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**A Novel Virulence Mechanism in
Staphylococcus aureus: FnBPA Cross-Linking
to Fibrin(ogen) and Fibronectin by Bacterially
Activated FXIII and TG2**

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Abstract

Staphylococcus aureus is a versatile opportunistic pathogen capable of causing a spectrum of infections, from superficial skin lesions to life-threatening systemic diseases. Its capacity to evade host immune responses and establish persistent infections is critically dependent on its ability to adhere to and colonize host tissues. Central to this process is the interaction of *S. aureus* surface proteins with extracellular matrix (ECM) components, particularly fibronectin (Fn) and fibrinogen (Fbg). This thesis investigates a novel pathogenic mechanism by which *S. aureus* exploits host transglutaminases, specifically Factor XIII (FXIII) and tissue transglutaminase 2 (TG2), to covalently anchor itself to host proteins through the multifunctional surface adhesin Fibronectin Binding Protein A (FnBPA), with the bacterial coagulase von Willebrand factor-binding protein (vWbp) playing a central activating role.

The study begins by examining the expression profile and localization of vWbp during *S. aureus* growth, revealing that the protein is secreted during the exponential phase and subsequently rebinds to the bacterial surface via interactions with lipoteichoic acid (LTA) and peptidoglycan (PPG). Functionally, surface-associated vWbp non-proteolytically activates prothrombin, leading to fibrin formation and subsequent activation of FXIII. In this environment, bacterially-activated FXIII catalyzes the formation of ϵ -(γ -glutamyl)-lysine isopeptide bonds between Fbg and FnBPA, embedding the bacterium within a stable fibrin scaffold.

Biochemical analysis identified Gln103 in the N1 subdomain of FnBPA's N-terminal Region A as the key reactive glutamine residue essential for vWbp-activated FXIII mediated crosslinking. Recombinant FnBPA mutants lacking Gln103 (Q103A) retained non-covalent binding to Fbg via the classical Dock, Lock, and Latch (DLL) mechanism but failed to form covalent complexes with fibrin(ogen). This specificity was confirmed using whole bacterial cells expressing Q103A FnBPA, which demonstrated impaired incorporation into fibrin matrices. Also, bacterial virulence was significantly reduced in a murine subcutaneous infection model in presence of the Q103A mutation, as evidenced by smaller dermonecrotic lesions and lower bacterial loads compared to wild-type strains. In addition, this study also establishes that TG2, a transglutaminase expressed in various tissues and induced during inflammation, can catalyze the same crosslinking reaction, broadening the physiological relevance of this mechanism beyond sites of coagulation.

Beyond Fbg, this work demonstrates that Fn is also a substrate for FnBPA-mediated covalent crosslinking to host proteins in the presence of vWbp-activated FXIII. Interestingly, this function is conferred not by the N1N2N3 region, but by the C-terminal repeat region (RR) of FnBPA, which forms high-molecular-weight complexes with both full-length Fn and the N-terminal 29 kDa fragment of Fn. The RR region is already known to mediate non-covalent interactions with Fn through a tandem β -zipper mechanism, highlighting its dual role in adhesive processes.

In summary, this thesis uncovers a novel virulence mechanism whereby *S. aureus* utilizes vWbp to hijack host transglutaminase activity, resulting in covalent stabilization of bacterial-host protein complexes via FnBPA. These findings not only deepen our understanding of *S. aureus* adhesion biology and immune evasion but also identify FnBPA-mediated covalent crosslinking as a promising therapeutic target, particularly in the context of antibiotic-resistant infections and biofilm-associated diseases.

Introduction

The Genus *Staphylococcus*

The *Staphylococcus* genus includes 45 species and 24 subspecies in the *Staphylococcaceae* family. The name comes from the Greek words staphylē (grape) and kókkos (granule) because they cluster in grape-like formations after Gram staining (Ryan & Ray, 2004) (fig. 1). Individual cocci are also common, so this morphological trait is reinforced. They are 0.5 to 1.5 μm in diameter and are adaptable, tolerating high salt and a wide temperature range of 18°C to 40°C (Murray et al., 2016). *Staphylococcus* are non-spore forming, non-motile and facultative anaerobes that can do both fermentative and respiratory metabolism (Wilkinson, 1997). Nutritionally, many species in this genus require organic nitrogen sources, often in the form of essential amino acids and B-complex vitamins (Kloos & Musselwhite, 1975; Wilkinson, 1997). *Staphylococcus* are catalase positive and oxidase negative (Wilkinson, 1997). Pathogenic species produce coagulase: an enzyme that helps form fibrin clots. This is a diagnostic marker to differentiate coagulase positive species like *Staphylococcus aureus* and *Staphylococcus intermedius* from coagulase negative species like *Staphylococcus epidermidis* (Kloos & Schleifer, 1975; Harris et al., 2002).

