; Jamal *et al.*, 2018; Pelling *et al.*, 2019; Tande & Patel, 2014). Biofilm-mediated infections tend to become chronic, reduce the benefits of the intervention, and often end with the removal of the implant (Malchau *et al.*, 2020).

## 3. *Staphylococcus aureus* as a leading cause of periprosthetic joint infection

Periprosthetic joint infections (PJI) are the most serious complication of arthroplasty (Izakovicova *et al.*, 2019). Many bacterial species can cause PJI. Amongst these, approximately 80% of orthopedic infections are generated by bacteria of the genus *Staphylococcus* spp., with *S. aureus* being the most frequent species causing PJI (Arciola, An, *et al.*, 2005; Veerachamy *et al.*, 2014).

The prevalence of PJI varies from 2 to 4%. However, because of factors such as the increase in primary procedures, the increase in life expectancy, the high prevalence of obesity, and the extension of surgical indications to younger patients in the coming years, an exponential growth in the number of arthroplasty procedures and likewise in the number of associated infections is expected (Izakovicova *et al.*, 2019; Patel *et al.*, 2015).

In Spain, the survey conducted through the PREVENCOT project, led by the Spanish Society of Orthopedic and Traumatology Surgery (SECOT), showed a strong adherence of medical staff to measures that prevent prosthetic infections. However, despite these efforts, the percentage of implanted prostheses that become infected remains relatively high (Castel-Oñate *et al.*, 2022). According to data provided by the Navarra Government, between 2016 and 2020, about 1500 arthroplasty procedures were performed annually in public health centers. These data include hip and knee arthroplasties, of which infections occurred in about 4% of the procedures. Table 1 summarizes this information.

**Table 1.** Arthroplasty procedures and associated infections between 2016-2020 in public health centers of Navarra (Spain).

| ***Number of medical procedures*** | | | | | |
|---|---|---|---|---|---|
| | **2016** | **2017** | **2018** | **2019** | **2020** |
| **Hip arthroplasty** | 805 | 853 | 898 | 807 | 649 |
| **Knee arthroplasty** | 732 | 717 | 732 | 687 | 529 |
| **Total** | 1537 | 1570 | 1630 | 1494 | 1178 |
| ***Number and percentage of associated infections*** | | | | | |
| | **2016** | **2017** | **2018** | **2019** | **2020** |
| **Hip arthroplasty** | 25 (3.1%) | 30 (3.5%) | 21 (2.3%) | 25 (3.1%) | 15 (2.3%) |
| **Knee arthroplasty** | 34 (4.6%) | 37 (5.2%) | 45 (6.1%) | 29 (4.2%) | 18 (3.4%) |
| **Total** | 59 (3.8%) | 67 (4.3%) | 66 (4.0%) | 54 (3.6%) | 33 (2.8%) |

Considering the aging of the population and the active lifestyle of the elderly, the forecasts for the future are that the number of implants will continue to increase and consequently, the number of infections associated with implants will also increase. The management of these infections is complex; it increases the cost of the treatment by up to twenty-four times, requires multiple revision surgeries and prolonged therapy with antimicrobials, and causes high morbidity, low joint functionality or disability, considerably deteriorating the life quality of patients and ultimately causing death (Hosny & Keenan, 2018; Izakovicova *et al.*, 2019). For this reason, medical device-associated infections represent an issue of great relevance in public health and every effort to

understand the molecular mechanisms that regulate biofilm on medical surfaces is welcomed.

## 4. Genetic determinants involved in *Staphylococcus aureus* PJI

A key step in the development of implant-associated infections is the initial adherence of bacteria to the implant surface (Veerachamy *et al.*, 2014). The orthopedic surgeon Anthony G. Gristina defined the process of implant colonization as a race between host eukaryotic cells and bacteria toward the implant surface, arguing that when host cells win the race for the surface, the probability of bacterial colonization is very low (Gristina *et al.*, 1988). Bacterial adhesion can occur on the naked surface before implantation, being this adhesion mediated by physicochemical properties of the bacterial and biomaterial surfaces. In addition, non-specific interactions of staphylococcal autolysins (AtlA) and other adhesins with the hydrophobic and electrostatic forces of the naked surface can play a fundamental role the initial attachment (Donlan, 2001; Montanaro *et al.*, 2011). Thus, biomaterial researchers are doing major efforts to improve the properties of biomaterials and prevent bacteria from binding to the surface (Donlan, 2001; Francolini & Donelli, 2010; Zander & Becker, 2018). Many of these biotechnological efforts focus on chemical and physical surface modifications to promote tissue integration and avoid bacterial adherence (Ficai & Ficai, 2017). However, modification of the surface properties is only effective before the implantation of the medical device. As soon as the implant is inserted, it becomes coated with plasma and host extracellular matrix proteins (ECM), such as vitronectin (Vn), fibrinogen (Fg), and fibronectin (Fn).

The interaction of the implant surface with these proteins changes the physicochemical characteristics of the surface, thus reducing the effectiveness of the anti-biofilm properties of the biomaterial surface (Rodes, 2001). At this moment, the interaction between the bacteria and the coated surface is established through the recognition of surface adhesins, from the bacterial cell wall and the ECM and plasma proteins coating the surface of the implants (Clarke & Foster, 2006; Foster, 2019a; Otto, 2018). Once bacteria are attached to the implant surface, they start producing the biofilm matrix. The biofilm matrix produced by *S. aureus* is composed of the PIA/PNAG exopolysaccharide (Cramton *et al.*, 1999; O'Gara, 2007), eDNA (Montanaro *et al.*, 2011), and proteins (Speziale *et al.*, 2014). However, completely PIA/PNAG-independent biofilms, with proteins mediating intercellular accumulation, have been described (Geoghegan *et al.*, 2010; Merino *et al.*, 2009; O'Neill *et al.*, 2008; Valle *et al.*, 2012; Vergara-Irigaray *et al.*, 2009).

Methicillin-resistant *S. aureus* (MRSA) strains are especially prone to produce a protein-mediated biofilm matrix (O'Neill *et al.*, 2008). In this respect, some studies describe the importance of proteins of the LPXTG family, such as SasG, the biofilm-associated surface protein (Bap), and fibronectin-binding proteins (FnBPs), as mediators of biofilm formation in *S. aureus* (Corrigan *et al.*, 2009; Cucarella *et al.*, 2001; Vergara-Irigaray *et al.*, 2009). In the following sections, specific determinants influencing *S. aureus* biofilm formation on medical surfaces are comprehensively described (summary in figure 2).

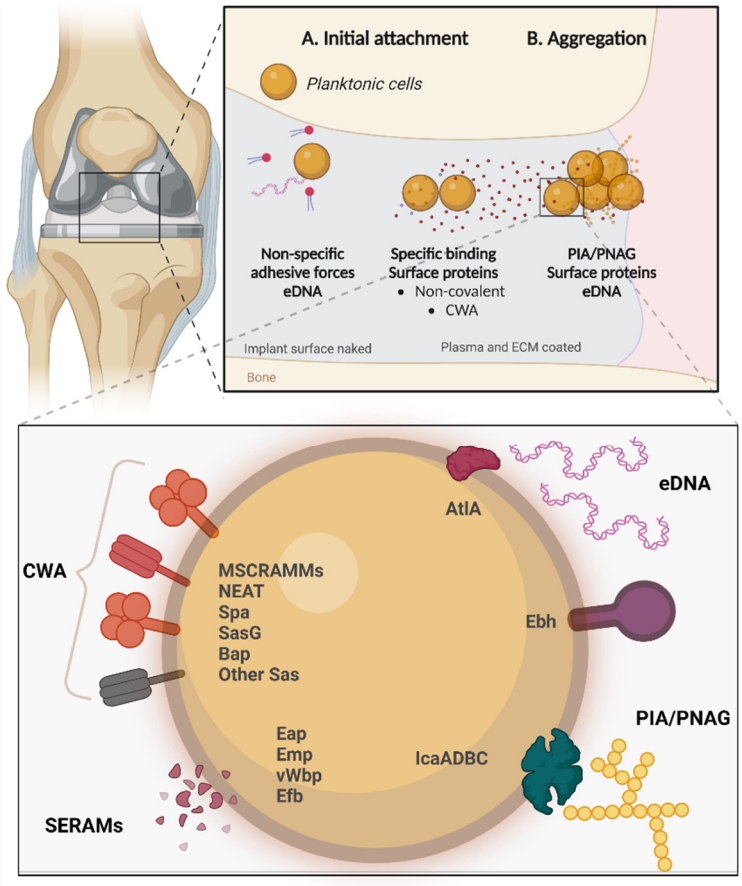



**Figure 2. Determinants of *S. aureus* colonization and biofilm formation on implanted prostheses. (A)** Initial attachment is mediated by cell-wall-anchored proteins (CWA) from the LPXTG family of proteins, or by non-covalent associated proteins (SERAMs, Ebh, and AtlA) that mediate the adhesion to both, specific ligands coating the surface and the naked surface. **(B)** Biofilm aggregation is mediated by major elements expressed by the bacteria depending on environmental cues and the genetic background: PIA/PNAG, encoded by the *icaADBC* locus, mediates intercellular adhesion and is the main component of the biofilm matrix; surface adhesins, including CWA and non-covalently anchored proteins (SERAMs and AtlA), also contribute to the accumulation of the biofilm matrix by promoting eDNA release and intercellular adhesion through specific or unspecific interactions. Created with Biorender.com.

### 4.1 Primary adhesion

*S. aureus* strains express a profile of adhesion-related proteins that are covalently anchored or non-covalently associated with the cell wall (Otto, 2018). Because of their exposition to the surface, these proteins interact with the host and have multiple functions, including binding to ECM and plasma proteins that coat the surface of implanted medical devices (Donlan, 2001; Paharik & Horswill, 2016).

#### 4.1.1 Cell-wall-anchored proteins

*S. aureus* can express several cell-wall-anchored proteins (CWA) (Foster, 2019a; Roche, Massey, *et al.*, 2003). Some of these proteins are highly conserved and present in all the *S. aureus* isolates whereas other proteins are only present in some strains and absent in others. Thus, the combination and the number of CWAs present in the cell wall of a *S. aureus* isolate is characteristic of each strain. Besides, the expression levels of the CWA proteins may also be different between strains and in the same strain depending on the growth conditions (limitation of nutrients such as iron, stationary or exponential phase) (Mazmanian *et al.*, 2001; Roche, Massey, *et al.*, 2003; Speziale *et al.*, 2014). Very often, CWA proteins have functional redundancy. One protein can recognize several ligands and different proteins can recognize the same ligand. This functional redundancy implies that when one protein is missing, others can compensate for its function (Foster *et al.*, 2014; Zapotoczna *et al.*, 2016). In some cases, the host cell ligand recognized by CWA proteins is not always known and therefore the function remains undefined.

The structure that characterizes CWA proteins is the existence of an LPXTG (Leu-Pro-X-Thr-Gly) recognition domain at the C-terminal extreme, which allows them to anchor covalently to the cell wall peptidoglycan via the sortase A (SrtA) activity; this recognition domain is followed by a secretory, Sec-dependent signal sequence, and a ligand-binding domain in the N-terminal region. Foster *et al.* classified the LPXTG family into five distinct groups of proteins based on the presence of motifs that structural and functional analyses have defined (Foster, 2019b, 2019a; Foster *et al.*, 2014) (Figure 3).

On the other hand, some confusion in CWA proteins' nomenclature exists. Some authors refer to them as to the first-assigned function, while in other cases proteins are named as *S. aureus* surface proteins (Sas) (Mazmanian *et al.*, 2001). To avoid confusion, in this thesis, we have adopted the Sas nomenclature. In the following paragraphs, we outline the understanding of these proteins according to their structural classification. Table 2 summarizes the different names ascribed to these proteins, their assigned functions, and known ligands.

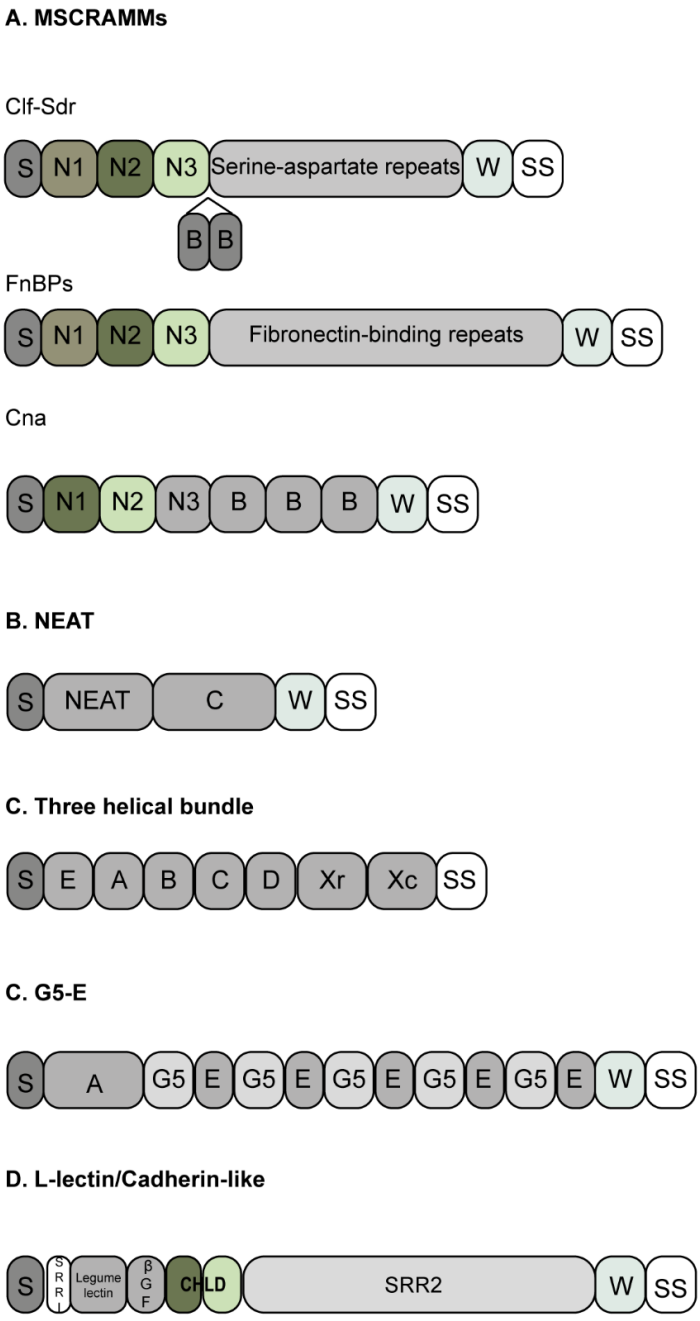



**Figure 3. Cell-wall-anchored proteins classification based on their structural motifs.** CWA proteins are classified into five groups according to their structure. **(A)** Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). **(B)** Near iron transporter (NEAT) motif protein family. **(C)** Three-helical bundle motif protein A. **(D)** G5-E repeat family. **(E)** Legume-lectin, cadherin-like domain protein. Figure adapted from (Foster, 2019a).

***MSCRAMMs***

The MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) family of proteins is the major group of proteins anchored to the *S. aureus* surface (Foster, 2019a). Their primary function is to bind to ECM proteins and the host cellular components, thus playing a key role in the process of adhesion to surfaces covered by these components (Foster & Höök, 1998).

All the proteins of the LPXTG family are included in this group because initially, this appellation was given to all those proteins that could bind to elements of the ECM and other ligands present in the plasma, such as Fg, Fn, and collagen (Patti *et al.*, 1994). However, on account of the proposed classification by Foster, and adopted for this thesis, the term MSCRAMMs should be restricted to adhesins characterized by containing at least two IgG-like folds and employing a ligand binding mechanism known as "dock, lock, and latch" (Foster *et al.*, 2014). These proteins are classified into three groups according to their architecture: i) the clumping factor (Clf)-serin aspartate repeat (Sdr) family, including the bone sialoprotein-binding protein (Bbp), an isoform of the SdrE protein (Tung *et al.*, 2000); ii) the FnBPs; iii) and the collagen-binding protein (Cna) (Figure 3a).

***NEAT domain motif family***

The NEAT (near iron transporter) membrane-anchored proteins include the iron-regulated surface proteins (Isd). Isd surface proteins contain one (SasE/IsdA), two (SasJ/IsdB), or three (SasI/IsdH) NEAT motifs, which allow them to bind to the heme group of hemoglobin, facilitating their transport into

the bacterial cell (Ellis-Guardiola *et al.*, 2021). These proteins have a role in colonization, adhesion, and promotion of biofilm formation under iron-limiting conditions (Clarke *et al.*, 2004; Corrigan *et al.*, 2009; Ellis-Guardiola *et al.*, 2021; Missineo *et al.*, 2014).



***Triple helix bundle***

To this group belongs the extensively investigated protein A (Spa). The structure of Spa consists of an Fc-partial-region, the X-region, and the C-terminal region (X. Wang, 2020). Spa is known for its ability to bind to the Fc region of IgG and inhibit opsonophagocytosis. It comprises five homologous IgG-binding domains that fold into a three-helix bundle. In addition, protein A can bind to the von Willebrand factor (VWF) and promote aggregation (Hartleib *et al.*, 2000). In this regard, previous studies in our group have described that besides cell aggregation, Spa promotes biofilm formation (Merino *et al.*, 2009).

***G5-E***

In *S. aureus*, SasG and the plasmin-sensitive protein (Pls) belong to this family and play a relevant role in adhesion to the nasal epithelium and biofilm formation (Roche, Meehan, *et al.*, 2003). SasG and Pls structures possess a G5 domain consisting of five glycine residues. The Pls protein is associated with the mobile genetic element *SCCmec* type I cassette, which confers resistance to β-lactam antibiotics. It is, therefore, only present in some MRSA strains. At the same time, the SasG protein, which is associated with biofilm formation and

aggregation, is carried only by certain isolates and its presence is lineage-dependent (Corrigan *et al.*, 2007; Geoghegan *et al.*, 2010).

### *L-lectin-like Cadherin-like*

The protein SasA/SrAp glycoprotein, which is characterized by containing serine-rich repeats, belongs to this group. It is involved in the pathogenesis of infective endocarditis through its ligand-binding region (BR) that promotes binding to human platelets (Yang *et al.*, 2014). In addition, this protein binds to the saliva agglutinin Gp340 via the N-acetylneuraminic acid moiety (NeuAc), which is present in many of the glycoproteins expressed in human tissues (Kukita *et al.*, 2013).

### *5′-nucleotidase*

Formerly named SasH, adenosine synthase A (AdsA) is an important virulence factor that facilitates immune evasion through the generation of adenosine (Ado) and deoxyadenosine (dAdo), which dampen pro-inflammatory immune responses and prevent macrophage infiltration of infected tissues, respectively (Soh *et al.*, 2020).

### *Biofilm-associated surface protein*

Bap was the first example of a surface protein capable of inducing biofilm development, described in an isolate that had caused a biofilm-associated mastitis (Cucarella *et al.*, 2001). The *bap* gene in *S. aureus* was initially identified

in a mobile pathogenicity island (SaPIbov2) that has never been found in human isolates of *S. aureus* (Úbeda *et al.*, 2003).

### *Other proteins of the LPXTG family*

In addition to those mentioned above, other Sas proteins have been identified (Mazmanian *et al.*, 2001), whose structure or function need further characterization. This group includes SasD and SasF containing a modified LPXTG motif, whereas proteins SasB, SasC, SasJ, Sask, SasL, and SasX contain the canonical LPXTG motif (Roche, Massey, *et al.*, 2003). SasC structure has been previously described; it contains a N-terminal motif that is found in various architectures (FIVAR; 54 aa, starting at N-590 and ending at D-643) (Schroeder et al., 2009), and is associated with bacterial primary attachment and biofilm accumulation; however, SasC does not bind to normal host components such as thrombospondin 1, VWF, or platelets, thus the specific ligand for SasC is still unknown (Heilmann, 2011; Schroeder *et al.*, 2009). In terms of the other Sas proteins, it is assumed that they interact with ECM proteins of the host to promote adhesion to the surface and intercellular adhesion between bacteria, but this has not been experimentally confirmed.

**Table 2.** *Staphylococcus aureus* cell-wall-anchored proteins[a].

| Subfamily | Protein | Gene | Ligand | Function | References |
|---|---|---|---|---|---|
| MSCRAMMs family | | | | | |
| Clf-Sdr | ClfA | *clfA* | Fg-γ, CFI, VWF-A1. | Adhesion, immune system evasion, and aggregation induction. Binding to the VWF A1 domain in the presence of vWbp. | (Claes *et al.*, 2017; Hair *et al.*, 2008; Herman-Bausier *et al.*, 2018; Viljoen *et al.*, 2021) |
| | ClfB | *clfB* | Fg-α, Ck 10, loricrin. | Adhesion to squamous epithelial cells and nasal colonization. | (Mulcahy *et al.*, 2012; O'Brien *et al.*, 2002; Walsh *et al.*, 2004, 2008) |
| | SdrC | *sdrC* | Neurexin-1. | Adhesion to squamous epithelial cells and nasal colonization. Aggregation and biofilm formation. | (Barbu *et al.*, 2010, 2014; Corrigan *et al.*, 2009; J. Wang *et al.*, 2021) |
| | SdrD | *sdrD* | $Ca^{2+}$, Dsg1. | Adhesion to the squamous epithelial cells. | (Askarian *et al.*, 2016; Corrigan *et al.*, 2009; Josefsson *et al.*, 1998) |
| | SdrE Bbp[b] | *sdrE bbp* | CFH–CCP20, C4BP, BSP, Fg-α. | Adhesion, colonization, and immune system evasion. | (Luo *et al.*, 2017; Sharp *et al.*, 2012; Tung *et al.*, 2000; Vazquez *et al.*, 2011; Zhang *et al.*, 2017) |
| FnBPs | FnBPA | *fnbA* | Fn, Fg, elastin. | Adhesion, colonization, internalization, endothelial invasion. Biofilm promotion. | (Gries *et al.*, 2020; Keane *et al.*, 2007; Piroth *et al.*, 2008) |
| | FnBPB | *fnbB* | Fn, elastin, loricrin. | | (da Costa *et al.*, 2022; Peacock *et al.*, 1999) |
| Cna | Cna | *cna* | Collagen, C1q. | Adhesion and colonization. | (Kang *et al.*, 2013; Zong *et al.*, 2005) |

[a] Adapted from (Foster, 2019a); [b] Allelic variant of SdrE.