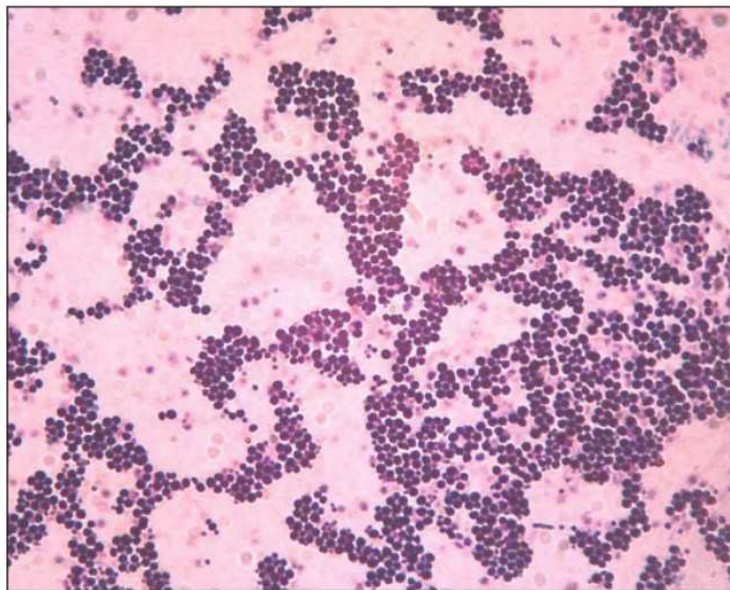


Figure 1. *S. aureus* colonies under Gram staining at 1000x magnification. The bacteria appear as purple, spherical cocci arranged in irregular grape-like clusters, characteristic of Gram-positive organisms. The intense violet coloration results from the retention of crystal violet dye due to the thick peptidoglycan layer in the bacterial cell wall (Ramadhani et al., 2024).

Pathogenic *Staphylococcus* species

Among the species that can infect humans *Staphylococcus aureus* and *Staphylococcus epidermidis* are the most common, especially in hospital acquired infections (de Vor et al.,

2020). *S. aureus* is more often associated with disease but both species can cause a wide range of clinical manifestations from acute (abscesses and sepsis) to chronic infections especially those related to implanted medical devices. These bacteria can evade the immune system, adhere to host tissue and produce tissue damaging enzymes and toxins (Murray et al., 2016). They can also form biofilms which contributes to immune evasion and chronic infection. Understanding these virulence strategies is key to developing new treatments that can treat antibiotic resistant infections (de Vor et al., 2020). In particular, *Staphylococcus epidermidis* is a coagulase-negative staphylococcus and a leading cause of biomaterial-associated infections, particularly in prosthetic joints, catheters and heart valves. Its pathogenicity stems from biofilm formation mediated by polysaccharide intercellular adhesin (PIA), and its ability to evade immune responses and resist antibiotics (Otto, 2014). There are also other relevant species that can cause a large variety of human diseases. *Staphylococcus lugdunensis* is an aggressive species, frequently causing severe skin and soft-tissue infections, intravenous catheter-associated bacteremia, osteomyelitis and destructive endocarditis on native and prosthetic valves (Koumaki et al., 2024; Renzulli et al., 2000). Clinical series show that up to two-thirds of *S. lugdunensis* infections manifest as invasive abscesses or deep-tissue infections requiring surgical drainage and prolonged antibiotics, with mortality rates and complication rates similar to *S. aureus*. The pathogen's virulence is linked to fibrinogen-binding adhesins (e.g., Fbl), robust biofilm formation and tissue-degrading enzymes that facilitate implant colonization and osteoarticular infection (Liu et al., 2012). *Staphylococcus saprophyticus* is a well-established cause of community-acquired urinary tract infections in young women, adhering to urothelium via specific adhesins and causing "honeymoon cystitis" symptoms. Another opportunist, *S. haemolyticus*, is found in bloodstream and device-associated infections in hospitalized patients; its pathogenicity is due to potent biofilm formation, capsule-mediated neutrophil phagocytosis evasion, multidrug resistance and toxin production (Raz et al, 2005; Eltwisy et al, 2022). *Staphylococcus capitis* is also emerging in neonatal sepsis and prosthetic device infections, forming biofilms and causing endocarditis (Heath et al., 2023)

Staphylococcus aureus

S. aureus is an opportunistic pathogen found on skin and mucosal surfaces. Although it's part of the normal flora, it can cause a range of diseases from mild, superficial infections to severe, life threatening conditions like sepsis and pneumonia (Ryan & Ray, 2004). Colonial pigmentation is gold or yellow and is due to staphyloxanthin. Staphyloxanthin is a carotenoid with antioxidant properties that enhances bacterial resistance to oxidative stress and immune defenses (Xue et al., 2019; Liu et al., 2005). This pigment is crucial shield the bacterium also from neutrophil-mediated killing, thus enhancing virulence and survival during infection (Liu et al., 2005). Staphyloxanthin's conjugated double bonds absorb visible light and gives *S. aureus* colonies their yellow to orange color on agar plates (Clauditz et al., 2006) (fig. 2). Morphologically, *S. aureus* forms smooth, round, convex and hemolytic

colonies 2-4 mm in diameter after 24 hours at 37°C. The colonies often show β -hemolysis on blood agar due to secreted hemolysins (Zhang et al., 2016). *S. aureus* grows well on non-selective media like tryptic soy agar (TSA) and nutrient agar and on selective and differential media like mannitol salt agar (MSA) which has high salt concentration (7.5% NaCl) that inhibits most other bacteria and has mannitol and phenol red to distinguish *S. aureus* by its ability to ferment mannitol, turning the medium yellow (Forbes et al., 2007). These features help in clinical identification and isolation.

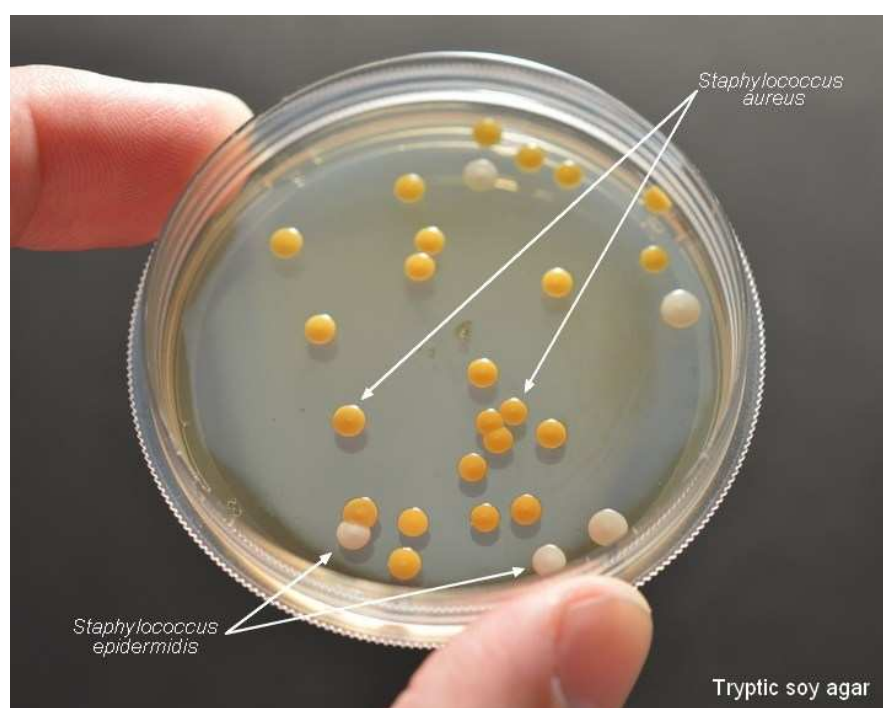


Figure 2. Colony morphology of *S. epidermidis* and *S. aureus* cultivated on tryptic soy agar (TSA) for 24 hours at 37 °C. *S. aureus* produces medium-sized, smooth, golden-yellow colonies due to the carotenoid pigment staphyloxanthin, with antioxidant properties. In contrast, *S. epidermidis* forms smaller, round, white to cream-colored colonies. (microbiologyinpictures.com, 2015)

Genomic organization

The *S. aureus* genome is a circular chromosome that is approximately 2.8 to 2.9 Mbp in size (Crossley and Archer, 1997). The first whole *S. aureus* genome was sequenced in 2001 with two methicillin resistant strains, N315 and Mu50 (Kuroda et al., 2001). Since then many other *S. aureus* strains have been sequenced (Baba et al., 2008) but a large portion of strains remain unsequenced. The genome can be divided into the core genome which is the bulk of the genetic material and various accessory and mobile genetic elements. The core genome encodes the housekeeping genes which are essential for basic cellular functions such as metabolism, DNA replication and maintenance of cellular integrity.