**Table 2.** *Staphylococcus aureus* cell-wall-anchored proteins[a]. (cont.)

| Subfamily | Protein | Gene | Ligand | Function | References |
|---|---|---|---|---|---|
| NEAT | | | | | |
| | IsdA (SasE) | *isdA* | Fg, Fn, fetuin, Hb, TF, hemin, CK 10, loricrin. | Adhesion, iron absorption. Transport of the heme group of hemoglobin. | (Clarke *et al.*, 2004; Corrigan *et al.*, 2009; Ellis-Guardiola *et al.*, 2021) |
| | IsdB (SasJ) | *isdB* | Hb, hemin, Vn. | | (Ellis-Guardiola *et al.*, 2021; Pietrocola *et al.*, 2020) |
| | IsdC | *isdC* | Hemin. | Transport of the heme group of hemoglobin. | (Ellis-Guardiola *et al.*, 2021) |
| | IsdH (SasI) | *isdH* | Haptoglobin, Hb complex. | | (Ellis-Guardiola *et al.*, 2021) |
| Three-helix bundles | | | | | |
| | SpA | *spa* | IgG Fc, IgM, VWF, TNFR1. | Opsonophagocytosis inhibition, cell aggregation, and biofilm formation. | (Hartleib *et al.*, 2000; Merino *et al.*, 2009) |
| L-lectin-like Cadherin-like | | | | | |
| | SraP (SasA) | *sasA* | Gp340, Platelets. | Adhesion, colonization, and internalization. | (Kukita *et al.*, 2013) |
| G5-E | | | | | |
| | SasG | *sasG* | Epithelial cells, unknown receptor. | Adhesion, nasal colonization, and biofilm formation. | (Roche, Meehan, *et al.*, 2003) |
| | Pls | *pls* | Epithelial cells, cellular lipids. | Adhesion, nasal colonization, and biofilm formation. Strains carrying the *Sccmec* type I. | (Huesca *et al.*, 2002; Roche, Meehan, *et al.*, 2003) |
| FIVAR motif | | | | | |
| | SasC | *sasC* | Unknown. | Cell aggregation, biofilm formation, and colonization. | (Schroeder *et al.*, 2009) |

[a] Adapted from (Foster, 2019a).

**Table 2.** *Staphylococcus aureus* cell-wall-anchored proteins[a]. (cont.)

| Subfamily | Protein | Gene | Ligand | Function | References |
|---|---|---|---|---|---|
| **EF-hand motif (calcium binding structural domain)** | | | | | |
| | Bap | *bap* | Gp96. | Biofilm formation in bovine mastitis. | (Cucarella *et al.*, 2001; Valle *et al.*, 2012) |
| **CWA proteins without well-characterized structure or function** | | | | | |
| | SasB, SasD, SasJ, SasK, SasL | *sasB, sasD, sasJ, sasK, sasL* | Unknown. | Unknown. | (Roche, Massey, *et al.*, 2003) |
| | SasX | *sasX* | Unknown. | Encoded by a lysogenic bacteriophage. | (Li *et al.*, 2012) |
| | SasF | *sasF* | Unknown. | Resistance to bactericidal effects of long chain fatty acids. Virulence. | (Kenny *et al.*, 2009) |

[a] Adapted from (Foster, 2019a).

### 4.1.2 Non-covalently associated surface proteins

The cell-wall of *S. aureus* also includes proteins that remain bound to the cell wall through ionic interactions. These proteins also play relevant functions in colonization and biofilm formation. As well as LPXTG proteins, non-covalently associated proteins can either attach to the naked surface, through non-specific interactions, or bind to specific proteins in the plasma and the ECM proteins coating the implant. In the following paragraphs, we describe the most important proteins of this group involved in *S. aureus* adhesion and colonization.

### *Secreted Expanded-Repertoire Adhesive Molecules*

*S. aureus* expresses a range of Secreted Expanded-Repertoire Adhesive Molecules (SERAMs) with binding properties to the ECM and plasma that ensure tissue-specific tropism and colonization. SERAMs bind to the cell-wall by mechanisms that are not well characterized. This group of adhesins includes the von Willebrand factor binding protein (vWbP), the extracellular fibrinogen binding protein (Efb), the extracellular matrix binding protein (Emp), and the extracellular adhesion protein (Eap). These proteins are involved in different processes related to virulence and pathogenicity, and some of them are involved in biofilm formation under iron-limiting conditions (Chavakis *et al.*, 2005; Johnson *et al.*, 2008). Table 3 summarizes the binding ligands and functions.

### *Major cell wall autolysin*

The major *S. aureus* autolysin (AtlA) is a murein hydrolase with amidase and glycosaminidase domains responsible for the separation of the daughter cells (Porayath *et al.*, 2018). Due to its ability to promote the binding to hydrophobic surfaces and induce cell lysis and eDNA release, AtlA can mediate the initial adhesion to naked surfaces and promote biofilm formation in the early stages (Bose *et al.*, 2012). In addition, AtlA binds to ECM and plasma proteins, such as Fg, Fn, and Vn, which contributes to the accumulation of the biofilm matrix (Bose *et al.*, 2012; Paharik & Horswill, 2016; Porayath *et al.*, 2018).

### *Membrane-spanning proteins*

In *S. aureus* the giant extracellular matrix-binding protein homologue (Ebh) is encoded by the largest gene in *S. aureus* (1.1 mDa) (Clarke *et al.*, 2002). Ebh contains Fn-binding domains that may be involved in adhesion to endothelial cells (Clarke & Foster, 2006; Heilmann, 2011), a C-terminal membrane-spanning domain that seems to attach Ebh to the cell membrane, and a putative peptidoglycan-binding repeat, which binds it ionically to the cell wall peptidoglycan (Clarke *et al.*, 2002).

**Table 3.** Non-covalently anchored protein functions and ligands.

| Protein | Gene | Ligand | Function | References |
|---|---|---|---|---|
| vWbp | *vwb* | VWF A1-ClfA. | Coagulase. | (Bjerketorp *et al.*, 2004) |
| Eap | *eap* | DNA,<br>Cn1. | Adhesion and immunomodulation. Iron-limiting conditions biofilm. Suppresses the formation of "neutrophil extracellular traps". | (Bur *et al.*, 2013; Eisenbeis *et al.*, 2018; Geraci *et al.*, 2017; Johnson *et al.*, 2008) |
| Emp | *emp* | Fg, Fn Vn,<br>Cn1. | Iron-limiting conditions biofilm. Adhesion to skin and cartilage. | (Geraci *et al.*, 2017; Johnson *et al.*, 2008) |
| Efb | *efb* | Fg,<br>C3,<br>P-selectin. | Inhibits the interaction of platelets with leukocytes.<br>Immune system evasion. | (Ko *et al.*, 2011; Wallis *et al.*, 2022) |
| AtlA | *atlA* | Fn, heparin, gelatin. | Cell wall degradation and daughter cell separation. | (Porayath *et al.*, 2018) |

**Table 3.** Non-covalently anchored protein functions and ligands. (Cont.)

| Protein | Gene | Ligand | Function | References |
|---|---|---|---|---|
| Ebh | *Ebh* | Fn. | Adhesion to endothelial cells. | (Clarke & Foster, 2006) |



### 4.1.3 Regulation of the expression of surface adhesins

The regulatory network controlling the production of surface adhesins and biofilm formation involves several regulatory elements such as quorum sensing (QS) or two-component systems (TCS), and their effectors. These elements, in response to different environmental, physicochemical and host signals, modulate the expression of adhesins and biofilm determinants at different stages (Schilcher & Horswill, 2020).

In *S. aureus*, the QS *agr* and *luxS* are key regulatory elements (Filmer *et al.*, 2000; Kong *et al.*, 2006). The locus composed of *agrBDCA* encodes two distinct transcripts from two promoters. P2 encodes RNAII transcript, which comprises the QS module, while P3 encodes the RNAIII, the main effector of *agr* (Jenul & Horswill, 2019). The signal sensed by the *agr* QS is an autoinducing peptide (AIP) that activates the system when it accumulates in the extracellular environment to an adequate concentration (Jenul & Horswill, 2019). As for adhesins expression, *agr* leads to their downregulation and thus, the initial attachment stage requires conditions with low *agr* activity; moreover, *agr* inactivation has been suggested to promote pathogen success during medical device-related infections (Filmer *et al.*, 2000; Kong *et al.*, 2006). In this respect, host proteins, as well as a low pH can inhibit *agr* activity (Jenul & Horswill, 2019; Paharik & Horswill, 2016). In addition, *agr* controls dispersion factors, PSMs

(phenol-soluble modulins), and proteases, playing an important role in the biofilm detachment stage (Jenul & Horswill, 2019).

Other elements that influence adhesins expression in response to environmental signals are the SaeRS TCS and the SarA family of regulators. SaeRS is activated by perturbations in the membrane, the presence of $H_2O_2$ and α-defensins, and is inhibited by low pH, salt and the presence of free fatty acids (DeMars *et al.*, 2021; Geiger *et al.*, 2008). SaeRS is known to induce the expression of FnBPA and FnBPB adhesins that have a relevant role in a proteinaceous-dependent biofilm formation process (Vergara-Irigaray *et al.*, 2009). Regarding the SarA family of regulators, SarA, Rot and MgrA, also regulate the expression of some adhesins (Chan & Foster, 1998). SarA and Rot promote adhesion and the production of a FnBPs-dependent biofilm by inducing the expression of surface proteins while repressing protease activity (Jenul & Horswill, 2019). In contrast, MgrA which is also an effector of the ArlRS TCS, acts as a repressor of several adhesins (Jenul & Horswill, 2019), and indirectly regulates adhesion and clumping by controlling the expression of giant surface adhesins (Ebh, SasA and SasG), which interfere with other adhesins binding their host ligands (Chavakis *et al.*, 2005). Together, *agr* QS, SaeRS TCS, and SarA also respond to low-iron conditions upregulating the expression of some adhesins and promoting biofilm formation under iron-limiting conditions (Johnson *et al.*, 2008; Paharik & Horswill, 2016).

Another global regulator involved in adhesins expression and biofilm formation is the alternative sigma factor SigB. SigB, in response to stress conditions, upregulates some adhesins (FnBPA and ClfA) and represses

proteases and nucleases production, needed for biofilm dispersion (Kullik *et al.*, 1998; Schilcher & Horswill, 2020). Moreover, SigB acts over the regulators described above, depending on the environmental signals or the genetic background; SigB reduces RNAIII expression, the main effector of the *agr* QS, increases *sarA* expression and downregulates the *saeRS* TCS expression (Bischoff *et al.*, 2001; Geiger *et al.*, 2008; Horsburgh *et al.*, 2002). Finally, it is also known that SOS response proteins (LexA and RecA) that induce gene repair and recombination in response to persistent damage, regulate FnBP-dependent biofilm production (Bisognano *et al.*, 2004; Vergara-Irigaray *et al.*, 2009).

### 4.2 PIA/PNAG exopolysaccharide

PIA/PNAG is an exopolysaccharide composed of N-acetyl glucosamine polymers linked via a β-1,6 linkage (O'Gara, 2007). It is synthesized by the *icaADBC* operon consisting of four genes, *icaA, icaB*, *icaC*, and *icaD*, which are present in all *S. aureus* isolates, but whose expression can vary significantly amongst them (Cramton *et al.*, 1999; Götz, 2002; Nguyen *et al.*, 2020). The PIA/PNAG exopolysaccharide plays a fundamental role in intercellular adhesion and evasion of the immune response in certain isolates (Arciola *et al.*, 2015). Its production results in a high energy cost for the bacteria and thus requires tight regulation of *ica* operon expression (Nguyen *et al.*, 2020). It has been shown that PIA production and *ica* expression depend on environmental conditions, i.e. salt, iron, alcohol, oxygen availability, or antibiotics (Nguyen *et al.*, 2020). Many systems regulate PIA/PNAG production, including the transcriptional regulatory protein IcaR, whose gene is adjacent to the *icaADBC* genes, and binds

specifically to the promoter of the *ica* operon, preventing its expression (Jeng *et al.*, 2008). Also, the *luxS*/AI-2 dependent QS system decreases *S. aureus* biofilm formation in an *icaR*-dependent manner (Yu *et al.*, 2012).

Other transcriptional regulators of PIA/PNAG expression are TcaR, SarA, and ArlR. These regulators bind to the intergenic region (IGR) between *icaADBC* and *icaR* to regulate PIA/PNAG production (Jefferson *et al.*, 2004; Ouyang *et al.*, 2019; Valle *et al.*, 2003; Wu *et al.*, 2012). TcaR negatively regulates PIA/PNAG production, however to a much smaller extent than IcaR (Jefferson *et al.*, 2004). SarA positively controls *ica* operon transcription and indirectly regulates Bap-dependent and FnBPs-dependent biofilm through the action of proteases (Trotonda *et al.*, 2005; Valle *et al.*, 2003). Another important elements regulating PIA/PNAG production are the ArlRS TCS that increases *icaADBC* expression, at least through down regulation of *icaR* expression (Burgui *et al.*, 2018; Toledo-Arana *et al.*, 2005), and the SigB RNA polymerase subunit. In the presence of SigB, the turnover of Ica proteins is accelerated, reducing the synthesis of PIA/PNAG exopolysaccharide and consequently the PIA/PNAG-dependent biofilm formation capacity which represses PIA/PNAG synthesis (Valle *et al.*, 2019).

## 5. Evolution and identification of pathogenic *Staphylococcus aureus* isolates

Since *S. aureus* colonizes the skin and nares of the healthy population, it is challenging to distinguish between highly aggressive and commensal isolates. The adaptation of *S. aureus* occurs through changes in the genome that provide

an advantage to adapt to specific anatomical niches, resist stress conditions, evade the immune system, and overcome antimicrobial treatments (Fitzgerald, 2014). Numerous epidemiological tools have been developed to differentiate *S. aureus* lineages associated with different infection capacities. *S. aureus* typing, such as *spa* typing and MLST typing (multi-locus sequence type system), are the most used methods for correlating their genetic characteristics with important phenotypes, including the acquisition of virulence and antibiotic resistance determinants, or adaptation to host niches (Enright *et al.*, 2000; X. Wang, 2020).The *spa* gene contains a region containing a variable number of 24 bp repeat sequences in the X-region that is used as a typing method to detect clinically relevant *S. aureus* isolates. This is a highly discriminatory and rapid characterization method that accurately predicts the lineage relative to other typing methods such as MLST, ribotyping, and pulsed-field gel electrophoresis (PFGE) (X. Wang, 2020). According to the sequence of seven housekeeping genes (MLST), *S. aureus* can be classified into clonal complexes (CC) and sequence types (STs). Some CCs are dominant or common, while others are rare. Those strains identical in all seven housekeeping genes belong to the same ST, while those strains containing five or six identical housekeeping genes sequences belong to different STs, but are grouped in the same CC. Thus, the strains containing a conserved core genome sequence belong to the same lineage. Finally, strains containing less than five identical sequences are classified within different CCs (Lindsay, 2019).

MLST typing was one of the first approaches of genomic analysis to identify clinically relevant isolates (Bougnoux *et al.*, 2002; Enright *et al.*, 2000;

Loughman *et al.*, 2008). Nowadays, the significant decrease in the prize of bacterial genome sequencing is replacing the classical typing techniques by genome-wide association studies (GWAS). This method studies the entire genome of a large group of strains searching for small variations, called single nucleotide polymorphisms or SNPs, associated with specific phenotypes.

In the database of the National Center for Biotechnology Information, (NCBI), at the date of writing this thesis, 931 complete genomes of *S. aureus* were available, being one of the most sequenced species. As previously stated, *S. aureus* is a very versatile microorganism that can adapt to different niches and cause many pathologies (Cheung *et al.*, 2021). It is assumed that *S. aureus* evolves through point mutations, acquisition or loss of individual genes, or variability in coding regions (Lindsay, 2019). The contribution is easier to determine when the differences between strains are associated with the absence/presence of genes encoding virulence determinants or antibiotic resistance genes, or when variations in coding regions lead to amino acid changes that change protein structure or function. However, polymorphisms do not only occur in coding sequences, and they may also arise at IGRs. The fact that a mutation occurs in an IGR does not mean that it is silent. Certain point mutations at IGRs may affect the promoter strength by promoting or preventing the RNA polymerase from binding to this sequence, influencing the expression of neighboring genes or regulatory non-coding RNA molecules. In this case, the causal link between the SNPs and the host-adaptive trait is less recognizable, and functional validation assays are needed to support the relationship between the genotype and the phenotype (Pascoe *et al.*, 2015; Sheppard *et al.*, 2013).

Studies in *Salmonella* Typhimurium*, Pseudomonas aeruginosa, S. aureus,* and *Mycobacterium tuberculosis*, have demonstrated that some genetic variations in IGRs are not neutral and mutations in IGRs may have an adaptative and evolutionary significant role in connection to bacterial pathogenesis, making its study necessary (Khademi *et al.*, 2019; Osborne *et al.*, 2009; Thorpe *et al.*, 2016, 2017).

In this thesis, we have studied whether variability in the IGRs upstream genes encoding factors important for colonization of medical implants contribute to generate differences in the capacity of *S. aureus* to cause PJI. For that, we have focused our attention in two major players for surface colonization in *S. aureus*: (i) the *icaADBC* operon, responsible for the formation of a polysaccharide-dependent biofilm and considered as the main polysaccharide composing the biofilm matrix because of its structural role; and (ii) genes encoding adhesin-functional proteins. Specifically, we set out to study the genetic variability in the regulatory regions of the *icaADBC* operon and genes encoding adhesin-functional proteins, including LPXTGs and SERAMs, in a collection of 71 clinical isolates from PJI and wound-associated infections. Our hypothesis was that genetic variations in the regulatory regions upstream these genes modulate the capacity of *S. aureus* isolates for biofilm formation and adhesion to medical devices.

# Objectives

The specific objectives of this thesis are:

a. To analyze how genetic variability in the intergenic region upstream of the *icaADBC* operon of *S. aureus* clinical isolates is associated with strains causing PJI and how it affects *ica* expression and PIA/PNAG production.

b. To investigate how the variability in the intergenic regions upstream of the genes encoding surface adhesins affects their expression profile and subsequently the capacity of isolates to cause PJI infections.

c. To implement a methodology in order to simultaneously compare the capacity of several strains to colonize medical implants in a murine catheter infection model.

# Materials and Methods

### *Bacterial strains, plasmids, oligonucleotides, and culture conditions*

Bacterial strains, plasmids, and oligonucleotides used in this work are listed in Tables 4-9. The forty-five PJI *S. aureus* strains used in this study were isolated from patients admitted at Sahlgrenska University Hospital (Mölndal, Sweden) with PJI of the hip or knee (Malchau *et al.*, 2021; Trobos *et al.*, 2022). Twenty-six strains from wound infections, isolated between 1966 and 2010, were obtained from the Culture Collection University of Gothenburg (CCUG) (Morales-Laverde *et al.*, 2022; Turner *et al.*, 2022). *Escherichia coli* and *S. aureus* strains were routinely grown in Luria-Bertani medium (LB; Conda-Pronadisa, Madrid, Spain) and Trypticase soy broth (TSB; Conda-Pronadisa, Madrid, Spain), respectively, at 37°C. Bacteriological agar was used as a gelling agent (VWR, Radnor, PA, USA). When required, growth media were supplemented with antibiotics at the following concentrations: erythromycin (Ery), 10 μg/mL or 2.5 μg/mL; ampicillin (Amp), 100 μg/mL.



### *DNA manipulations*

Routine DNA manipulations were performed using standard procedures unless otherwise indicated (Sambrook *et al.*, 1989). Oligonucleotides were synthesized by STABVIDA, Lda. (Caparica, Portugal). FastDigest restriction enzymes, Phusion DNA polymerase, and the rapid DNA ligation kit (Thermo Scientific, Waltham, MA, USA) were used according to the manufacturer's instructions. Plasmids were purified using a plasmid purification kit from Macherey-Nagel (Allentown, PA, USA) according to the manufacturer's protocol. Plasmids were transformed in *E. coli* by electroporation (1 mm cuvette; 200 Ω,

25 µF, 1250 V; Gene Pulser X-Cell electroporator). *S. aureus* competent cells were generated as previously described (Schenk & Laddaga, 1992). Plasmids were transformed in *S. aureus* by electroporation (1mm cuvette; 100 Ω, 25 µF, 1250 V; Gene Pulser X-Cell electroporator). All constructed plasmids were confirmed by Sanger sequencing at STABVIDA Lda (Caparica, Portugal).

***Construction of isogenic mutants***

To construct the MW2 *icaR* V176E mutant, two DNA fragments were amplified with the primer pairs Au56/LM9 and LM10/Au73 (Table 6) from the MW2 wild type strain. The two PCR fragments were fused through overlapping PCR using primers Au56 and Au73, cloned into the pJET 1.2 vector and then subcloned into the pMAD vector (Arnaud *et al.*, 2004), digested with SalI and BamHI, generating plasmid pMAD::*icaR*V176E. This plasmid was purified from *E. coli* IM01B (Monk *et al.*, 2015) and transformed into the MW2 wild type strain by electroporation.

To construct the sortase A (*srtA*) mutants, two DNA fragments were amplified with the primer pairs BG_STAP79/LM97 and LM98/BG_STAP86 (Table 9) from the MW2 wild type strain. The two PCR fragments were fused through overlapping PCR using primers BG_STAP79 and BG_STAP86, cloned into the pJET 1.2 vector and then subcloned into the pMAD vector (Arnaud *et al.*, 2004) digested with NcoI and BamHI, generating plasmid pMAD::*srtA*$_{AD}$. This plasmid was transformed into the MIC 6947, MIC 6981 (purified from *S. aureus* RN4220)(Kreiswirth *et al.*, 1983), and MIC 6982 (purified from *E. coli* IM30B) wild-type strains by electroporation.

Homologous recombination experiments were performed as described (Schilcher & Horswill, 2020). Ery-susceptible white colonies, which did not further contain the pMAD plasmid, were tested by PCR using the primers Au56 and Au73 and further digestion of the PCR product with ScaI (MW2 *icaR* V176E mutant) and using the primers BG_STAP86 and BG_STAP87 (*srtA*). Sanger sequencing was used to confirm the generated isogenic mutants.

***Whole-genome sequencing and genomic analysis***

Whole genome sequencing of PJI isolates was previously performed (Malchau *et al.*, 2021; Trobos *et al.*, 2022). Genomic DNA from the twenty-six *S. aureus* strains isolated from wounds was extracted with the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions for Gram-positive bacteria. DNA was used for library preparation using a Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA) followed by Illumina sequencing at MicrobesNG (University of Birmingham, UK) on a HiSeq2500 Illumina sequencer using a paired-end approach (2*250 bp). The Sequence Read Archives (SRAs) with detailed information of the strains was deposited in the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA765573.

Nodes containing the *ica* locus and twenty-three genes encoding adhesins were localized in each strain using the blastn tool from the NCBI website. Analysis of the SNPs and comparison between the strains were performed with SnapGene v5.3.3. All sequences containing the *ica* locus and

adhesin encoding genes were aligned and compared against the *S. aureus* MW2 reference genome (GenBank accession number NC_003923). Genetic analysis was focused on the regulatory sequences of the *icaADBC* operon including the 3′ UTR of *icaR*, the *icaR* coding sequence, and the IGR between *icaR* and *icaA*; and on the promoter region of adhesin-encoding genes *fnbA, clfA, clfB, sdrC, spa, sasC, sasE* (*isdA*), *sasF, sasH* (*adsA*), *sasI* (*isdH*), *sasJ* (*isdB*), *eap, emp, vw*b and *efb*, which were present in all strains. For PJI isolates, sequence typing (ST) was previously performed (Malchau *et al.*, 2021; Trobos *et al.*, 2022). We used the MLST 2.0 tool (https://cge.cbs.dtu.dk/services/MLST/) and the Bacterial Isolate Genome Sequence Database (BIGSdb) (https://pubmlst.org/) to group the isolates from wounds.

***Generation of transcriptional fusions of ica regulatory sequences, and adhesin-encoding genes IGRs with gfp***

To generate transcriptional fusions of *ica* regulatory sequences, with the *gfpmut2* gene, the region comprising the 3′ UTR of *icaR*, the *icaR* coding sequence, and the IGR between *icaR* and *icaA* was amplified from each representative strain of clusters one to six using the primers Au56 and Au76 (Table 6). For adhesins encoding genes transcriptional fusions, the region comprising two hundred nucleotides immediately upstream of each gene coding sequence was amplified from one representative strain of CC15 (MIC 6935), CC8 (MIC 6947), CC45 (MIC 6981), and CC30 (MIC 6982), using primers listed in Table 9 for each adhesin-encoding gene. The PCR products were cloned into the pJET 1.2 vector and then subcloned into the pCN52 plasmid (Charpentier *et al.*,

2004) digested with SalI and KpnI, giving plasmids pCN52::IGR*ica* C1 to C6, and pCN52::$P_{xx}$CC.

To generate transcriptional fusions of *ica* regulatory sequences containing a mutated *icaR* gene, with a premature stop codon after residue 36, two DNA fragments were amplified with primer pairs Au56/Au78 and Au77/Au76 (Table 6) from each representative strain of clusters one to six. The two PCR fragments were fused through overlapping PCR using primers Au56 and Au76, cloned into the pJET 1.2 vector and then subcloned into pCN52 digested with SalI and KpnI, giving plasmids pCN52::IGRStop*ica* C1 to C6.



***Western blot analyses***

To collect supernatant proteins, overnight cultures of one representative strain of CC15 (MIC 6935), CC8 (MIC 6947), CC45 (MIC 6981), and CC30 (MIC 6982) grown in 50 mL of TSB-glucose 0.25% (TSB-gluc) were pelleted by centrifugation at 4,500 rpm for 30 min, at 4°C and the supernatant was collected and centrifuged again. Culture supernatants were filtered and precipitated at 4°C using 10% (v/v) trichloroacetic acid (Sigma-Aldrich, St Louis, MO, USA) for 1 h. The samples were centrifuged twice at 4,000 rpm for 2 h at 4°C and the supernatant was discarded. The pellets were resuspended in 800 µL ice-cold ethanol (96%) and then centrifuged at 14,000 rpm for 20 min at 4°C. Resulting pellets were air dried at room temperature and proteins were solubilized with 100 µL of 8 M urea (Amresco, Dallas, TX, USA) for 30 min at room temperature. Protein concentration was quantified using a Bicinchoninic Acid Kit (BCA) (Sigma-Aldrich, St Louis, MO, USA).

CWA proteins from *S. aureus* clinical isolates were prepared as previously described (Arrizubieta *et al.*, 2004). In brief, overnight cultures of each of the above-selected strains grown in 5 mL of TSB-gluc were pelleted by centrifugation at 4,500 rpm for 30 min, at 4°C, washed with 1 mL of PBS and resuspended in 100 µL of isosmotic digestion buffer (phosphate-buffered saline (PBS) containing 26% [wt/vol] raffinose; Sigma-Aldrich, St Louis, MO, USA). CWA proteins were solubilized by adding 1.5 µL of a 1 mg/mL solution of lysostaphin (Sigma-Aldrich, St Louis, MO, USA) and incubation with shaking at 37°C for 2 h. Samples were centrifuged at 8,000 x g for 30 min with slow deceleration and the supernatant was taken as the cell wall fraction. Protein concentration was quantified using a Bicinchoninic Acid Kit (BCA) (Sigma-Aldrich, St Louis, MO, USA).

For detection of cytoplasmic GFP, overnight cultures of *S. aureus* 132 containing pCN52 plasmids were diluted 1:100 and cultured in 25 mL of TSB supplemented with 3% NaCl (TSB-NaCl: pCN52::IGR*ica* C1 to C6 and pCN52::IGRStop*ica* C1 to C6) or with 0.25% of glucose (TSB-gluc: pCN52::$P_{xx}$CC) at 37°C under static conditions for 5 h. Cells were pelleted by centrifugation at 4,500 rpm for 30 min at 4°C, washed with 1 mL of PBS, suspended in 400 µL PBS and lysed using a FastPrep-24™ 5G homogenizer (MP Biomedicals, LLC, Irvine, CA, USA). The total amount of protein was quantified using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA).

For western blot analysis, equal amounts of protein per sample were loaded on a 12% Stain-Free FastCast™ Acrylamide Kit, 12% (Bio-Rad, Hercules, CA, USA) after adding a volume of Laemmli buffer and boiling them for 5 min.

After the electrophoresis, the samples were transferred to Amersham™ Protran™ Premium 0.45 µm nitrocellulose blotting membranes (Cytiva, Marlborough, MA, USA) by electroblotting. Membranes were blocked overnight in PBS containing 0.1% Tween 20 and 5% skimmed milk under shaking conditions and incubated with rabbit anti-*S. aureus* polyclonal antibody that reacts with soluble and structural antigens of the whole bacterium (Bio-Rad, Hercules, CA, USA) at 1 µg/mL in blocking solution for 1 h at room temperature. For GFP detection, the membranes were incubated with anti-GFP antibodies (Living Colors A.v. monoclonal antibody JL-8; Clontech, Mountain View, CA, USA), diluted 1:2,500 in blocking solution for 2 h at room temperature. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Thermo Scientific, Waltham, MA, USA) diluted 1:5,000 in blocking solution was used as a secondary antibody and the subsequent chemiluminescence reaction was recorded with ECL Prime western blotting detection reagents (Cytiva, Marlborough, MA, USA) in the Chemidoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).



***Polysaccharide biofilm phenotypes and PIA/PNAG detection***

The biofilm formation assays were performed in sterile 96-well polystyrene microtiter plates (Thermo Scientific, Waltham, MA, USA) as described elsewhere (Heilmann *et al.*, 1996). Briefly, overnight cultures grown in TSB were diluted 1:100 in TSB-NaCl and incubated at 37 °C for 24 h. The wells were gently rinsed three times with water, dried, and stained with 0.1% crystal violet for 15 min. The wells were rinsed again, and the crystal violet was solubilized with 200 µL of ethanol-acetone (80:20, vol/vol). The $OD_{590nm}$ was

determined in an Epoch (BioTek, Winooski, VT, USA) microplate spectrophotometer. Biofilm formation experiments were performed in triplicate (*n*=3) with three technical replicates.

PIA/PNAG was extracted and detected by dot-blot as described elsewhere (Cramton *et al.*, 1999). Overnight cultures were diluted 1:100 in 2 mL TSB-NaCl and incubated at 37°C for 24 h in sterile 24-well cell culture plates from Costar (Corning, New York, NY, USA). Biofilm cells were recovered from each well, centrifuged and suspended in 50 µL of 0.5 M EDTA (pH 8.0). Cells were then incubated for 5 min at 100°C and centrifuged. The supernatant (40 µL) was incubated with 10 µL of proteinase K (20 mg/mL) for 30 min at 37 °C. After the addition of 10 µL of Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.4]) containing 0.01% bromophenol blue, 4 µL were spotted onto Amersham™ Protran™ Premium 0.45 µm nitrocellulose blotting membranes (Cytiva, Marlborough, MA, USA) using a Bio-Dot microfiltration apparatus (Bio-Rad, Hercules, CA, USA), dried and blocked overnight. The membrane was incubated with an anti-PNAG antibody diluted 1:10,000 at room temperature for 2 h (Kropec *et al.*, 2005). Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G antibodies (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA, USA).

### *Galleria mellonella* survival assay

*Galleria mellonella* larvae were obtained in bulk from Bichosa (Vigo, Spain), and stored at 16°C until used. Bacterial cultures were grown in TSB for 16 h at 37°C under shaking, and cultures were pelleted and suspended in 2 mL of PBS

to obtain a cell density of $1\times10^9$ CFU/mL. For infection, 10 µL of the bacterial suspensions ($1\times10^7$ CFU/larvae) were injected in the left lower abdominal proleg with a Micro Fine Insulin Syringe 0.3 mL, 30g (BD, Frajklin Lakes, NJ, USA), using a Burkard hand microapplicator (Bukard Scientific, Rickmansworth, UK). The control group was injected with 10 µL of sterile PBS. A group of 25 larvae was used for each strain. Infected larvae were transferred to a clean Petri dish lined with filter paper, incubated at 37°C, and scored for survival every 24 h for a total of 96 h. The survival experiments were performed with three biological replicates. Statistical analyses were performed with the GraphPad Prism v9.2.0 program. For single comparisons, data were analysed using an unpaired, two-tailed Student's t-test. Data corresponding to the *G. mellonella* survival assay were compared using a log-rank (Mantel–Cox) test. In all tests, *p* values of less than 0.05 were considered statistically significant (*$p$<0.05, **$p$<0.01, ***$p$<0.001).



***Co-infection experiments***

To evaluate *in vivo* colonization, we used a murine model of catheter-associated biofilm. Bacteria grown overnight in TSB were suspended in PBS to an $OD_{600nm}$ of 0.2 ($10^8$ CFU/mL). A total of 24 five-week-old ICR female mice (Envigo, Indianapolis, IN, USA) were anesthetized with isoflurane, and two 15 mm intravenous catheters (24G; B. Braun Medical, Bethlehem, PA, USA) were implanted into the subcutaneous interscapular of each mouse and co-infected by injection of 100 µL (a total of $10^7$ CFU) of a bacterial mixture, containing the same proportion of each *S. aureus* strain (CC15 (MIC 6935), CC8 (MIC 6947),

CC45 (MIC 6981), and CC30 (MIC 6982)). Five days after infection, animals were anesthetized by isoflurane inhalation and euthanatized by cervical dislocation. The catheters were removed aseptically and placed in Lysing Matrix E tubes (Thermo Scientific, Waltham, MA, USA) containing 1 mL of TES, and cells were lysed using a FastPrep-24™ 5G homogenizer (MP Biomedicals, LLC, Irvine, CA, USA) to isolate the DNA.

Extracted DNA was used for library preparation using Illumina Amplicon Library kit MS-102-3003 (Illumina, San Diego, CA, USA) followed by Illumina sequencing at Fisabio (Valencia, Spain) on a MiSeq System Illumina sequencer (San Diego, CA, USA) using a paired-end approach. The oligonucleotides LM63/LM64 and LM99/LM100 (Table 9) were designed for P*sasJ*, and P*clfB* amplicon sequencing, respectively. DNA amplicon libraries were generated using a limited cycle PCR: initial denaturation at 95°C for 3 min followed by 25 cycles of annealing (95°C 30 sec, 55°C 30 sec, 72°C 30 sec) and extension at 72°C 5 min, using a KAPA HiFi HotStart ReadyMix (KK2602) (Sigma-Aldrich**,** St Louis, MO, USA). Then, Illumina sequencing adaptors and dual-index barcodes (Nextera XT index kit v2, FC-131-2001) were added to the amplicons. Libraries were normalized and pooled before sequencing. Quality assessment was performed by the use of the fastp program (Chen *et al.*, 2018) applying the following parameters: min_length: 50; trim_qual_right: 30; trim_qual_type: mean; and trim_qual_window: 10. R1 and R2 from Illumina sequences were joined using the *FLASH* program applying default parameters (Magoč & Salzberg, 2011). Reports, graphs, and statistics were obtained using the ea-utils programs suite (Aronesty, 2013). The MultiQC report was produced using the

*MultiQC* program (Ewels *et al.*, 2016). The SRAs with detailed information of the samples were deposited in NCBI under the BioProject accession number PRJNA834761.

Sequence mapping was carried out with the software BWA v.0.7.17-r1188 and the variants within the reads were located with the software ivar v.1.3.1. For the feature counts, a bash script was written to count the number of reads that mapped to each specific sequence. The number of reads was used to determine the percentage of read abundance. Statistical analyses were performed with the GraphPad Prism v9.2.0 program. One-way ANOVA was used followed by Tukey´s multiple comparisons. In all tests, *p* values of less than 0.05 were considered statistically significant: *$p$<0.05, **$p$<0.01, ***$p$<0.001, and ****$p$<0.0001.



***Biofilm formation phenotypes on protein precoated surfaces***

To determine differences in initial adhesion between strains, a biofilm formation assay on protein pre-coated polystyrene plates was performed. First, Fn, Fg and VWF were dissolved in buffer sodium carbonate 40 mM at a concentration of 5 µg/mL. Then, 100 µL of the corresponding coating was added to wells of a 48-well Nunclon™ Delta surface microtiter plate (Thermo Scientific, Waltham, MA, USA). After overnight incubation at 4°C under shaking, the plate was rinsed once with water and the biofilm formation assays were performed. Briefly, overnight cultures were adjusted to an $OD_{546nm}$ of 0.13 ($10^8$ CFU/mL) in TSB-gluc and further diluted 1:1000. An inoculum consisting of 1 mL of each diluted suspension ($10^5$ CFU/mL) was added to the pre-coated wells. After 5 h

of static incubation at 37°C, wells were rinsed once with water, dried, and stained with 1 mL of crystal violet for 5 min (VWR, Radnor, PA, USA). To quantify stained cells, 1 mL of ethanol-acetone (80:20, vol/vol) was added to each well, 500 µL of the suspension were transferred to a new 48-well plate and the $OD_{595nm}$ was recorded in a FLUOstar Omega (BMG LABTECH, Ortenberg, Germany) microplate reader. Six biological replicates with two technical replicates were used for the statistical analysis.

### *Ethics statement*

All animal studies were reviewed and approved by *the Comité de Ética para la Experimentación Animal* (CEEA) of the *Universidad de Navarra* (approved protocol 032-17). The experiments were performed at the *Centro de Investigación Médica Aplicada* building (ES312010000132) under the principles and guidelines described in the European Directive 2010/63/EU for the protection of animals used for experimental purposes.

**Table 4.** Bacterial strains used in chapter I.

| Strain | Relevant Characteristic(s) | MIC [a] | Reference |
|---|---|---|---|
| *E. coli* IM01B | *E. coli* K12 DH10B Δ*dcm* and containing the *hsdS* gene of MW2 integrated between the *essQ* and *cspB* genes. Used for cloning experiments and isolation of plasmids that are transformed into *S. aureus* CC1 strains at high efficiency. | 5694 | (Monk *et al.*, 2015) |
| *S. aureus* 15981 | MSSA clinical isolate; strong biofilm producer; PNAG-dependent biofilm matrix. | 0532 | (Valle *et al.*, 2003) |
| *S. aureus* MW2 | Community-acquired strain of MRSA, which was isolated in 1998 in North Dakota, USA | 3566 | (Baba *et al.*, 2002) |
| *S. aureus* MW2 *icaR* V176E | MW2 strain harbouring a mutation in the *icaR* gene, coding for a V176E IcaR variant. | 7983 | This study |
| *S. aureus* 132 *ica::tet* | *S. aureus* 132 containing a tetracycline resistance cassette that replaces *ica* genes. | 3343 | (Vergara-Irigaray *et al.*, 2009) |
| *S. aureus* clinical isolate | Sahlgrenska University Hospital (Sweden). Periprosthetic joint infection (hip). | 6924 | (Malchau *et al.*, 2021; Trobos *et al.*, 2022) |
| *S. aureus* clinical isolate | Sahlgrenska University Hospital (Sweden). Periprosthetic joint infection (hip). | 6934 | (Malchau *et al.*, 2021; Trobos *et al.*, 2022) |
| *S. aureus* clinical isolate | Sahlgrenska University Hospital (Sweden). Periprosthetic joint infection (hip). | 6935 | (Malchau *et al.*, 2021; Trobos *et al.*, 2022) |
| *S. aureus* clinical isolate | Sahlgrenska University Hospital (Sweden). Periprosthetic joint infection (hip). | 6936 | (Malchau *et al.*, 2021; Trobos *et al.*, 2022) |

[a] Identification number of each strain in the culture collection of the Laboratory of Microbial Pathogenesis, Navarrabiomed-*Universidad Pública de Navarra*.

**Table 4.** Bacterial strains used in chapter I. (cont.)

| Strain | Relevant Characteristic(s) | MIC [a] | Reference |
|---|---|---|---|
| *S. aureus* clinical isolate | Sahlgrenska University Hospital (Sweden). Periprosthetic joint infection (hip). | 6948 | (Malchau *et al.*, 2021; Trobos *et al.*, 2022) |
| *S. aureus* clinical isolate | Sahlgrenska University Hospital (Sweden). Periprosthetic joint infection (knee). Strong biofilm producer. | 7018 | (Malchau *et al.*, 2021; Trobos *et al.*, 2022) |
| *S. aureus* clinical isolates | Culture Collection at University of Gothenburg, (CCGU), Sweden. Wounds. | 7032-7050 7166-7172 | (Morales-Laverde *et al.*, 2022; Turner *et al.*, 2022) |

[a] Identification number of each strain in the culture collection of the Laboratory of Microbial Pathogenesis, Navarrabiomed-*Universidad Pública de Navarra*.

**Table 5.** Plasmids used in chapter I.

| Plasmids | Relevant Characteristics | Reference |
|---|---|---|
| pJET1.2 | Cloning vector. $Amp^R$. | Thermo Scientific |
| pMAD | *E. coli-S. aureus* shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria. $Amp^R$ $Ery^R$. | (Arnaud *et al.*, 2004) |
| pMAD::*icaR*V176E | pMAD plasmid containing the DNA sequence for *icaR* V176E mutation. | This study |
| pCN52 | *E. coli-S. aureus* shuttle vector with promoterless *gfpmut2*. $Ery^R$. | (Charpentier *et al.*, 2004) |
| pCN52::IGR$_{ica}$ C1 | 3'UTR *icaR-icaR*—IGR *icaR/icaA* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::IGR$_{ica}$ C2 | 3´UTR *icaR-icaR*—IGR *icaR/icaA* amplified from MIC 6924 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::IGR$_{ica}$ C3 | 3´UTR *icaR-icaR*—IGR *icaR/icaA* amplified from MIC 6934 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::IGR$_{ica}$ C4 | 3´UTR *icaR-icaR*—IGR *icaR/icaA* amplified from MIC 6936 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::IGR$_{ica}$ C5 | 3´UTR *icaR-icaR*—IGR *icaR/icaA* amplified from MIC 6948 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::IGR$_{ica}$ C6 | 3´UTR *icaR-icaR*—IGR *icaR/icaA* amplified from MIC 7018 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::IGRStop$_{ica}$ C1 | 3´UTR *icaR-icaR*$_{STOP}$—IGR *icaR/icaA* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::IGRStop$_{ica}$ C2 | 3´UTR *icaR-icaR*$_{STOP}$—IGR *icaR/icaA* amplified from MIC 6924 and fused to promoterless *gfpmut2* in pCN52. | This study |

**Table 5.** Plasmids used in chapter I. (Cont.)

| Plasmids | Relevant Characteristics | Reference |
|---|---|---|
| pCN52::IGRStop$_{ica}$ C3 | 3´UTR *icaR-icaR*$_{STOP}$—IGR *icaR*/*icaA* amplified from MIC 6934 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::IGRStop$_{ica}$ C4 | 3´UTR *icaR-icaR*$_{STOP}$—IGR *icaR*/*icaA* amplified from MIC 6936 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::IGRStop$_{ica}$ C5 | 3´UTR *icaR-icaR*$_{STOP}$—IGR *icaR*/*icaA* amplified from MIC 6948 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::IGRStop$_{ica}$ C6 | 3´UTR *icaR-icaR*$_{STOP}$—IGR *icaR*/*icaA* amplified from MIC 7018 and fused to promoterless *gfpmut2* in pCN52. | This study |

**Table 6.** Primers used in Chapter I.

| **Primers** | **Sequence** [a] |
|---|---|
| Au56 | ATGCCTGCAG**GTCGAC**CGAGTAGAAGCATCATCATTACTTGATT |
| Au73 | **GGATCC**TAAGCCATATGGTAATTGATAG |
| Au76 | ACGAATTCGAGCTC**GGTACC**TTTCTTTACCTACCTTTCGTTAGTTAGGTTG |
| Au78 | TTATTGATAACGCAATAACCTTATAAGGATCCTTTTCAGAGAAGGGGTATGACGG |
| Au77 | CCGTCATACCCCTTCTCTGAAAAGGATCCTTATAAGGTTATTGCGTTATCAATAA |
| LM9 | CAAAGATGAAG**AGTACT**CGCTACTAAATA |
| LM10 | TAGTAGCG**AGTACT**CTTCATCTTTGAATTG |

[a] Restriction enzymes sites and nucleotides for gene editing are indicated in bold and underlined, respectively.