In a comparative analysis of several *S. aureus* strains including MW2, N315 and Mu50 it was found that the overall G+C content of the genome is approximately 32.9%. Most of this G+C content is concentrated in the protein coding regions. The proportion of coding sequences

or open reading frames (ORFs) is more than 33% of the total genome in these strains with over 2500 protein coding genes. While the protein coding regions are fairly conserved the presence of accessory elements such as insertion sequences, transposons and genomic islands varies significantly across different strains. A small portion of the genome is dedicated to the encoding of ribosomal RNA (rRNA), transfer RNA (tRNA) and tmRNA genes which are essential for protein synthesis and cellular function (Baba et al., 2002).

Many *S. aureus* strains also harbour the staphylococcal cassette chromosome (SCC) a mobile genetic element that is normally integrated near the origin of replication. This element often contains the *mecA* gene which confers resistance to methicillin. When the SCC carries the *mecA* gene it is called SCCmec and its presence is directly associated with the development of methicillin resistant *S. aureus* (MRSA). Other mobile genetic elements are present in different *S. aureus* strains including pathogenicity islands such as superantigen pathogenicity islands (SaPIs) and genomic islands of the vSa family. *S. aureus* is also known for its ability to acquire genes through horizontal gene transfer especially those that confer antibiotic resistance. This process of lateral gene transfer has resulted to the evolution of a highly versatile and complex genome of *S. aureus* which allows it to survive in different environments and develop resistance to many antimicrobial agents (Shittu et al., 2007).

Morphology and Cell Wall Composition

The structure of *S. aureus* has several key components: a plasma membrane, a rigid cell wall and in some strains a polysaccharide capsule. The cell wall is mostly peptidoglycan which makes up almost half of the wall mass (Waldvogel et al., 1990). Both the peptidoglycan layer and the capsule are virulence factors that play a big role in the pathogen's ability to cause infections.

As a Gram positive bacterium the cell wall of *S. aureus* has the characteristic features of this group (fig. 3). The wall itself is 20-40 nm thick and homogeneous (Giesbrecht et al., 1998). Peptidoglycan is a complex heteropolymer made up of a disaccharide backbone, consisting of alternating units of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). On average the sugar chain is 10 disaccharide units long (Sidow et al., 1990). Each NAM unit is covalently linked to a highly conserved tetrapeptide which includes L-alanine, D-glutamine, L-lysine and D-alanine. These stem peptides are important for cross-linking to the stem peptides of neighboring chains. The linkage is facilitated by a pentaglycine bridge, a hallmark of staphylococcal species (Labischinski et al., 2013). Interestingly this pentaglycine rich structure is partly responsible for *S. aureus*'s vulnerability to lysostaphin, an enzyme that targets this glycine structure (Projan, Novick et al., 1997).

Despite this susceptibility to lysostaphin, *S. aureus* remains resistant to lysozyme, a key antimicrobial enzyme, thanks to a distinctive modification of the NAM molecule. Specifically, at position C6-OH, NAM is modified through O-acetylation by a specialized membrane-bound enzyme (Bera, Herbert et al., 2005). This modification is crucial for the bacterium's

survival, as lysozyme is commonly found in various host tissues and fluids. As a result, *S. aureus* can asymptotically colonize a variety of host environments, including the skin (Noble, 1969), mucosal surfaces (Williams, 1963), oral cavity (Smith, Jackson et al., 2001), and the gastrointestinal tract (Acton, Plat-Sinnige et al., 2009).