**Table 7.** Bacterial strains used in chapter II.

| Strain | Relevant characteristic(s) | MIC [a] | Reference |
|---|---|---|---|
| *E. coli* IM01B | *E. coli* K12 DH10B Δ*dcm* and containing the *hsdS* gene of MW2 integrated between the *essQ* and *cspB* genes. Used for cloning experiments and isolation of plasmids that are transformed into *S. aureus* CC1 strains at high efficiency. | 5694 | (Monk *et al.*, 2015) |
| *E. coli* IM30B | *E. coli* DH10B *Δdcm* and containing the *hsdS* gene of MRSA252 integrated between the *essQ* and *cspB* genes. Used for cloning experiments and isolation of plasmids that are transformed into *S. aureus* CC30 strains at high efficiency. | 7743 | (Monk *et al.*, 2015) |
| *S. aureus* RN4220 | Restriction-deficient mutant of 8325-4. Used for cloning experiments and isolation of plasmids that are transformed into *S. aureus* strains. | 0099 | (Kreiswirth *et al.*, 1983) |
| *S. aureus* MW2 | Community-acquired strain of MRSA, which was isolated in 1998 in North Dakota, USA. | 3566 | (Baba *et al.*, 2002) |
| *S. aureus* 132 | MRSA clinical strain; biofilm positive: alternates between a proteinaceous and an exopolysaccharidic biofilm matrix. | 3343 | (Vergara-Irigaray *et al.*, 2009) |
| *S. aureus* clinical isolate | Sahlgrenska University Hospital. Periprosthetic joint infection-hip. *S. aureus* CC15. | 6935 | (Malchau *et al.*, 2021; Trobos *et al.*, 2022) |
| *S. aureus* clinical isolate | Sahlgrenska University Hospital. Periprosthetic joint infection-hip. *S. aureus* CC8. | 6947 | (Malchau *et al.*, 2021; Trobos *et al.*, 2022) |
| *S. aureus* clinical isolate | Sahlgrenska University Hospital. Periprosthetic joint infection-hip. *S. aureus* CC45. | 6981 | (Malchau *et al.*, 2021; Trobos *et al.*, 2022) |

[a] Number of each strain in the culture collection of the Laboratory of Microbial Pathogenesis, *Navarrabiomed-Universidad Pública de Navarra*.

**Table 7.** Bacterial strains used in chapter II. (Cont.)

| Strain | Relevant characteristic(s) | MIC[a] | Reference |
|---|---|---|---|
| *S. aureus* clinical isolate | Sahlgrenska University Hospital. Periprosthetic joint infection-hip. *S. aureus* CC30. | 6982 | (Malchau *et al.*, 2021; Trobos *et al.*, 2022) |
| MIC 6947 △*srtA* | MIC 6947 strain with deletion of *srtA* gene. | 7705 | This study |
| MIC 6981 △*srtA* | MIC 6981 strain with deletion of *srtA* gene. | 7709 | This study |
| MIC 6982 △*srtA* | MIC 6982 strain with deletion of *srtA* gene. | 7710 | This study |

[a] Number of each strain in the culture collection of the Laboratory of Microbial Pathogenesis, *Navarrabiomed-Universidad Pública de Navarra*.

**Table 8.** List of plasmids used in Chapter II.

| Plasmids | Relevant characteristics | Reference |
|---|---|---|
| pJET1.2 | Cloning vector. $Amp^R$ | Thermo Scientific |
| pMAD | *E. coli-S. aureus* shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria. AmpR EryR | (Arnaud *et al.*, 2004) |
| pMAD::$srtA_{AD}$ | pMAD plasmid containing the DNA sequence for *srtA* deletion. | This study |
| pCN52 | *E. coli–S. aureus* shuttle vector with promoterless *gfpmut2*. $Ery^R$ | (Charpentier *et al.*, 2004) |
| pCN52::$P_{spa}$CC15 | Promoter region of *spa* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::$P_{spa}$CC8 | Promoter region of *spa* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::$P_{spa}$CC45 | Promoter region of *spa* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::$P_{spa}$CC30 | Promoter region of *spa* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::$P_{fnbA}$CC8/CC15 | Promoter region of *fnbA* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::$P_{fnbA}$CC30/CC45 | Promoter region of *fnbA* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::$P_{clfA}$CC15 | Promoter region of *clfA* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |

**Table 8.** List of plasmids used in Chapter II. (Cont.)

| Plasmids | Relevant characteristics | Reference |
|---|---|---|
| pCN52::P*clfA*CC8 | Promoter region of *clfA* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*clfA*CC45 | Promoter region of *clfA* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*clfA*CC30 | Promoter region of *clfA* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*clfB*CC15 | Promoter region of *clfB* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*clfB*CC8 | Promoter region of *clfB* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*clfB*CC45 | Promoter region of *clfB* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*clfB*CC30 | Promoter region of *clfB* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sdrC*CC15 | Promoter region of *sdrC* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sdrC*CC8 | Promoter region of *sdrC* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sdrC*CC45 | Promoter region of *sdrC* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |

**Table 8.** List of plasmids used in Chapter II. (Cont.)

| Plasmids | Relevant characteristics | Reference |
|---|---|---|
| pCN52::P*sdrC*CC30 | Promoter region of *sdrC* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasC*CC15 | Promoter region of *sasC* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasC*CC8 | Promoter region of *sasC* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasC*CC45 | Promoter region of *sasC* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasC*CC30 | Promoter region of *sasC* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasE*CC15 | Promoter region of *sasE* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasE*CC8 | Promoter region of *sasE* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasE*CC45 | Promoter region of *sasE* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasE*CC30 | Promoter region of *sasE* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasF*CC15 | Promoter region of *sasF* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasF*CC8 | Promoter region of *sasF* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |

**Table 8.** List of plasmids used in Chapter II. (Cont.)

| Plasmids | Relevant characteristics | Reference |
|---|---|---|
| pCN52::P*sasF*CC45 | Promoter region of *sasF* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasF*CC30 | Promoter region of *sasF* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasH*CC15 | Promoter region of *sasH* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasH*CC8 | Promoter region of *sasH* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasH*CC45 | Promoter region of *sasH* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasH*CC30 | Promoter region of *sasH* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasI*CC15 | Promoter region of *sasI* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasI*CC8 | Promoter region of *sasI* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasI*CC45 | Promoter region of *sasI* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasI*CC30 | Promoter region of *sasI* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasJ*CC15 | Promoter region of *sasJ* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |

**Table 8.** List of plasmids used in Chapter II. (Cont.)

| Plasmids | Relevant characteristics | Reference |
|---|---|---|
| pCN52::P*sasJ*CC8 | Promoter region of *sasJ* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasJ*CC45 | Promoter region of *sasJ* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*sasJ*CC30 | Promoter region of *sasJ* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*eap*CC15 | Promoter region of *eap* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*eap*CC8 | Promoter region of *eap* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*eap*CC45 | Promoter region of *eap* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*eap*CC30 | Promoter region of *eap* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*emp*CC15 | Promoter region of *emp* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*emp*CC8 | Promoter region of *emp* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*emp*CC45 | Promoter region of *emp* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*emp*CC30 | Promoter region of *emp* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |

**Table 8.** List of plasmids used in Chapter II. (Cont.)

| Plasmids | Relevant characteristics | Reference |
|---|---|---|
| pCN52::P*vwb*CC15 | Promoter region of *vwb* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*vwb*CC8 | Promoter region of *vwb* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*vwb*CC45 | Promoter region of *vwb* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*vwb*CC30 | Promoter region of *vwb* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*efb*CC15 | Promoter region of *efb* amplified from MIC 6935 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*efb*CC8 | Promoter region of *efb* amplified from MIC 6947 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*efb*CC45 | Promoter region of *efb* amplified from MIC 6981 and fused to promoterless *gfpmut2* in pCN52. | This study |
| pCN52::P*efb*CC30 | Promoter region of *efb* amplified from MIC 6982 and fused to promoterless *gfpmut2* in pCN52. | This study |

**Table 9.** List of oligonucleotides used in Chapter II.

| Gene | Primer | Sequence[a] |
|---|---|---|
| *fnbA* | LM16 | CC**GTCGAC**CATAAAATTAATGACAATCTTAACTTT |
| | LM17 | CC**GGTACC**TATAATATCTCCCTTTAAATGCAAA |
| | LM18 | CC**GGTACC**TTAATATCTCCCTTTAAATGCAAAA |
| *sasC* | LM21 | CC**GGTACC**TTCATTTTCCTCCTGGTTGTCTGTT |
| | LM22 | CC**GGTACC**TTCGATTTCCTCCTGGTTGTCTGTT |
| | LM23 | CC**GGTACC**TTCGTTTTCCTCCTGGTTGTCTATT |
| | LM24 | CC**GTCGAC**ACAATCTAAAGCGTTCTTGTTATGTTTT |
| *eap* | LM25 | CC**GGTACC**AAATTATCTCTCCTTTTTTATGTAATT |
| | LM26 | CC**GGTACC**AAATTATCTCTCCTTTTTTGTGTAATT |
| | LM27 | CC**GTCGAC**ATGAAGATTGTTATGACATTTAAGTTTGAA |
| *emp* | LM28 | CC**GTCGAC**TGTGGACTATTACTTTTTTCACTTTATATTA |
| | LM29 | CC**GGTACC**CTGTTATTTCTCCTTTATATAGACTCA |
| *sasE* | LM30 | CC**GGTACC**GTTGTTTTCCTCCTAAGGATACAAATTAT |
| | LM31 | CC**GTCGAC**ACATAATCCTCCTTTTTATGATTGCTTT |
| *sasH* | LM32 | CC**GTCGAC**TAAATGAGAGTCACCCTATAAAGTTCGGCA |
| | LM33 | CC**GGTACC**TTCGTTCCCTCCATTAACTGTCATAC |
| *sasI* | LM34 | CC**GGTACC**GTTGTTAACAACTCCTATAAGAT |
| | LM35 | CC**GTCGAC**AATTTGGCGAATCTAACGACAATGTTTGAA |
| *spa* | LM36 | GG**GGTACC**ATTAATACCCCCTGTATGTATTTGTA |
| | LM37 | CC**GTCGAC**GTCTTTTACTTCCTGAATAAATCTTT |
| *clfA* | LM38 | CC**GTCGAC**ATTAATAATAAAAAATGTTTGCAATGAAAT |
| | LM39 | GG**GGTACC**TTTATTCCCTCTTTTTAAAAAGTCATTTTA |
| *clfB* | LM40 | CC**GGTACC**AAATATTACTCCATTTCAATTTCTAGATTA |
| | LM41 | CC**GTCGAC**TAAGGTGATGAAAAATTTAGAACTTCTAAG |
| *sdrC* | LM42 | GG**GTCGAC**TAAAAATATAGATAAATAATTTAAAGCAAT |
| | LM43 | CC**GGTACC**TTAATAATACTCCTTTAAAATATCAAAATT |
| *vwb* | LM44 | CC**GTCGAC**GTAATAATGATATTAAATTAATCAT |
| | LM45 | CC**GGTACC**CTGTATTTTCTCCTTAATTTTCCCTAAT |
| *sasF* | LM46 | CC**GGTACC**CAACATACTCCTTCCTCACTTACTT |
| | LM47 | CC**GGTACC**CTGTATTTTCTCCTTAATTTTCCCTAAT |
| | LM48 | CC**GGTACC**CAACATACTCCTTTCTCACTTACTT |
| | LM49 | CC**GTCGAC**AGATTTGACGTAATATGATGTTAGCGAC |
| *sasJ* | LM50 | CC**GGTACC**GTTGTAGAAACAACTCCTAATTGTATATTATCAA |
| | LM51 | CC**GTCGAC**CATCGTCACACTCATAACTTAATATATTTT |

[a] Restriction enzymes sites are indicated in bold.

**Table 9.** List of oligonucleotides used in Chapter II. (Cont.)

| Gene | Primer | Sequence[a] |
|---|---|---|
| *sasJ* | LM52 | CC**GGTACC**GTTGTAGTAACAACTCCTAATTGTATATTATCAA |
| | LM53 | CC**GTCGAC**CATCGTCATACTCATAACTTAATATATTTT |
| *efb* | LM54 | CC**GTCGAC**AGACACCAACATTCAAATGGTGTCTTTTTTGTTGTGT |
| | LM55 | CC**GGTACC**GTTAATTATCCTCCAAATTATTATCTTACATT |
| | LM56 | CC**GGTACC**GTTAATTATCCTCCAAATTGTTGTCTTATAAT |
| P*sasJ* | LM63 | TGCAACGCCTAGTGATGACT |
| | LM64 | AACACTTGCCCTTTTAGGTTCATT |
| P*clfB* | LM99 | CTTGTGCTTGATGATTGCCTA |
| | LM100 | GAGGTCTAACGTACATTTATACGTT |
| *srtA* | BG_STAP79 | GGG**CCATGG**GAATTAACATGGTTGTCTTG |
| | LM97 | CTTTCGGAATTTGAGGTTTACGTTAAGGCTCCTTTTATACATT |
| | LM98 | AGCCTTAACGTAAACCTCAAATTCCGAAAGA |
| | BG_STAP86 | CCC**GGATCC**TTTTTCCCAAACGCCTGTCT |
| | BG_STAP87 | CTCAGCATGATTATCGTTTT |

[a] Restriction enzymes sites are indicated in bold.

# Chapter I

Experimental polymorphism survey in intergenic regions of the *icaADBCR* locus

**Summary**

Identification of polymorphisms in bacterial genomes can help to establish associations between sequence variants, and a specific phenotype and outcome of infections. *Staphylococcus aureus* is a leading cause of prosthetic joint infections (PJI) characterized by its biofilm formation and recalcitrance to immune-mediated clearance and antibiotics. The molecular events behind PJI infection have not yet been unraveled. In this chapter, we report an experimental nucleotide-level survey specifically aimed at the intergenic regions (IGRs) that regulate the expression of the major exopolysaccharide of the *S. aureus* biofilm matrix in a collection of strains sampled from PJI and wounds. IGRs controlling *icaADBC* operon expression were highly conserved and no PJI-specific SNPs were found. Moreover, polymorphisms in these IGRs did not significantly affect transcription of the operon under *in vitro* laboratory conditions. In contrast, a SNP in the *icaR* gene, resulting in a V176E change in the transcriptional repressor IcaR, led to a significant increase in *icaADBC* operon transcription, PIA/PNAG overproduction and a reduction in *S. aureus* virulence, which led to increased survival of *Galleria mellonella*. In conclusion, SNPs in *icaADBC* IGRs of *S. aureus* isolates from PJI are not associated with *icaADBC* expression, PIA/PNAG production and adaptation to PJI.

## Results

### *The SNPs occurrence rate is low in the regulatory IGRs of the icaADBCR locus*

Most studies of the host adaptation have focused on genetic variations within coding regions, whereas the role of SNPs in IGRs has remained disregarded (Khademi *et al.*, 2019). We hypothesized that SNPs in the regulatory IGRs of the *icaADBCR* locus might reflect differences in the capacity of an *S. aureus* strain to produce implant-associated infections. To explore this hypothesis, we first compared the *ica* locus sequence conservation among 1000 complete genomes of *S. aureus* available at the NCBI database using blastn. The *ica* locus consists of the regulatory gene, *icaR*, located upstream and divergently transcribed from the biosynthetic operon *icaADBC*. For the analysis, the locus sequence was divided into the following segments: the 3' untranslated region (3'UTR) of *icaR*, involved in the post-transcriptional regulation of *icaR* expression (Ruiz de los Mozos *et al.*, 2013); the coding sequence of *icaR*; the entire *icaR-icaADBC* IGR containing *icaR* and *icaADBC* promoters and the 5´UTRs, and the coding sequences of *icaA*, *icaD*, *icaB*, and *icaC*. In addition, the flanking IGRs outside of the *ica* locus were included (Figure 1). The results revealed a variation of 8.2% in the 3'UTR of *icaR*, 9.3% in the *icaR-icaA* IGR and variation rates that ranged from 3.4% to 12% in the *icaR* and *icaADBC* coding sequences. Contrary to this low degree of variation along the *ica* locus, the variation rates of the IGRs flanking the *icaADBCR* locus were 21.2% and 22.9%. Together, these data showed that the regulatory sequences of the *icaADBCR* locus (3'UTR of *icaR* and *icaR-icaA* IGR) were highly conserved and less prone to evolutionary changes than other IGRs.

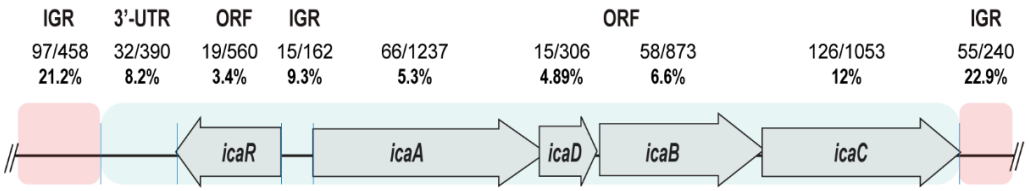



**Figure 1. Analysis of nucleotide variation rates along the *icaADBCR* locus.** The 3'UTR of *icaR* and the *icaR-icaA* IGR show nucleotide variation rates similar to *ica* coding sequences. The flanking IGRs, highlighted in red boxes, show high variation rates. The nucleotide variation rate in each region was calculated using 1000 genomic sequences available at the NCBI web page and the percentage represents the ratio between the total number of nucleotide changes in at least one *S. aureus* genome, and the length of the analysed sequence. ORF, open reading frame; IGR, intergenic region.

### *Identification of genetic variations in the regulatory IGRs of the icaADBCR locus*

The fact that the regulatory IGRs of the *icaADBCR* locus are highly conserved suggests that SNPs in these regions might have a significant impact on the expression of the *icaADBC* operon and on the ability of certain strains to cause PJI. To evaluate the association between particular SNPs and adaptation to PJI, we compared the sequence of *ica* regulatory IGRs of a collection of *S. aureus* clinical strains isolated from PJI (*n*=43) and wounds (*n*=26). We used, as a reference, the *S. aureus* MW2 genome and focused on the 3'UTR of *icaR* and the IGR between *icaR* and *icaA*. In addition, the *icaR* gene was included in the analysis since changes within the coding region might affect IcaR activity and therefore *ica* operon expression. A collection of 69 strains were clustered in seventeen groups according to the SNPs found (Figure 2). Wound isolates displayed high sequence similarity and twelve of them (46% of wound isolates)

did not harbor any SNP when compared to *S. aureus* MW2 (cluster 1). On the other hand, only nine PJI isolates (21% of PJI isolates) showed a sequence identical to that of *S. aureus* MW2 (cluster 1). Cluster 2 included twelve PJI isolates (28% of PJI isolates) and only one wound isolate, containing a total of eleven SNPs. From these, seven SNPs were located in the 3'UTR of *icaR*, three SNPs in the 5'UTR of *icaR* and one SNP in the 5'UTR of *icaA*. Cluster three included seven PJI isolates (16% of PJI isolates) and five wound isolates (19% of wound isolates) containing five SNPs that are common to the regulatory IGRs of cluster two. Cluster 4 included three PJI isolates and no wound isolates, also containing a total of five SNPs that are present in IGRs of cluster two and that differ in one SNP with IGRs of cluster three. The rest of the clusters included from one to three isolates of the same origin, either PJI or wound, that contain from one to twelve SNPs, most of them present in IGRs of cluster two. Interestingly, 48 out of the total 69 strains (70% of total isolates) did not show any SNP in the promoter and 5'UTR of the *icaADBC* operon, confirming that regulation of the operon imposes strong restrictions to sequence variations. As regards the *icaR* coding sequence, fourteen synonymous and two non-synonymous SNPs were detected. Specifically, cluster five included one PJI isolate that contains one SNP that leads to a G112E change, while cluster six included two PJI isolates containing a SNP that leads to a V176E change in the carboxyl-terminal domain of the transcriptional repressor IcaR. All in all, the fact that 70% of *S. aureus* isolates from wounds clustered together with strains from PJI strongly suggests that mutations in IGRs of the *icaADBCR* locus do not contribute to adaptation to PJI.

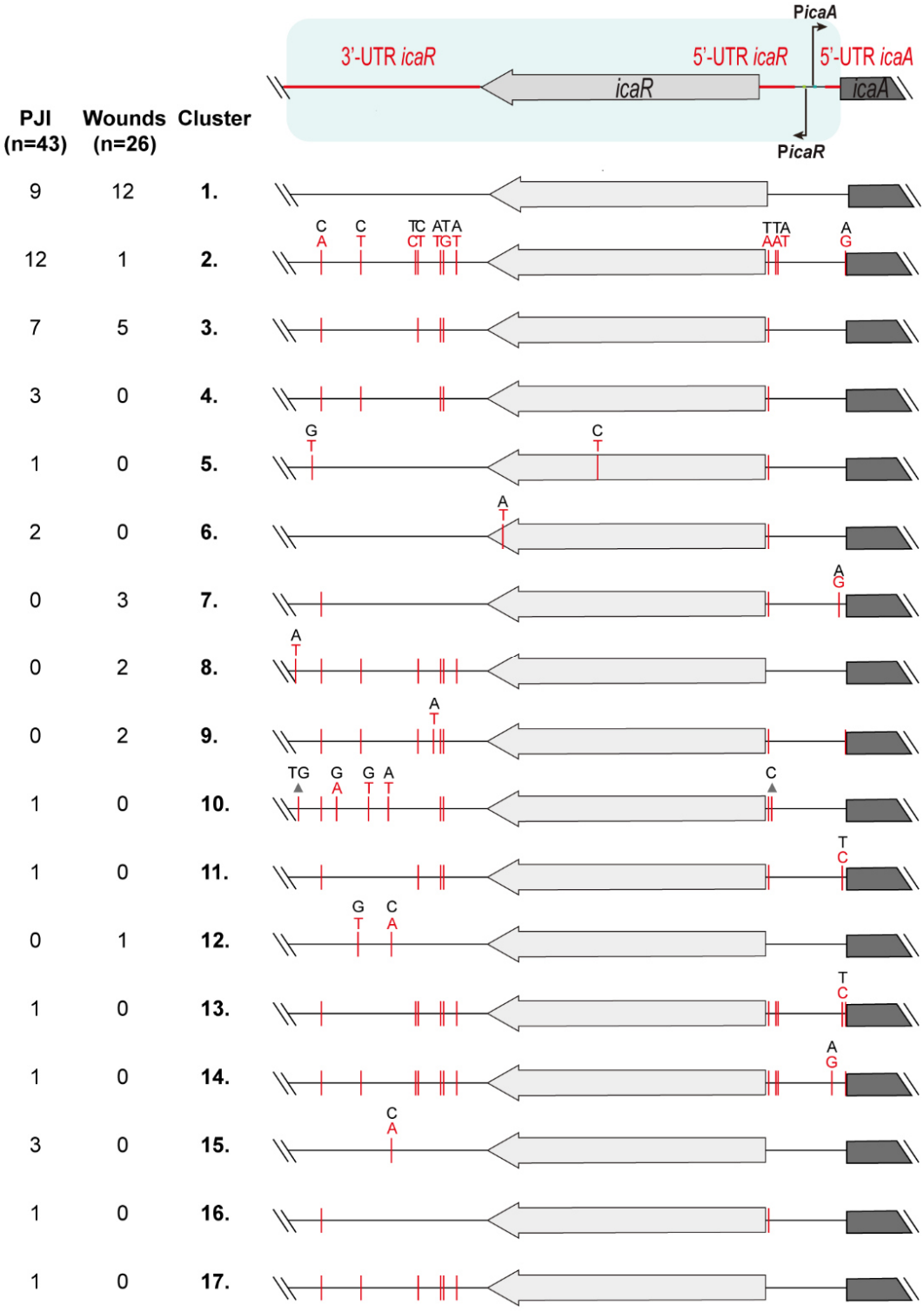


**Figure 2. Analysis of genetic variations in IGRs controlling expression of the *icaADBCR* locus.** PJI and wound isolates were grouped into seventeen different clusters according to SNPs present in the 3'UTR of *icaR*, the *icaR* coding sequence, and the IGR between *icaR* and *icaA* when compared to the sequence of the reference strain MW2 (cluster 1). The red lines show the SNPs or indels found. All sequence variations found in cluster two are depicted (nucleotide changes from a black to a red nucleotide in the upper strand). In the rest of the clusters, only the sequence variations that are different from the ones found in cluster two are detailed.