Another important feature of the *S. aureus* cell wall is the presence of teichoic acids (TAs), which contribute significantly to the cell wall's mass (about 40%) and are crucial for a variety of cellular functions (Knox and Wicken, 1973). Teichoic acids are large polymeric molecules that contain phosphate groups, and they can be classified into two types based on their attachment. Wall-Teichoic Acids (WTA) are covalently linked to the peptidoglycan layer, while Lipo-Teichoic Acids (LTA) are anchored to the cytoplasmic membrane. Due to their high negative charge, these molecules attract cations, which can help in the binding of proteins to the cell surface, thus facilitating several biological processes. Their roles in bacterial pathogenesis and colonization are well documented. For instance, TAs are involved in the activation of macrophages, promoting the release of pro-inflammatory cytokines and contributing to the inflammatory response (Ruhland and Fiedler, 1990; Deininger, Stadelmaier et al., 2003). Additionally, WTAs have been implicated in the adhesion of *S. aureus* to host tissues (Majcherczyk et al., 2003), and they play a critical role in the pathogenesis of septic shock and organ failure, often working synergistically with peptidoglycan (De Kimpe et al., 1995).

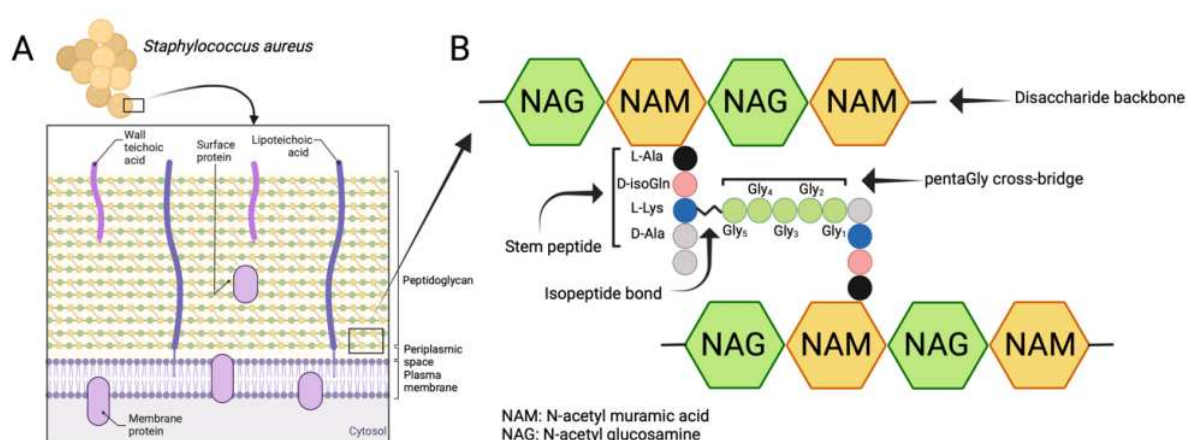


Figure 3. Structure of the cell wall peptidoglycan in *S. aureus*. (A) Schematic overview of the *S. aureus* cell wall, illustrating key components including the thick peptidoglycan layer, teichoic acids, and associated proteins. (B) Detailed structure of the peptidoglycan, showing glycan strands cross-linked by peptide bridges that provide rigidity and shape to the bacterial cell (Kong et al., 2016).

Epidemiological Relevance

S. aureus has become one of the most common bacterial pathogens with its clinical significance amplified by its ability to cause both local and systemic infections in various host populations. *S. aureus* can infect many anatomical sites (skin, soft tissues, lungs, bloodstream, bones, gastrointestinal tract) but its impact is largely determined by the strain and the host environment (DeLeo et al., 2009; Tong et al., 2015). Infections can manifest as

toxin-mediated syndromes or invasive diseases, from mild skin lesions like impetigo and folliculitis to life threatening illnesses like bacteremia, infective endocarditis, osteomyelitis, septic arthritis and toxic shock syndrome (Tong et al., 2015).

At the heart of its pathogenicity is *S. aureus*'s ability to produce a range of virulence factors. Many of these are toxins secreted during defined growth phases: post-exponential and early stationary phases. They help in host tissue invasion and destruction. These include hemolysins, leukotoxins, exfoliative toxins, enterotoxins and toxic shock syndrome toxin-1 (TSST-1). In parallel, many virulence determinants are embedded in the bacterial surface to aid in adherence, immune modulation and persistent colonization (Kong et al., 2016; Jusk et al., 2014).