***Contribution of SNPs in the regulatory IGRs of the icaADBCR locus to PIA/PNAG production capacity***

SNPs and small indels within IGRs can have major phenotypic consequences. To identify a biofilm phenotypic alteration associated to the SNPs found in the regulatory IGRs of the *icaADBCR* locus, we selected one PJI isolate of the most representative clusters (clusters one to four), and also one isolate of clusters five and six, encoding *icaR* mutants. Next, we determined their biofilm formation capacity *in vitro*, including as a control the *S. aureus* 15981 strain that forms a strong PIA/PNAG dependent biofilm (Valle *et al.*, 2003). All strains were very weak biofilm formers, with no significant differences in their ability to form a biofilm, except for MIC 7018 (cluster 6), that showed a very high biofilm-forming capacity (Figure 3A). Next, we analyzed the production of PIA/PNAG exopolysaccharide by dot-blot. Results showed a direct correlation between the amount of PIA/PNAG produced by each strain and its biofilm formation capacity (Figure 3B). These results suggested that the SNPs found in the regulatory IGRs of the *icaADBCR* locus do not influence the *in vitro* biofilm forming ability of PJI strains. Furthermore, our analyses indicated that a single mutation in the IcaR coding sequence could have a major effect on the *S. aureus* biofilm formation capacity.

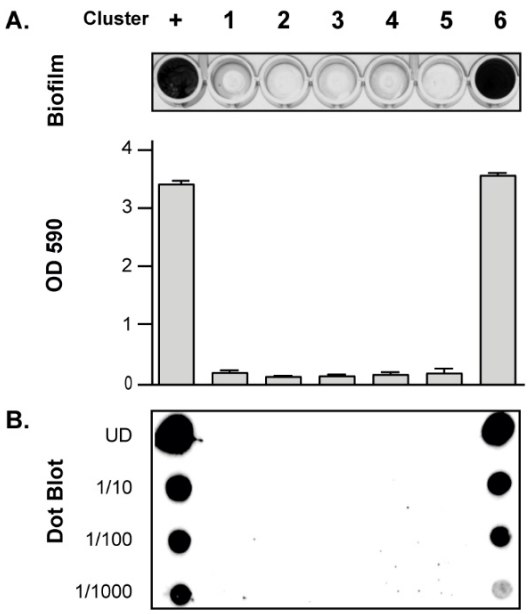



**Figure 3. Biofilm formation capacity and PIA/PNAG production analyses of one representative *S. aureus* PJI isolate of clusters one to six.** Cluster 1: MIC 6935; cluster 2: MIC 6924; cluster 3: MIC 6934; cluster 4: MIC 6936; cluster 5: MIC 6948; cluster 6: MIC 7018. **(A)** Biofilm phenotype on polystyrene microtiter plates after 24 h of growth at 37°C in TSB-NaCl medium. Bacterial cells were stained with crystal violet, and biofilms were quantified by solubilizing the crystal violet with alcohol-acetone and determining the absorbance at $OD_{590nm}$. The error bars represent the standard deviations of the results of three independent experiments. **(B)** Quantification of PIA/PNAG exopolysaccharide biosynthesis by dot-blot. Samples were analysed after 16 h of static incubation in TSB-NaCl at 37°C. Serial dilutions (1/10) of the samples were spotted onto nitrocellulose membranes and PIA/PNAG production was detected with specific anti-PIA/PNAG antibodies. UD; undiluted sample. +; a strong PIA/PNAG dependent biofilm forming strain was included as a control.

To further explore the potential relationship between IGR mutations and PIA/PNAG production, we aimed at quantifying the influence of IGR mutations on transcription of the *icaADBC* genes. To this end, we constructed transcriptional fusions of the intergenic alleles amplified from each representative PJI isolate with the *gfp* gene in the pCN52 plasmid (Figure 4A). The resulting plasmids were transformed into the same recipient strain, that is the *icaADBC* mutant *S. aureus* 132 *ica::tet* (Vergara-Irigaray *et al.*, 2009), in order to avoid differences in genetic background that might interfere with *ica* operon expression. It is important to note that the *S. aureus* 132 strain was chosen because of its ability to produce a polysaccharidic-dependent biofilm when grown in TSB-NaCl (Vergara-Irigaray *et al.*, 2009). As a positive control, we used a reporter plasmid containing the *ica* intergenic allele of the *S. aureus* 15981 strain, which includes the *icaR* gene with a synonymous mutation that results in a premature stop codon. This makes the protein less efficient, leading to a higher expression of the *ica* genes. In agreement with the above results concerning biofilm formation and PIA/PNAG production, GFP was only detectable in *S. aureus* 132 *ica::tet* carrying the reporter constructed from the cluster six isolate, which encodes for a V76E mutant in the carboxyl-terminal region of IcaR (Figure 4A). These results strongly suggested that IcaR is the dominant regulatory component in our experimental conditions and that the contribution of the SNPs in IGRs to *ica* expression, if any, might be unnoticeable in the presence of IcaR.

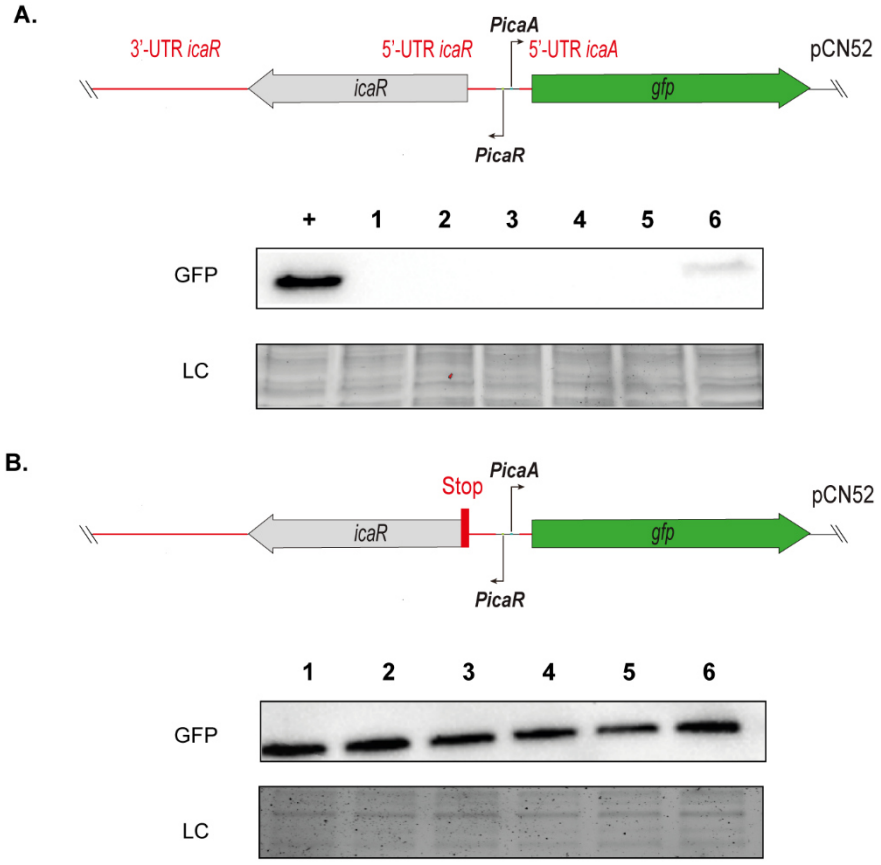



**Figure 4. GFP protein levels in *S. aureus* 132 *ica::tet* expressing *ica* intergenic alleles-*gfp* reporter fusions. (A)** Schematic representation of the transcriptional fusions of the *ica* intergenic alleles amplified from each representative PJI isolate with the *gfp* gene in the pCN52 plasmid. The western blot shows GFP protein levels in the *S. aureus* 132 *ica::tet* strain expressing the reporter fusions. +; a reporter plasmid containing the *ica* intergenic allele of the *S. aureus* 15981 strain was used as a positive control. LC; a stain-free gel portion is included as loading control. **(B)** Analysis using reporter fusions containing a stop codon in the *icaR* coding sequence.

To assess the influence of IGRs on *ica* expression independently of IcaR, we then constructed new reporters of each intergenic allele containing a stop codon in the *icaR* coding sequence (Figure 4B). In this case, *gfp* expression was indeed detected with all the reporters. However, no relevant differences in GFP levels were observed. Together, these results demonstrated that the SNPs present in IGRs of the *icaADBCR* locus found in PJI isolates do not cause variability in the expression of the *icaADBC* operon, at least under the conditions evaluated.

***Increasing PIA/PNAG production leads to a reduction in virulence***

Next, we explored the direct consequences that de-regulation of *icaADBC* operon expression has for the capacity of *S. aureus* to colonize and survive in the host. For that, we used a *G. mellonella* infection model in which larvae were challenged with $10^7$ CFU of the *S. aureus* MW2 wild type strain and its derivative strain MW2 *icaR* V176E. The strain MW2 *icaR* V176E is an isogenic mutant of *S. aureus* MW2 containing a T to A mutation at nucleotide 527 of the chromosomal copy of *icaR*, which is the same mutation present in isolate MIC 7018 (cluster 6). This isogenic mutant strain exhibited a significantly increased PIA/PNAG-dependent biofilm formation capacity compared to the MW2 wild type strain. (Figure 5). Survival assays revealed a significant reduction in virulence of MW2 *icaR* V176E strain ($p$<0.01) compared to the wild type strain (Figure 6). These results showing that an increase in PIA/PNAG production is unfavorable for bacterial survival in the host confirmed that *ica* operon expression and

PIA/PNAG production have to be tightly regulated for *S. aureus* success as a pathogen.

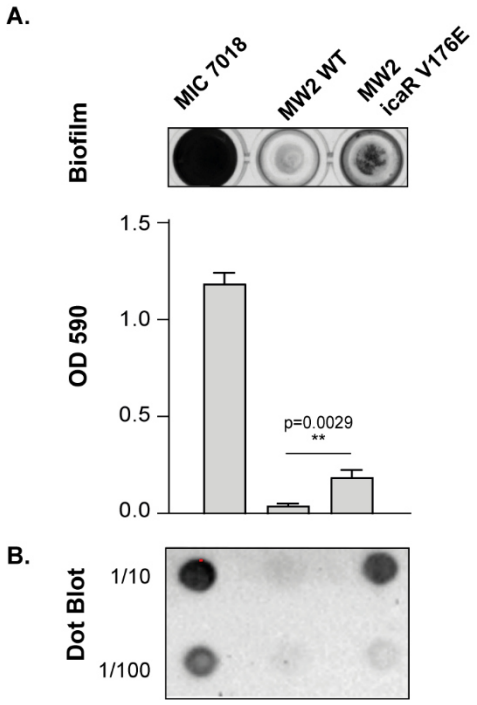



**Figure 5. Biofilm formation capacity and PIA/PNAG production of the MW2 *icaR* V176E mutant. (A)** For comparison, the MIC 7018 strain (cluster 6) and the MW2 wild type strains were included in the analysis. Biofilm phenotype on polystyrene microtiter plates was visualized after 24 h of growth at 37°C in TSB-NaCl medium. The biofilm was stained with crystal violet, and the biofilm biomass was quantified by solubilizing the crystal violet with alcohol-acetone and determining the absorbance at $OD_{590nm}$. The data are shown as mean ± SD of three independent experiments. Statistical analysis was performed by two-tailed unpaired t-test**. (B)** Quantification of PIA/PNAG exopolysaccharide biosynthesis by dot-blot. Samples were analysed after 16 h of static incubation in TSB-NaCl at 37°C. Serial dilutions (1/10) of the samples were spotted onto nitrocellulose membranes and PIA-PNAG production was detected with specific anti-PIA-PNAG antibodies.

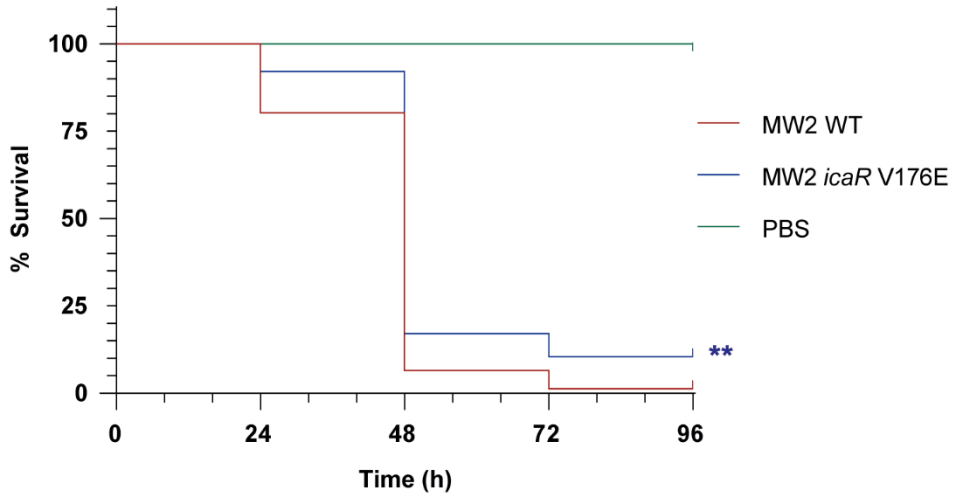



**Figure 6. *Galleria mellonella* infection with *S. aureus* MW2 wild type and MW2 *icaR* V176E mutant strains.** Groups of larvae (25/group) were inoculated with 10 µL PBS (uninfected control group) or bacterial suspensions containing $10^7$ CFU of the corresponding strain, into the last proleg and incubated at 37°C. Worms were checked daily, and any deaths were recorded every 24 h, for a total of four days. Three independent experimental trials were performed. Survival data were plotted using the Kaplan-Meier method and expressed as a percentage of survival versus time. Statistically significant differences were determined using the log rank test (**, $p<0.01$).

# Chapter II

## Functional analysis of intergenic regulatory regions of genes encoding surface adhesins

**Summary**

*Staphylococcus aureus* is a leading cause of prosthetic joint infections (PJI). Surface adhesins play an important role in the primary attachment to plasma proteins that coat the surface of prosthetic devices after implantation. Previous efforts to identify a genetic component of the bacterium that confers an enhanced capacity to cause PJI have focused on gene content, kmers, or single-nucleotide polymorphisms (SNPs) in coding sequences. Here, using a collection of *S. aureus* strains isolated from PJI and wounds, we investigated whether genetic variations in the regulatory region of genes encoding surface adhesins lead to differences in their expression levels and modulate the capacity of *S. aureus* to colonize implanted prosthetic devices. The data revealed that *S. aureus* isolates from the same clonal complex (CC) contain a specific pattern of SNPs in the regulatory region of genes encoding surface adhesins. As a consequence, each clonal lineage shows a specific profile of surface proteins expression. Co-infection experiments with representative isolates of the most prevalent CCs demonstrated that some lineages have a higher capacity to colonize implanted catheters in a murine infection model, which correlated with a greater ability to form a biofilm on coated surfaces with plasma proteins. Together, results indicate that differences in the expression level of surface adhesins may modulate the propensity of *S. aureus* strains to cause PJI. Given the high conservation of surface proteins among staphylococci, our work lays the framework for investigating how diversification at intergenic regulatory regions affects the capacity of *S. aureus* to colonize the surface of medical implants.

## Results

### *Identification of genetic variations in the regulatory regions of genes encoding surface-associated adhesins*

Surface-associated adhesins play an important role in the initial attachment to plasma proteins that cover the surface of prosthetic devices after implantation (Anderson *et al.*, 2008; Clarke & Foster, 2006; Foster, 2019b). We explored if the differences in the presence and/or expression levels of genes encoding surface adhesins in clinical isolates of *S. aureus* might modulate the initial attachment and capacity of *S. aureus* to colonize implanted prosthetic devices. To explore this hypothesis, we examined a collection of clinical strains isolated from PJI (*n*=45) and wounds (*n*= 26) (Trobos *et al.*, 2022) (Figure 1) for the presence/absence of twenty-three genes encoding CWA proteins and non-covalently associated surface proteins of the SERAM family.

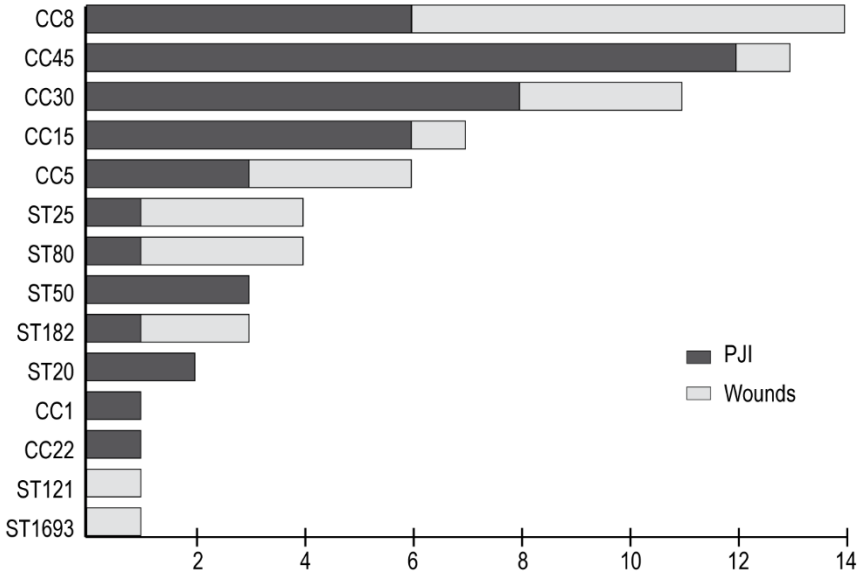



**Figure 1. *Staphylococcus aureus* diversity between PJI and wounds isolates.** Seventy-one clinical isolates from PJI (*n*=45) and wounds (*n*=26) were clustered in genetically related groups according to multi-locus sequence typing (MLST) classification.

The results revealed that the genes *fnbA, clfA, clfB, sdrC, spa, sasC, sasE* (*isdA*), *sasF, sasH* (*adsA), sasI* (*isdH*), *sasJ* (*isdB*), *eap, emp, vwb* and *efb* were present in all strains, whereas the genes *fnbB, cna, sdrD, sdrE, sasA* (*srAp*), *sasD, sasG* and *fmtB* (*sasB*) were absent in some of them (Table 1). For instance, isolates from lineages CC15 and CC5 and most of the isolates from CC8 (93%) contained *sasG*, whereas none of the isolates from CC30 and CC45 contained this gene. On the contrary, the *cna* gene was present in all the isolates from CC45 and CC30 and was absent in CC15, CC5 and most of the CC8 (86%) isolates (Table 1). With respect to genes whose presence varied within the same lineage, no correlation was observed between their presence/absence and the source of infection (PJI or wounds).

**Table 1.** Prevalence of genes encoding surface adhesins in PJI and wounds isolates[a].

| **PJI** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **CC (*n*)** | ST (*n*) | Prevalence (%) | | | | | | | |
| | | *fnbB* | *cna* | *sdrD* | *sdrE* | *sasA* | *sasD* | *sasG* | *fmtB* |
| **CC45 (12)** | ST45 (12) | 75 | 100 | 83 | 83 | 100 | 100 | 0 | 100 |
| **CC30 (8)** | ST30 (8) | 88 | 100 | 100 | 88 | 100 | 100 | 0 | 100 |
| **CC8 (6)** | ST8 (1) | 100 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| | ST630 (4) | 75 | 0 | 100 | 75 | 0 | 100 | 100 | 100 |
| | ST789 (1) | 100 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| **CC15 (6)** | ST15 (5) | 100 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| | ST3457 (1) | 100 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| **CC5 (3)** | ST5 (3) | 100 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| **CC22 (1)** | ST22 (1) | 0 | 100 | 100 | 0 | 0 | 0 | 100 | 100 |
| **CC1 (1)** | ST1 (1) | 100 | 0 | 100 | 100 | 100 | 100 | 0 | 100 |
| | ST50 (2) | 100 | 100 | 100 | 100 | 100 | 100 | 0 | 100 |
| | ST20 (2) | 50 | 0 | 50 | 100 | 100 | 100 | 100 | 100 |
| | ST25 (1) | 100 | 100 | 100 | 100 | 100 | 100 | 0 | 100 |
| | ST80 (1) | 100 | 0 | 100 | 0 | 100 | 100 | 0 | 100 |
| | ST50 (1) | 100 | 0 | 0 | 100 | 100 | 100 | 100 | 100 |
| | ST182 (1) | 100 | 100 | 0 | 0 | 100 | 0 | 100 | 0 |
| **Wounds** | | | | | | | | | |
| **CC8 (8)** | ST8 (2) | 100 | 0 | 100 | 100 | 100 | 100 | 50 | 100 |
| | ST254 (1) | 100 | 0 | 100 | 0 | 100 | 100 | 100 | 100 |
| | ST247 (3) | 33 | 0 | 100 | 33 | 100 | 100 | 100 | 100 |
| | ST239 (2) | 100 | 100 | 100 | 50 | 100 | 100 | 100 | 100 |
| **CC30 (3)** | ST30 (3) | 33 | 100 | 100 | 100 | 100 | 100 | 0 | 100 |
| **CC5 (3)** | CC5/ST5 (1) | 100 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| | CC5/ST225 (1) | 100 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| | CC5/ST1649 (1) | 100 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |

[a] The *fnbA, clfA, clfB, sdrC, spa, sasC, sasE, sasF, sasH, sasI, sasJ, eap, emp, vw*b and *efb* genes are not included because they are present in all strains.

**Table 1.** Prevalence of genes encoding surface adhesins in PJI and wounds isolates[a]. (Cont.)

| **Wounds** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **CC (*n*)** | ST (*n*) | Prevalence (%) | | | | | | | |
| | | *fnbB* | *Can* | *sdrD* | *sdrE* | *sasA* | *sasD* | *sasG* | *fmtB* |
| **CC45 (1)** | ST45 (1) | 0 | 100 | 100 | 100 | 100 | 100 | 0 | 100 |
| **CC15 (1)** | ST15 (1) | 100 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| | ST25 (3) | 100 | 0 | 100 | 100 | 100 | 100 | 0 | 100 |
| | ST80 (3) | 33 | 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| | ST182 (2) | 100 | 100 | 0 | 0 | 100 | 0 | 100 | 0 |
| | ST121 (1) | 100 | 100 | 100 | 100 | 100 | 0 | 0 | 100 |
| | ST1693 (1) | 0 | 100 | 100 | 100 | 100 | 0 | 0 | 100 |

[a] The *fnbA, clfA, clfB, sdrC, spa, sasC, sasE, sasF, sasH, sasI, sasJ, eap, emp, vw*b and *efb* genes are not included because they are present in all strains.

We next explored the possibility that genetic variations between isolates might be located in the regulatory regions controlling the expression of adhesin proteins. Thus, we compared a sequence of 200 nt upstream the first codon of each of the 15 genes encoding surface adhesins present in all the strains with the corresponding sequence in the reference genome of *S. aureus* MW2 strain (CC1). The conservation rate of the sequence in the regulatory regions was highly variable depending on the gene (Figure 2 and Supplementary figure S1). The sequence of the regulatory region of *sasE* and *fnbA* genes was highly conserved with variation rates below 5%. In contrast, the sequence of the regulatory region of *sdrC* showed a variation rate of 28% (Table 2). Notably, the SNPs were highly conserved between isolates of the same sequence type, regardless of whether the strain was isolated from PJI or wounds. Taken

together, these results indicated that SNPs variations in the regulatory region of surface adhesin genes are characteristic of each *S. aureus* ST lineage.

**Table 2.** Variation rates in the regulatory region of genes encoding surface adhesins.

| Gene name | Number of SNPs | Variation Rate (%)[a] |
|---|---|---|
| *sasE* (*isdA*) | 5 | 3 |
| *fnbA* | 7 | 5 |
| *vwb* | 11 | 6 |
| *emp* | 13 | 7 |
| *clfB* | 14 | 7 |
| *sasF* | 17 | 9 |
| *sasJ* (*isdB*) | 22 | 11 |
| *efb* | 22 | 11 |
| *spa* | 22 | 14 |
| *sasC* | 34 | 17 |
| *sasI* (*isdH*) | 39 | 20 |
| *eap* | 42 | 21 |
| *clfA* | 44 | 22 |
| *sasH* (*adsA*) | 47 | 24 |
| *sdrC* | 56 | 28 |

[a] The nucleotide variation rate in each region was calculated using the genomic sequences of the 71 PJI and wound isolates, and the percentage represents the ratio between the total number of nucleotide changes in at least one *S. aureus* isolate, and the length of the analysed sequence multiplied by 100. The promoter sequence analysed was 200 bp long except for *fnbA* and *spa*, which were 134 and 155 bp, respectively.

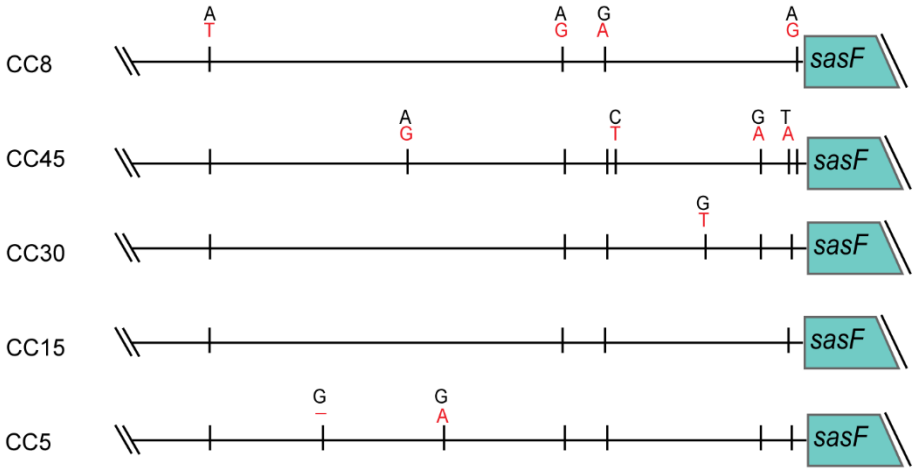



**Figure 2. Analysis of genetic variations in the regulatory region controlling the expression of surface-associated adhesins.** The region controlling *sasF* expression is shown as an example. PJI and wound isolates were grouped into twelve different clusters according to the SNPs present in the region comprising 200 nt upstream of the *sasF* gene compared to the sequence of the reference strain MW2. The most representative sequence variant for the most prevalent clonal complexes is shown. The black lines show the SNPs or indels found. All sequence variations found in CC8 are depicted (nucleotide changes from a black to a red nucleotide in the upper strand). In the rest of CCs, only sequence variations that are different from the ones found above are detailed. Eleven SNPs were identified among the representative sequence variants; none of the strains contained the same sequence as the MW2 reference strain.

***Contribution of SNPs to surface adhesins expression***

We next wondered whether the SNPs present in IGRs may have an impact on surface adhesins expression levels. For that, we first used a commercial polyclonal antibody (Bio-Rad, Hercules, CA, USA) raised against soluble and structural antigens of the whole bacterium to compare the overall expression levels of CWA and secreted proteins between selected strains from different CCs: CC15 (MIC 6935), CC8 (MIC 6947), CC45 (MIC 6981), and CC30 (MIC 6982). To identify the CWA proteins, we constructed sortase A (*srtA*) mutants for each strain. Our reasoning was that those bands that disappear in the absence of the SrtA activity would correspond to CWA proteins; regarding secreted proteins, no differences between wild-type and isogenic *srtA* mutants should be observed, therefore secreted proteins profiles are shown as a control. We generated *srtA* mutants for CC18, CC45 and CC30 strains. However, we were unable to genetically manipulate and generate the *srtA* mutant in the CC15 strain. Western blot analysis revealed differences in the levels of CWA proteins between isolates of different CCs (Figure 3).

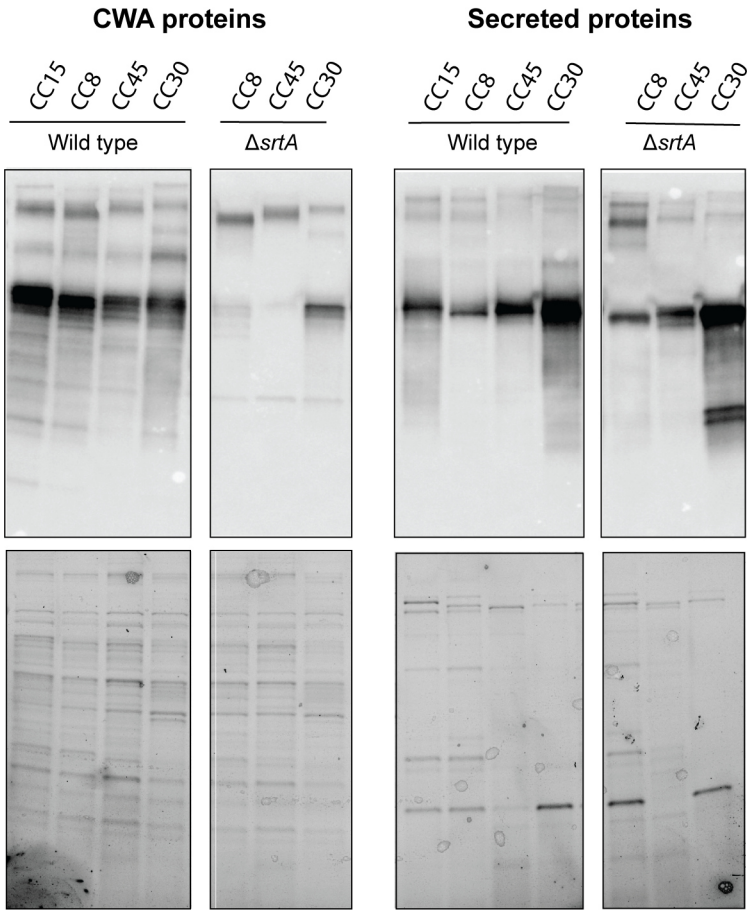



**Figure 3. Profiles of cell-wall-anchored and secreted proteins from *S. aureus* clinical isolates from different CCs.** Western immunoblotting results showing the CWA and secreted proteins in clinical isolates of *S. aureus*. Samples from CC15, CC8, CC45 and CC30, and their corresponding isogenic *srtA* mutants, grown in TSB-gluc were separated on 12% acrylamide gels (upper panel) and probed with a commercial *S. aureus* polyclonal antibody (Bio-Rad, Hercules, CA, USA) that reacts with soluble and structural antigens of the whole bacterium**.** Stain-free gels are shown as loading controls (lower panel). Note that we were unable to genetically manipulate strain MIC 6935 (CC15) and thus, its *srtA* mutant could not be included in the analysis.

To specifically investigate the contribution of the SNPs in IGRs to the expression of surface adhesins without the interference of particular regulators that control the expression of surface adhesins in each strain, the region encompassing 200 nt upstream of the first codon of each adhesin-encoding gene present in the representative PJI isolates of CC15, CC8, CC45 and CC30 was fused with the *gfp* gene in the pCN52 plasmid. Then, the resulting plasmids were inserted in the *S. aureus* 132 strain, which is able to produce a proteinaceous-biofilm matrix when grown in TSB-gluc medium (Vergara-Irigaray *et al.*, 2009). The effect of the genetic variations in IGRs on GFP expression levels was analyzed by western blot. The results revealed large differences in the strength of the promoters controlling the expression of each surface adhesin. Of note, in general, expression from the regulatory region of *spa, clfA, clfB*, *vwb* and *efb* genes was high when compared to other regions such as the ones controlling expression of *sdrC*, *sasC*, *sasE*, *sasI*, *sasJ*, *eap* and *emp* (Figure 4).

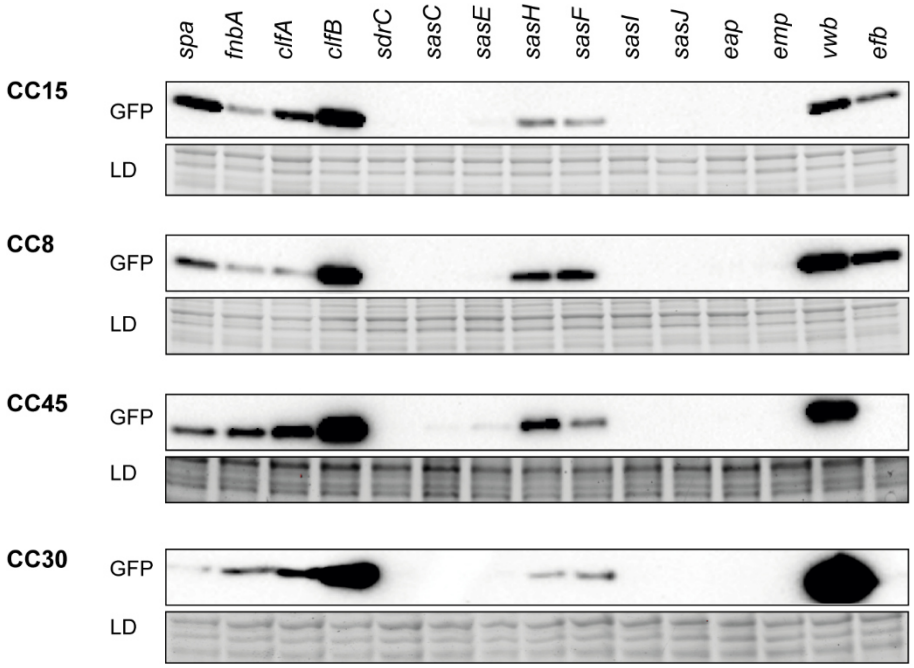



**Figure 4. Comparison of surface adhesins expression levels within the same strain.** Western blots show GFP protein levels in *S. aureus* 132 strain transformed with plasmids containing reporter fusions of 200 nt upstream the initial codon of 15 surface adhesin-encoding genes, amplified from the same isolate, with the *gfp* gene. One representative *S. aureus* PJI isolate of each of the four most abundant clonal complexes CC15, CC8, CC45 and CC30 was used for amplification of regulatory sequences. Bacteria were grown until the exponential phase and proteins were transferred to nitrocellulose membranes, incubated with anti-GFP monoclonal antibodies and developed using peroxidase-conjugated goat anti-mouse antibodies and a bioluminescence kit. Stain-free gels are shown as loading controls (LD).

Next, we compared the expression of *gfp* under the regulatory region of each surface adhesin-encoding gene amongst the four different CCs. The results showed variability in the expression pattern of *spa, sdrC, sasC, sasF, sasH, sasI, sasJ, emp, eap, vwb* and *efb* genes, indicating that the SNPs present in the regulatory regions of the genes encoding for surface adhesins affect their transcription levels (Figure 5). The level of expression of *fnbA, clfA, clfB* and *sasE* genes remained unchanged among the isolates of different CCs, indicating that the SNPs present in the regulatory region of these genes were not involved in transcriptional and/or post-transcriptional regulation of such genes expression. Collectively, these results provided robust evidence that surface adhesin expression levels vary among *S. aureus* ST lineages due to sequence variations in IGRs of the corresponding genes.

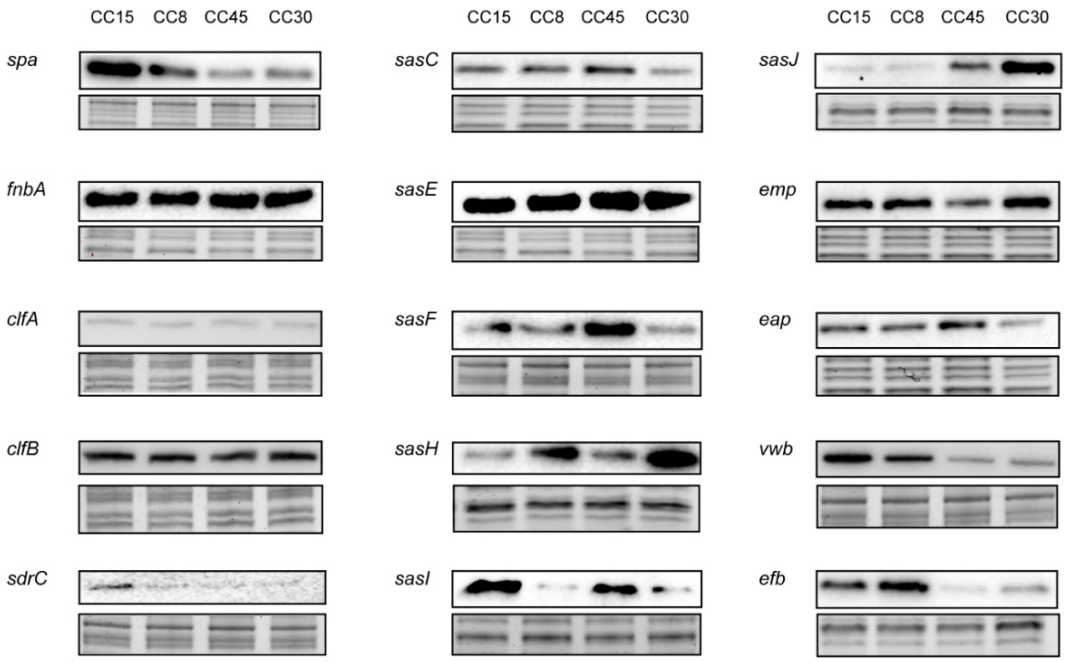



**Figure 5. Comparison of surface adhesins expression levels in strains from different CCs.** Western blots show GFP protein levels in *S. aureus* 132 strain transformed with plasmids containing reporter fusions of 200 nt upstream the initial codon of each surface adhesin-encoding genes, amplified from representative *S. aureus* PJI isolates of the four most abundant CCs, with the *gfp* gene. Bacteria were grown until the exponential phase and proteins were transferred to nitrocellulose membranes, incubated with anti-GFP monoclonal antibodies and developed using peroxidase-conjugated goat anti-mouse antibodies and a bioluminescence kit. Membrane exposure was adjusted in each case to allow band visualization in all cases. Stain-free gels are shown as loading controls.

***Comparison of the in vivo colonization capacity of S. aureus isolates of different CCs***

Taking into account the above results demonstrating that the strength of the promoters encoding for surface adhesins varies between *S. aureus* isolates of different CCs, we hypothesized that differences in the capacity to colonize implanted prostheses might occur. To investigate this possibility, we carried out an *in vivo* catheter colonization assay with a mixture containing four strains representative of the four CCs that most frequently caused prosthesis infections in our PJI collection. An intrinsic difficulty in co-infection experiments is that a selective analytical method is required in order to distinguish and quantify each strain in the mixture. To address this methodological challenge, we amplified a 200 nt region upstream of the *sasJ* and *clfB* genes that contain the specific SNPs of each CC (Figure 6A) (Zahariev *et al.*, 2018). To validate the selected DNA signatures, we prepared *in vitro* mock mixtures containing different proportions of the four isolates, total DNA was purified, PCR amplified and sequenced. The reads were processed and mapped to *sasJ* and *clfB* regions. The total number of reads that passed a quality filter was higher than 99% for both P*sasJ* and P*clfB*, respectively. The results confirmed that the number of mapped reads obtained corresponding to each DNA signature was proportional to the amount of each bacterium in the original mixture (Figure 6B). We next used this strategy to determine the proportion of each isolate on the surface of catheters implanted in the subscapular space of mice.

A mixture of equal numbers ($10^7$ CFU) of the four representative strains was used to colonize the implanted catheters (Figure 7) and after five days,

catheters were recovered, DNA was purified, and the amount of each bacterium was quantified based on the abundance of reads for each DNA signature. The results revealed that read abundance corresponding to strains from CC15 and CC8 was significantly higher than the reads corresponding to strains from CC45 and CC30 (Figure 6C). No significant differences were found between the number of reads corresponding to CC15 and CC8 strains and CC30 and CC45 strains. Together, these results evidenced that *S. aureus* isolates show CC dependent variation of the capacity to colonize an implanted catheter.

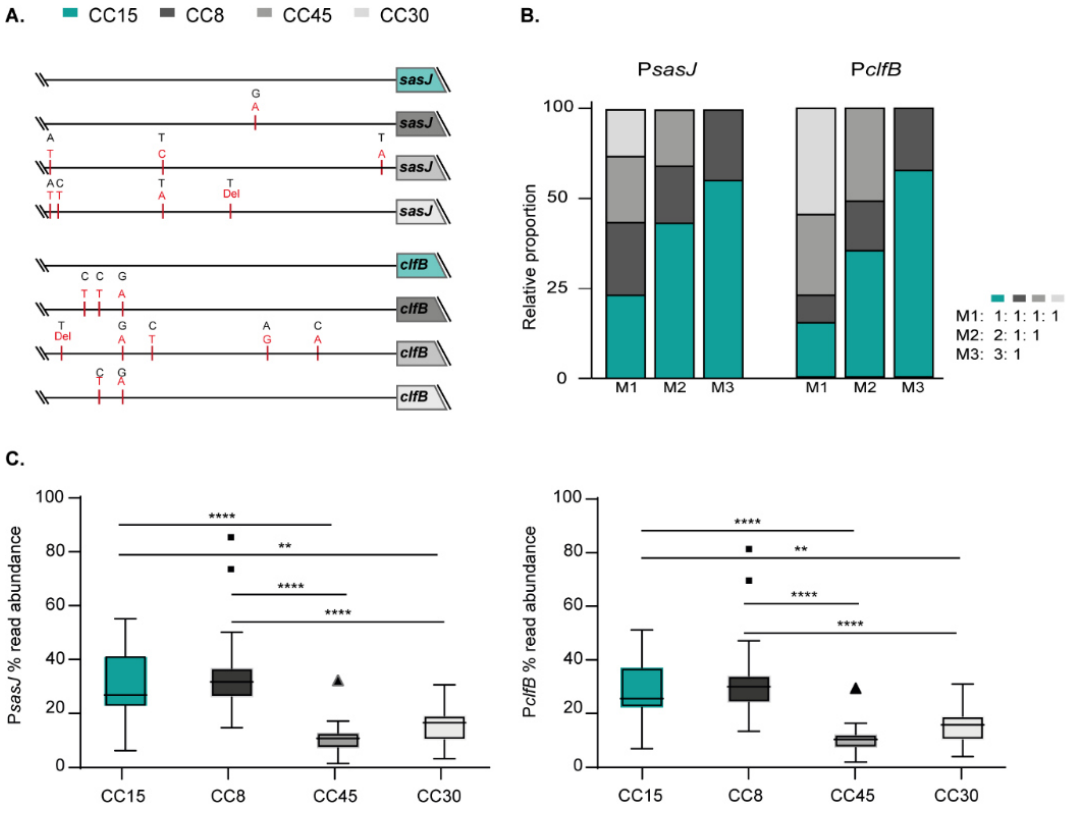


**Figure 6. DNA signatures for quantifying isolates from different clonal lineages during co-infection experiments of catheters in a mouse model. (A)** Natural sequence variants of the regulatory regions of *sasJ* and *clfB* genes from representative strains ascribed to CC15, CC8, CC45, and CC30. The red lines show the SNPs or indels found compared with the CC15 sequence (nucleotide changes from a black to a red nucleotide in the upper strand). **(B)** Relative proportion of each isolate based on the number of reads corresponding to sequence variants. The results represent the mean of three independent *in vitro* experiments ($n$=3). M1: Mixture one (equal proportion of each of the four strains); M2: Mixture two (double proportion of CC15 strain than CC8 and CC45); M3: Mixture three (triple proportion of CC15 strain than CC8). **(C)** Differences in colonization capacity of *S. aureus* isolates from different clonal complexes in a murine model of catheter colonization. Percentage of reading abundance after 5 days of infection according to the number of reads of sequence variants of *sasJ* and *clfB* regulatory regions. Catheters were coinfected with equal amounts ($10^7$ CFU) of representative *S. aureus* PJI isolates ascribed to the most prevalent clonal complexes. CC15: MIC 6935; CC8: MIC 6947; CC45: MIC 6981; CC30: MIC 6982. The boxes indicate the range between the first and third quartiles ($25^{th}$ and $75^{th}$ percentiles). The horizontal line inside the box is the median. The size of the box represents the interquartile range (IQR). The whiskers indicate the spread of data outside the box at up to 1.5 times the IQR from the edge of the box (1.5 times the size of the box below the $25^{th}$ percentile or above the $75^{th}$ percentile). Data further than 1.5 times the IQR from the box are designated outliers and plotted individually. Statistically significant differences were determined using one-way ANOVA, followed by Tukey´s multiple comparison (**, $p$<0.01), (***, $p$<0.001),and (****, $p$<0.0001).

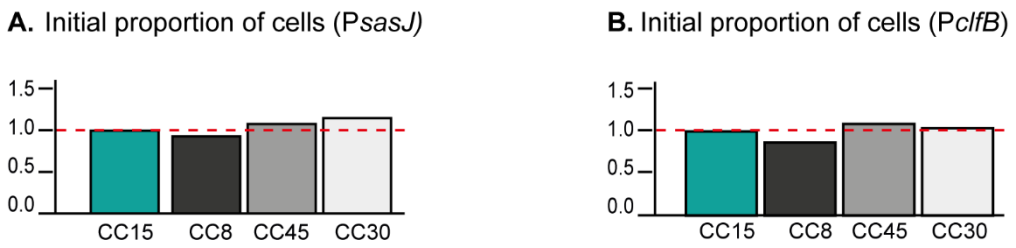



**Figure 7. Validation of the initial proportion of strains in the inoculum used for coinfection experiments.** DNA extracted from the initial mixture was sequenced, and the proportions were calculated based on the percentage of relative abundance.

If differences in the ability of the strains from the different clonal groups to adhere to implanted catheters were due to differences in their adhesion to plasma proteins coating the catheter surface, similar variances in their capacity to adhere to abiotic surfaces covered with plasma proteins *in vitro* could be expected. To explore this hypothesis, we performed primary attachment assays on polystyrene pre-coated with fibronectin (Fn), fibrinogen (Fg) and the von Willebrand factor (VWF) (Figure 8). The results revealed that the representative strains from CC15 and CC8 showed a significantly higher capacity than isolates from CC30 and CC45 to adhere to polystyrene surfaces coated with Fn and Fg, whereas in the case of surfaces coated with VWF, all isolates showed a similar primary adhesion capacity. Interestingly, isolates from CC15 and CC8 lineages also showed a higher capacity to adhere to uncoated polystyrene surfaces than isolates from CC30 and CC45. Notably, adhesion of CC15 and CC8 isolates to the uncoated surface was lower than to Fn and Fg coated surfaces. Together, these results demonstrated that the strains winning the race for colonization of the

surface of the implant *in vivo* also displayed a higher capacity to colonize abiotic surfaces coated with plasma proteins *in vitro*.

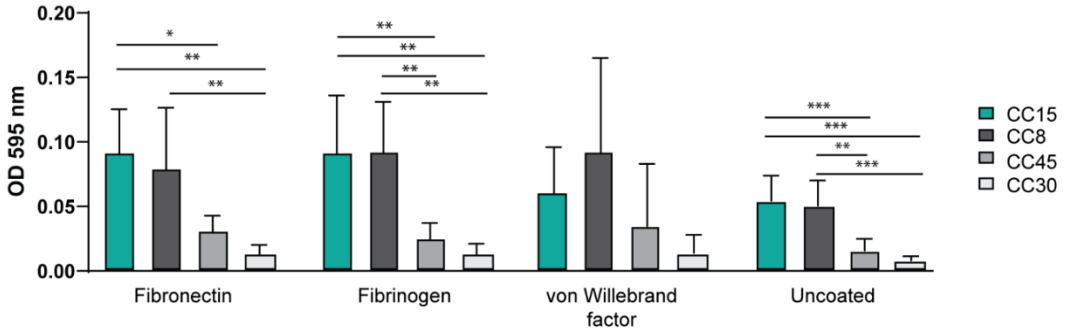
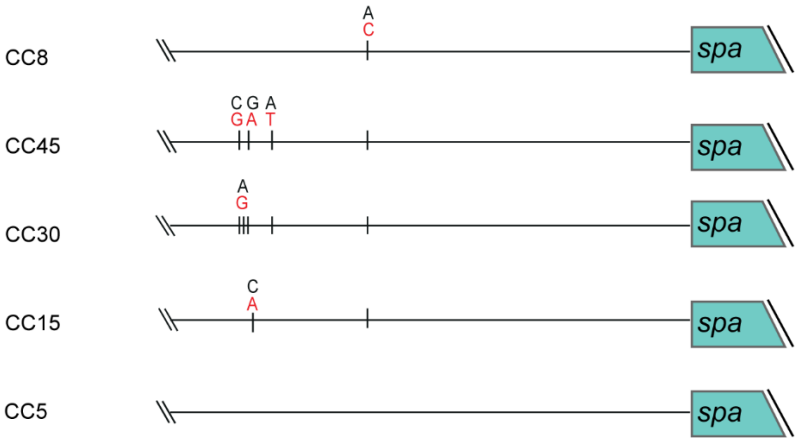
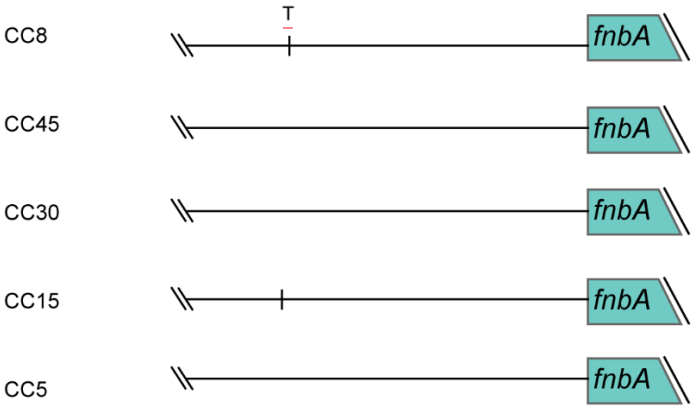



**Figure 8. Biofilm biomass formed by representative *S. aureus* PJI isolates from CC15, CC8, CC45 and CC30 on protein coated surfaces.** The wells of polystyrene plates were pre-coated with 5 µg/mL of fibronectin, fibrinogen, and the von Willebrand factor and kept overnight at 4°C. Static biofilms were grown in TSB-gluc on the pre-coated 48-well plates for 5 h. Biofilm formation was quantified by crystal violet staining, followed by 80:20 ethanol: acetone elution and $OD_{595nm}$ measurement. The data represent the mean of six biological replicates ($n$=6) with two technical replicates; error bars represent ± SD. Statistically significant differences were determined using one-way ANOVA, followed by Tukey´s multiple comparisons (*, $p$<0.05), (**, $p$<0.01), (***, $p$<0.001).

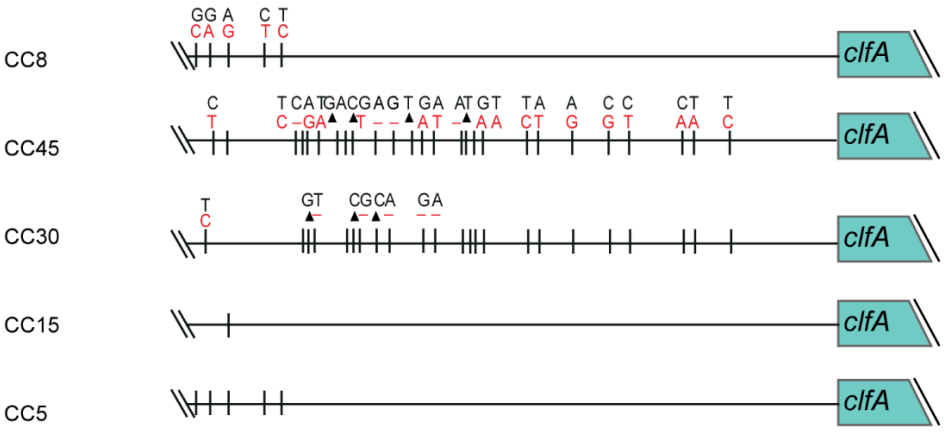
**A. *spa***



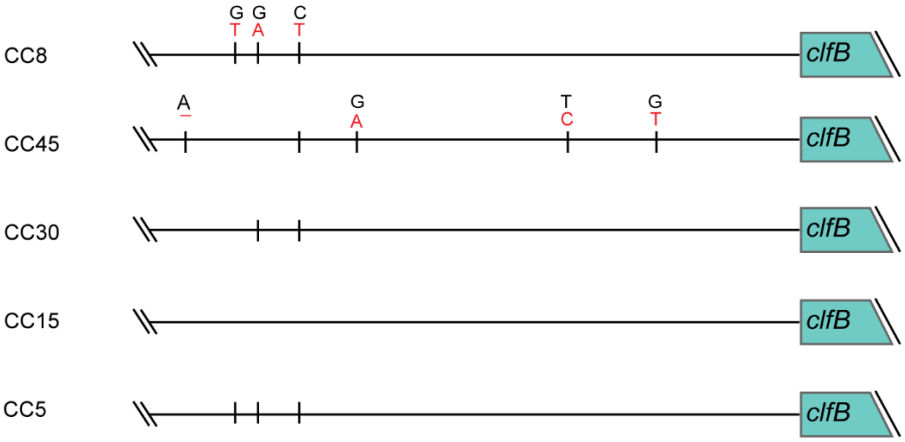

**B. *fnbA***



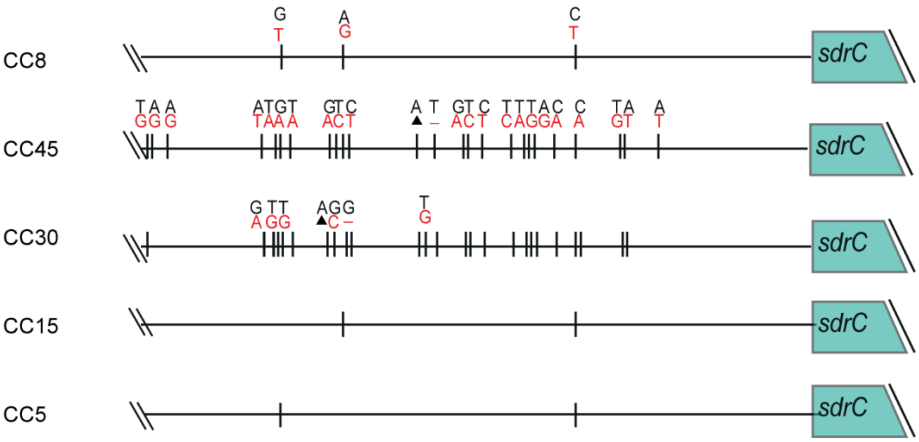

**C. *clfA***



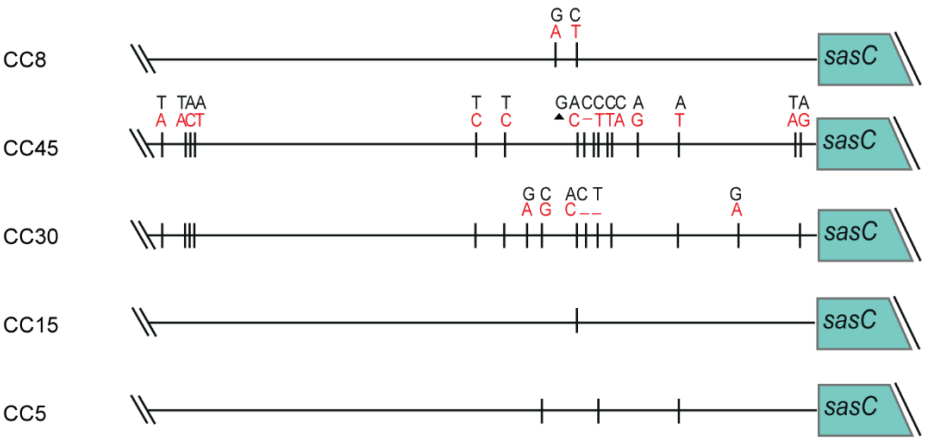
**D. *clfB***




**E. *sdrC***




**F. *sasC***



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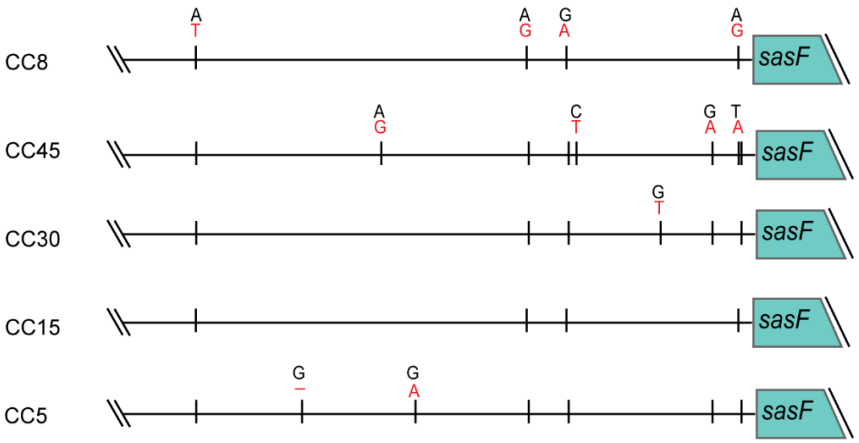


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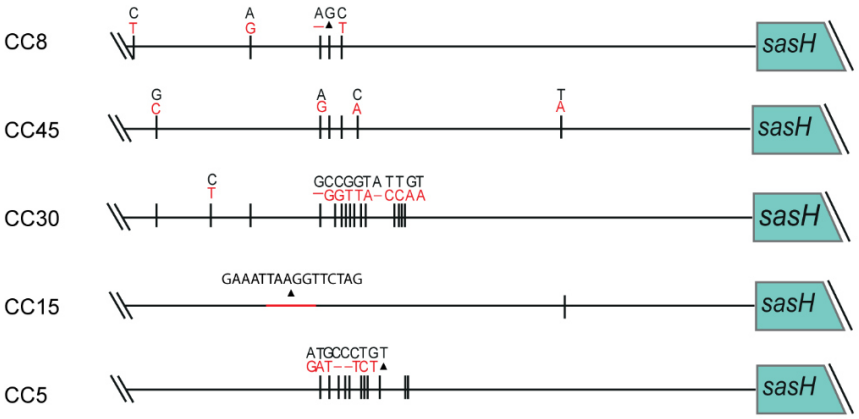
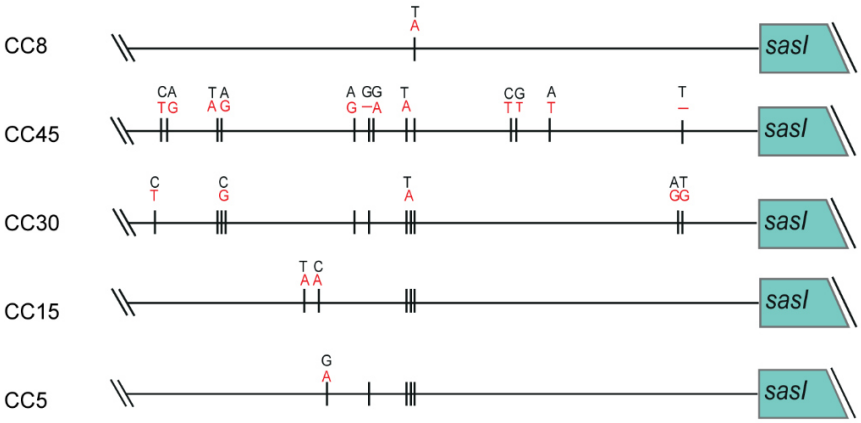
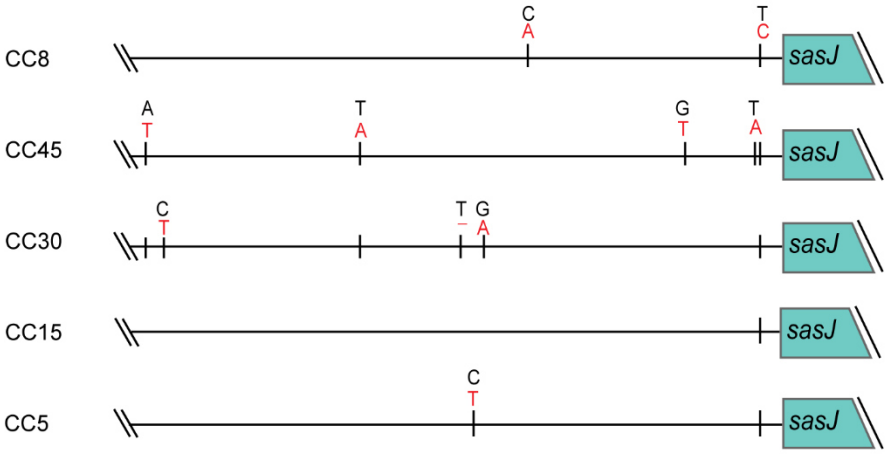


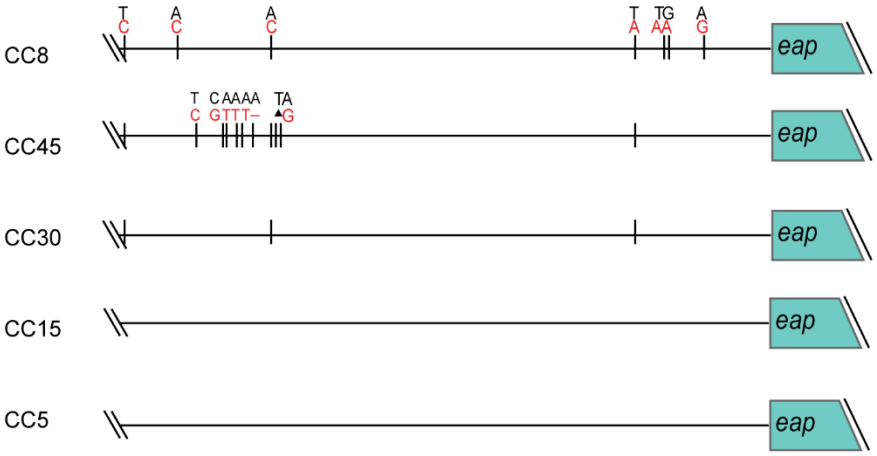

**I. *sasI***



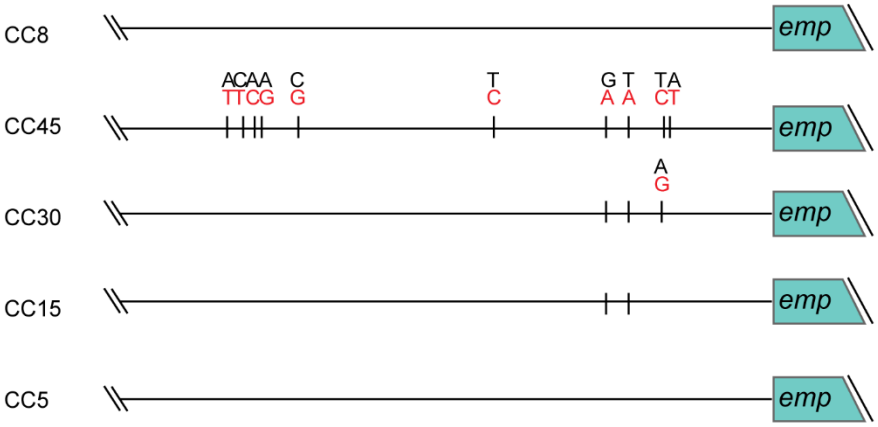
**J. *sasJ***



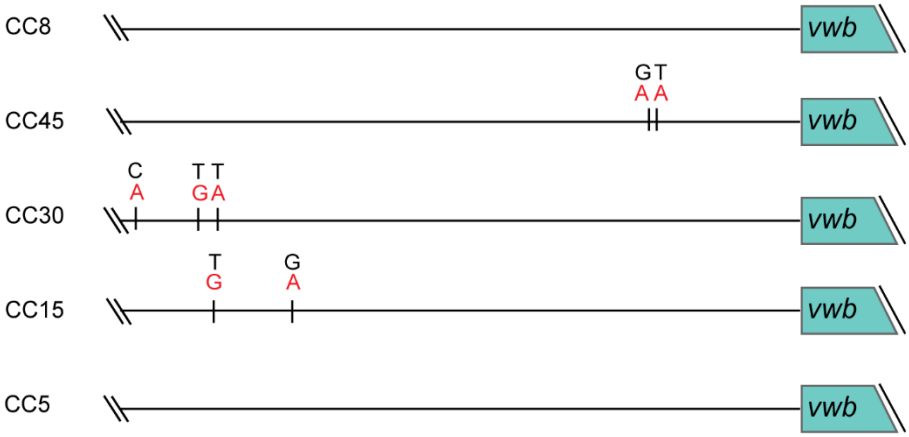

**K. *eap***



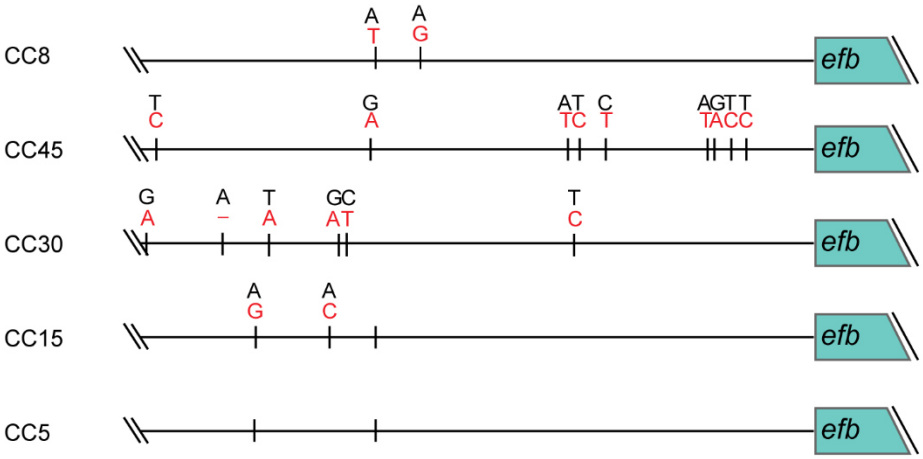

**L. *emp***



**M. *vwb***




**N. *efb***



**Supplementary figure S1. Representative sequence variants in IGRs controlling expression of each adhesin encoding gene present in strains of the most prevalent clonal complexes.** PJI and wound isolates were grouped into different clusters according to the SNPs present in the region comprising 200 nt upstream of each gene (except for *fnbA* and *spa*; 134 and 155 bp, respectively) compared to the sequence of the reference strain MW2. Sequence variations were highly conserved between isolates of the same sequence type. The black lines show the SNPs or indels found. All sequence variations found in CC8 are depicted (nucleotide changes from a black to a red nucleotide in the upper strand). In the rest of CCs, only sequence variations that are different from the ones found above are detailed.

# Discussion and Future Prospects

Infections related to joint prostheses are currently a major public health issue that has a significant impact on morbidity in most developed countries. Surgeons use titanium implants to treat bone diseases of the hip, knee and, to a lesser extent, shoulder. Implant infection can result in deficient osseointegration, that is, a poor structural and functional connection between the implant and the patient's bone. Very often, these patients have to undergo repeated interventions and prolonged antibiotic therapy. Eighty percent of PJI are caused by *Staphylococcus* spp. (Arciola, An, *et al.*, 2005), and in particular, *S. aureus* is the leading cause due to its ability to adhere to and establish biofilms on abiotic surfaces (Otto, 2018; Rosteius *et al.*, 2018). *Staphylococcus* spp. bacteria are usually acquired during surgery or in the early postoperative period, but can also occur by haematogenous seeding. The staphylococcal isolate responsible for the infection can come from the patient's skin, or the skin of the healthcare personnel responsible for their care.

The frequency of a particular staphylococcal isolate causing a prosthesis infection may depend on the number of people colonized by this isolate, which will certainly increase the chances that such bacteria encounter the implant, and/or on the ability of that isolate to colonize the implant every time it meets the surface. The hypothesis behind the second scenario is that some isolates have a greater capacity to adhere and cause infection than others. Following this reasoning, in this thesis, we wondered whether staphylococcal strains recovered from medical devices have adaptative features that make them particularly efficient at causing PJI. Several studies have previously tried to correlate the genetic determinants in *S. aureus* with PJI outcomes (Eichenberger

*et al.*, 2015; Ma *et al.*, 2021; Wildeman *et al.*, 2020). In this respect, a previous study showed no differences in the clonality and prevalence of virulence genes among *S. aureus* strains isolated from PJI and nares, indicating that any clonal complex (CCs) is equally prone to cause PJI (Wildeman *et al.*, 2020). CCs are established based on the nucleotide sequences of internal fragments of a standard set of metabolic housekeeping *loci*, and therefore, CC analysis cannot detect adaptative mutations that occur within IGRs (Enright *et al.*, 2000). On the other hand, in a very recent study, we showed that *S. aureus* strains that produce strong biofilms and *S. epidermidis* strains with resistance to several antibiotics were both significantly associated with unresolved PJI (Trobos *et al.*, 2022). These associations were based on core-genome multilocus sequence typing (cgMLST), phenotypic and genomic traits of strains, and patient infection outcomes. However, again, these epidemiological approaches do not consider the changes that take place in the IGRs as possible sources of variation that make some strains more likely to cause PJI. In this regard, recent studies have shown that changes in IGRs are not necessarily silent (Thorpe *et al.*, 2016, 2017). Ma *et al.* observed an accumulation of SNPs in IGRs adjacent to genes encoding surface adhesins during the PJI outcome (Ma *et al.*, 2021).

One particular difficulty related with the presence of SNPs in IGRs is that confirming their contribution to a particular phenotype requires experimental validation. In the present work, we investigated genetic signatures at the regulatory region of genes important for biofilm formation on medical devices. We validated their contribution to the expression of the genetic determinants involved in biofilm production following a methodological approach based on

reporter sequences containing the regulatory regions of these genetic determinants fused to the *gfp* gene.

In **chapter I,** we focused on mutations particularly placed at the IGRs that control the expression of the *icaADBC* operon, responsible for the production of the PIA/PNAG exopolysaccharide, a major constituent of the biofilm matrix. Interestingly, our results firstly showed that the IGR sequence between *icaR* and *icaADBC* genes is highly conserved, indicating strong restrictions on mutations of functional nucleotides (Blanka *et al.*, 2015; Cui *et al.*, 2014; Khademi *et al.*, 2019). The *icaR* gene and the *icaADBC* operon are transcribed in divergent orientations and therefore the IGR region encompasses the promoters, 5' UTRs and ribosome-binding sites of both *icaR* and *icaADBC*. It has been established that within IGRs, the strength of selective constraint appears to be particularly high immediately upstream of genes (Thorpe *et al.*, 2017). However, in the case of the *icaADBCR* IGR, we observed that the SNP density decreases as a function of the distance from gene start codons, indicating that a strong selective constraint particularly affects the promoter regions of *icaR* and *icaADBC*. This is in agreement with the fact that several transcriptional regulators (SarA, TcaR, IcaR and ArlR) modulate the production of PIA/PNAG exopolysaccharide at a transcriptional level through binding to these promoter regions.

To investigate the potential functional relevance of the SNPs in the *icaADBCR* IGR to the transcription of the *icaADBC* operon, we quantified the impact of a subset of intergenic mutations on the transcription of the *gfp* reporter gene, using transcriptional fusions of both a control sequence and

mutant intergenic alleles. The results showed that mutations in the *icaADBCR* IGR do not affect the transcriptional levels of the downstream *icaADBC* operon. Therefore, the polymorphisms found do not appear to be involved in the regulatory evolution of PIA/PNAG synthesis that might favor biofilm development. On the contrary, our study revealed that a single mutation in the coding region of *icaR* causes a significant increase in PIA/PNAG production. Jeng *et al*. previously described significant differences in the IcaR-binding affinity to its cognate DNA domain when some N-terminal residues (Leu23, Lys33 and Ala35) were substituted with those present in other TetR family transcriptional repressors (Jeng *et al.*, 2008). To the best of our knowledge, this is the first description of a single aminoacid substitution in the carboxyl-terminal domain of IcaR that leads to a significant increase in PIA/PNAG production.

To evaluate the consequences of harboring such an IcaR allele, we mutated the *icaR* gene in the *S. aureus* MW2 reference strain and performed a virulence assay using the *G. mellonella* model. With this approach, we were able to demonstrate that the single *icaR* V176E mutation reduces *in vivo* fitness and, therefore, increases the survival of *G. mellonella*. These results are in agreement with previous studies showing that PIA/PNAG overproduction is associated with a high fitness cost (Brooks & Jefferson, 2014). In particular, Brooks & Jefferson found that an isogenic PIA/PNAG-negative MN8 strain shows an *in vitro* fitness gain compared to the PIA/PNAG-overproducing MN8 strain. Similarly, non-mucoid mutants were selected over time from cystic fibrosis patients firstly colonized with highly mucoid *S. aureus* clones that over produced PIA/PNAG (Schwartbeck *et al.*, 2016). Altogether, these results support the view

that mutations inside coding genes may increase the fitness in a particular trait (in this case, biofilm formation) at the expense of a fitness decrease in another trait (in this case, virulence). On the other hand, adaptative mutations in noncoding DNA may favor subtle changes in the expression of downstream genes while maintaining responsiveness to environmental cues, such as those present on the implanted prosthesis (Coombes, 2013).

One limitation of our experimental approach is that we have evaluated the influence of the SNPs in IGRs under one specific environmental condition. To completely exclude that these SNPs found are not playing any role in *ica* operon expression, it would be necessary to repeat the experiments under different environmental conditions. Furthermore, as stated above, *ica* locus expression is regulated by many different regulators. Therefore, future experiments should also consider variations in IGRs upstream of these regulators as a possible source of variability between PJI isolates in the production of PIA/PNAG. In addition, and since the carboxyl-terminal domain of IcaR is involved in protein dimerization (Jeng *et al.*, 2008), further studies should be conducted to clarify the structural reasons behind the PIA/PNAG hyper-producer phenotype linked to the V176E mutation. Finally, the frequency of such mutation in additional collections of *S. aureus* isolates from PJI should be investigated.

In **Chapter II**, this analysis was extended to the IGRs flanking adhesins encoding genes, which are involved in biofilm development and the colonization of prostheses.

A simple mechanism to generate variability in the capacity to colonize implants between different isolates may be due to variations in the presence/absence of adhesin-encoding genes (McCarthy & Lindsay, 2010; Rasmussen *et al.*, 2013). For instance, SasG, a protein important in promoting biofilm formation during the accumulation phase, is present in strains of CC15 and CC8 whereas it is absent in strains from CC30 and CC45 (Corrigan *et al.*, 2007; Geoghegan *et al.*, 2010). On the contrary, the presence of the collagen-binding protein (Cna) has been mainly associated with strains from CC30 and CC45 (Arciola, Campoccia, *et al.*, 2005). The analysis of the genome sequences of our collection of PJI isolates confirmed the presence of *fnbA, clfA, clfB, sdrC, spa, sasC, sasE, sasF, sasH, sasI, sasJ, eap, emp, vwb* and *efb* in all the isolates. In agreement with previous studies (Rasmussen *et al.*, 2013), the *sasG* gene was found in all PJI strains from CC8 and CC15 and was not detected in any CC30 and CC45 isolate, whereas *cna* was present in all strains from CC30 and CC45 and absent in all CC15 and CC8 PJI isolates. Our results revealed that the selected strains from CC8 and CC15 showed a higher capacity to colonize catheters *in vivo* and a higher ability to form a biofilm on protein precoated surfaces than isolates from CC30 and CC45, and therefore we cannot exclude that the presence of SasG might contribute to an increased *S. aureus* propensity to colonize and accumulate on the surface of implanted prostheses. Thus, further work that implies the generation of mutants in the *sasG* gene in CC8 and CC15 isolates and the analysis of their *in vivo* colonization capabilities is needed in order to determine the real contribution of SasG to PJI. Following the same reasoning,

our findings suggest that the presence of Cna may not be as important when competing to colonize a surface.

A second source of variability in the ability of *S. aureus* to colonize implants can be generated by changes in the sequence of adhesin proteins. It is well known that the exposure of the bacteria to the pressure of the host immune system contributes to the accumulation of polymorphisms within surface proteins (Lindsay, 2019). Studies on *S. aureus* have revealed that clinical isolates accumulate SNPs in their surface proteins as the infection progresses (Ma *et al.*, 2021; Young *et al.*, 2017). Ma *et al*. showed that increasing genotypic variation in adhesin-encoding genes between the first and later isolates from PJI outcomes correlates with changes in the ability to bind to plasma proteins. These changes might confer advantages to successfully colonizing surfaces and/or evading the immune system. Similarly, several studies have evidenced that polymorphisms in FnBPA-binding repeats in isolates causing infection of cardiovascular devices are associated with an enhanced capacity to adhere to Fn (Casillas-Ituarte *et al.*, 2012; Lower *et al.*, 2011; Piroth *et al.*, 2008).

A third level of variability can be generated through mutations at the IGRs of certain genes that cause changes in the expression levels of compounds important for implant colonization. As mentioned above, most studies dedicated to investigating how bacteria adapt to the host environment have focused on changes that occur within coding regions, whereas the role of intergenic mutations has remained mostly disregarded. Importantly, several evolution studies have shown that mutations in the regulatory elements upstream of transcriptional start sites cause changes in the transcription levels of genes

important for evolution of pathogenic phenotypes, including essential genes that are less permissive to accumulate mutations in the coding sequence (Blanka *et al.*, 2015; Cui *et al.*, 2014; Khademi *et al.*, 2019).

A fourth level of variability can be generated by changes in the expression levels of global transcriptional regulators controlling the expression of the surface adhesins. Several regulatory elements, such as *agrAC, saeRS, arlRS, mgrA*, and *sarA* are known to directly or indirectly regulate the expression of staphylococcal adhesins in response to different environmental conditions (Paharik & Horswill, 2016). For instance, the *agr* system represses adhesins expression and its activity is inhibited by proteins found in human serum and blood (Jenul & Horswill, 2019). Similarly, SaeRS, Agr, and SarA, upregulate the expression of some SERAMs such as *eap* and *emp* under low-iron conditions (Johnson *et al.*, 2008; Paharik & Horswill, 2016). SarA also responds to $O_2$ or $CO_2$ levels and can induce (*fnbA and fnbB*) or repress (*spa*) the expression of some adhesins either in an *agr*-dependent or independent way (Chan & Foster, 1998). Moreover, the repression of the protease activity by SarA can affect the accumulation of some staphylococcal proteins (Jenul & Horswill, 2019). Taking the above into account, we decided to follow a strategy in order to differentiate between the contribution of the SNPs in IGRs from the contribution of other superimposed regulatory factors to the expression of staphylococcal adhesins that consisted in analysing the expression of the reporter gene in the same bacterial strain. This guaranteed that the differences in the levels of expression of surface adhesins found in our study were only dependent on variations in IGRs.

The comparison of the IGRs upstream 15 adhesin-encoding genes of the 71 isolates under study revealed strong differences in conservation rates. In particular, the IGR upstream the *fnbA* gene was highly conserved, only comprising seven SNPs, whereas the IGR upstream *sdrC* accumulated 56 SNPs that resulted in a 28% variation rate. The high degree of conservation in the IGR upstream *fnbA* was somehow unexpected because the FnBPA amino acid sequence varies considerably between *S. aureus* lineages (Loughman *et al.*, 2008) and polymorphisms associated with binding mechanisms have been described in isolates from cardiovascular devices infections (Lower *et al.*, 2011). When we analysed the potential functional relevance of the SNPs in the IGRs of the 15 adhesins encoding genes to their transcription using transcriptional fusions with the *gfp* reporter gene, we found that each CC shows a characteristic profile of adhesins expression. The Spa, vWbp and Efb reporters showed a higher level of expression in CC15 and CC8 isolates compared to CC30 and CC45 strains. Protein A (Spa) has been shown to promote catheter-associated infection (Merino *et al.*, 2009), and the secreted VWF-binding protein (vWbp) promotes ClfA-mediated adhesion to VWF (Viljoen *et al.*, 2021). The Extracellular Fibrinogen-binding Protein (Efb) is a very large secreted protein that binds host Fg and complement C3 and plays an important role in the evasion of the immune system (Ko *et al.*, 2011). On the other hand, the presence of SNPs in the IGRs upstream of genes encoding for FnBPA, ClfA, and ClfB did not affect expression levels, suggesting that the SNPs were silent, at least in the conditions tested.

Based on the notion that *S. aureus* colonization capacity of prosthetic implants is very likely due to the expression profile of the whole family of surface adhesins, we explored if *S. aureus* isolates from different CCs, with a particular profile in the presence and SNPs of adhesins genes, might show an advantage in colonizing an implant, using a catheter infection model in mice. At the same time, aiming to apply the reduction principle for ethical use of animals in scientific research (Russell & Burch, 1960), we coinfected each catheter with a mixture containing equal numbers of representative strains of four CCs. Because the four isolates were genetically very closely related, it was necessary to design oligonucleotides that amplify DNA regions containing enough number of SNPs to unambiguously distinguish and quantify the number of bacteria corresponding to each isolate on the surface of the catheter after the infection process. Once we selected the regions comprising IGRs of *sasJ* and *clfB* genes, we applied the well-established methodology used to sequence and characterize microbiomes using 16S amplification products. Interestingly, isolates from CC15 and CC8 showed a higher capacity to colonize the catheters' surface than isolates from CC30 and CC45. These results corresponded to the ability of each isolate to adhere to abiotic surfaces coated with individual plasma proteins, strongly suggesting that differences in the capacity to colonize implanted devices might result from the sum of adhesion properties of each individual adhesin.

We are aware that to unambiguously determine that the SNPs in IGRs of surface adhesins are ultimately responsible for the differences observed in implant colonization, more functional experiments are needed. In particular, a systematic exchange of SNPs or IGRs between strains of different CCs might be

carried out. However, this is a titanic effort since the number of adhesins is very high, genetic manipulation of clinical *S. aureus* isolates is not always possible due to restriction-modification barriers, and individual SNPs will very likely account for only a small percentage of the PJI phenotype. Thus, such functional analyses could only be performed for the most prominent differences observed.

As regards the *in vivo* methodology established in this thesis, we anticipate that such an experimental approach for quantifying and detecting closely related bacterial isolates will be very helpful to identify competitive advantages for certain clinical bacterial isolates in specific stages of the infectious process or in polymicrobial biofilm research. In our experimental setup, we used mixtures of four strains. Nevertheless, in the future, it would be interesting to test which is the maximum level of complexity that this method allows to monitor.

# Conclusions

1. *S. aureus* strains from PJI or wound infections share similar SNPs in the intergenic region upstream of the *icaADBC* operon. These SNPs do not generate variability in the expression levels of the *icaADBC* operon and the production of the PIA/PNAG exopolysaccharide.

2. Overexpression of the PIA/PNAG exopolysaccharide due to a single mutation in the *icaR* gene reduces *S. aureus* virulence in a *Galleria mellonella* infection model, underlining the importance of keeping the expression of the *icaADBC* operon under strict control.

3. *S. aureus* strains from PJI or wound infections share similar SNPs in the intergenic region upstream of genes encoding surface adhesins. These SNPs are characteristic of each CC, and they generate variability in the expression levels of these proteins. As a consequence, isolates of each CC show a characteristic expression profile of surface adhesins.

4. Differences in the expression levels of surface adhesins are associated with a different capacity to adhere and form a biofilm on abiotic surfaces coated with plasma proteins.

5. An amplicon-based sequencing method has been stablished to simultaneously detect and quantify *S. aureus* isolates from different CCs in a murine catheter coinfection model.

6. Analysis of genetic variations in the intergenic regions upstream of genes encoding factors involved in biofilm development may help to understand the differences in the capacity of *S. aureus* isolates to cause PJI.

# *Conclusiones*

1. Cepas de *S. aureus* aisladas de infecciones periprotésicas o heridas comparten SNPs similares en la región intergénica adyacente al operón *icaADBC*. Estos SNPs no generan variabilidad en los niveles de expresión del operón *icaADBC* ni en la producción del exopolisacárido PIA/PNAG.

2. La sobre-expresión del exopolisacárido PIA/PNAG, debida a un SNP en el gen *icaR*, reduce la virulencia de *S. aureus* en un modelo de infección de *Galleria mellonella*, destacando la importancia de mantener la expresión del operón *icaADBC* bajo un control estricto.

3. Cepas de *S. aureus* aisladas de infecciones periprotésicas o heridas comparten SNPs similares en la región intergénica adyacente a los genes que codifican adhesinas de superficie. Estos SNPs son característicos de cada complejo clonal y generan variabilidad en los niveles de expresión de estas proteínas. Como consecuencia, los aislados de cada complejo clonal muestran un perfil de expresión característico de adhesinas de superficie.

4. Las diferencias en los niveles de expresión de las adhesinas de superficie se asocian a una diferente capacidad para adherirse y formar un biofilm sobre superficies abióticas recubiertas de proteínas plasmáticas.

5. Se ha establecido un método de secuenciación basado en amplicones para detectar y cuantificar simultáneamente aislados de *S. aureus* de diferentes complejos clonales en un modelo murino de coinfección de catéter.

6. El análisis de las variaciones genéticas en las regiones intergénicas adyacentes a los genes que codifican factores implicados en el desarrollo del biofilm podría ayudar a entender las diferencias en la capacidad de los aislados de *S. aureus* de causar infecciones periprotésicas.

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**Diseño de portada: Servicio de Comunicación y Diseño. Navarrabiomed, 2022.**

Pamplona 05 de enero de 2023

A quien corresponda,

Teniendo en cuenta las sugerencias realizadas por el revisor externo Dr. RODRIGO BACIGALUPE, informamos de los siguientes cambios realizados a la memoria de la Tesis Doctoral "*Analysis of the association between polymorphisms in intergenic regions of Staphylococcus aureus genes involved in biofilm formation and periprosthetic joint infections*" presentada por la Doctoranda LILIANA ANDREA MORALES LAVERDE:

Página 62

Memoria anterior: Nowadays, the significant decrease in the prize of bacterial genome sequencing is replacing the classical typing techniques by genome-wide association studies (GWAS). This method studies the entire genome of a large group of strains searching for small variations, called single nucleotide polymorphisms or SNPs, associated with specific phenotypes.

**Memoria nueva: Nowadays, the significant decrease in the prize of bacterial genome sequencing is replacing the classical typing techniques by genome-based approaches. These approaches, such as Genome wide Association studies (GWAS), study the entire genome to accurately searching for essential genes or small variations, called single nucleotide polymorphisms or SNPs, associated with specific phenotypes.**

Página 73

Memoria anterior: The Sequence Read Archives (SRAs) with detailed information of the strains was deposited in the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA765573.

**Memoria nueva: The sequence reads, with detailed information of the strains, were deposited in the Sequence Read Archive (SRA) in the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA765573.**

Página 81

Memoria anterior: The SRAs with detailed information of the samples were deposited in NCBI under the BioProject accession number PRJNA834761.

**Memoria nueva: The sequencing reads, with detailed information of the samples, were deposited in the SRA in NCBI under the BioProject accession number PRJNA834761.**

Sin otro particular y para que así conste a los efectos oportunos,

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