A hallmark of *S. aureus* infection is abscess formation, particularly in skin and soft tissue infections. These abscesses are a complex interplay between the host immune response and bacterial virulence. Neutrophils, the dominant infiltrating leukocytes, act as the frontline defenders by releasing antimicrobial agents. But this defensive response often induces collateral tissue damage, allowing abscess maturation. *S. aureus* contributes to this process by molecules that recruit neutrophils, induce cytolysis and help in fibrin capsule formation around the infection site (Kobayashi et al., 2015). From an epidemiological perspective, asymptomatic carriage of *S. aureus* is key to its transmission and infection risk. The anterior nares is the primary site of colonization but other mucosal areas like the throat, gastrointestinal tract and vaginal epithelium are also common reservoirs (Wertheim, Van Kleeef et al., 2006). In the general population, 20% are persistent carriers, 60% are intermittent carriers and 20% are non-carriers (Kluytmans et al., 1997). Humans are the main reservoir for *S. aureus*, colonization is a precursor for both community acquired and healthcare associated infections (Chambers, 2005).

The increasing burden of methicillin-resistant *S. aureus* (MRSA) has raised public health concerns especially in clinical settings. A study conducted in various emergency departments across the US found that MRSA was the most common pathogen in skin and soft tissue infections. Among patients presenting with such conditions, *S. aureus* was isolated in 76% of cases and among these isolates 78% were methicillin-resistant. The frequency of MRSA varied significantly across study sites ranging from 15% to 74% making it the most common pathogen in these clinical scenarios (Moran et al., 2006).

Immunocompromised individuals are at heightened risk for severe *S. aureus* infections. In particular, patients with compromised mucosal barriers or impaired immune systems (such as those living with HIV) experience higher rates of colonization, recurrent infections, and mortality. Within hospital environments, *S. aureus* is now recognized as one of the most frequent nosocomial pathogens, disproportionately affecting vulnerable populations including HIV-positive individuals (A. Senthilkumar, 2001; J. N. Sheagren, 1984).

Antibiotic Resistance

S. aureus is considered a global threat because of its ability to become multi-drug resistant. A prime example is methicillin-resistant *S. aureus* (MRSA), which was first identified about 60 years ago, just after methicillin was introduced as a medicine. Initially MRSA was confined to hospital environments and was the poster child for hospital-acquired infections. But over time MRSA has spread beyond its nosocomial origins and is now a pathogen in the community where it has been involved in many outbreaks in otherwise healthy people worldwide (DeLeo and Chambers, 2009).

The spread of MRSA in the community and its ability to spread rapidly and become resistant to multiple antibiotics has complicated treatment and narrowed down the treatment options (DeLeo and Chambers, 2009). When methicillin resistance emerged, vancomycin was used as a treatment for MRSA. But when vancomycin-resistant *S. aureus* (VISA) strains emerged the medical community was alarmed, it was a critical moment in the management of antibiotic resistant pathogens (Stefani et al., 2012; Chiu et al., 2010).

Several theories have been proposed to explain the increasing MRSA in hospital settings. One theory is that there is an antibiotic resistant microbial reservoir in the local flora of the hospital environment that can horizontally transmit to patients. Another theory is that antimicrobial pressure in hospitalized patients selects for resistant strains from their endogenous microbiota (Chiu et al., 2010). The mechanism of methicillin resistance in *S. aureus* is in the complex process of cell wall biosynthesis. The cell wall is made of peptidoglycan which is composed of N-acetylglucosamine and N-acetylmuramic acid. These disaccharides are polymerized via β -1,4-glycosidic linkages through a transglycosylation reaction. Then a transpeptidation step links adjacent peptidoglycan strands through cross-bridging of stem peptides. Both steps are essential for cell wall integrity and are catalyzed by penicillin-binding proteins (PBPs), β -lactam antibiotics target the transpeptidation step (Giesbrecht et al., 2018).

Unlike the well-studied plasmid-mediated penicillin resistance in many bacterial species, MRSA's resistance determinant is chromosomally encoded. Early studies showed that the gene responsible for methicillin resistance was present only in resistant strains and absent in susceptible ones (Dyke et al., 1966; Sjöström et al., 1975). Further functional analysis involving cloning and heterologous expression in *Escherichia coli* led to the identification of a novel penicillin-binding protein, PBP2a, which retains transpeptidase activity even in the presence of β -lactam antibiotics (Stewart and Ronsenblum, 1980; Matsushashi et al., 1986).

The genetic locus encoding PBP2a, known as *mecA*, is situated on a mobile genetic element termed the staphylococcal cassette chromosome *mec* (SCC*mec*). This element includes recombinase genes (*ccr*) that enable site-specific integration and excision at the chromosomal locus *attB_{scc}* in *S. aureus* (Wijaya et al., 2006). Integration of SCC*mec* into the bacterial genome converts methicillin-susceptible strains into MRSA, rendering them

resistant to nearly all β -lactam antibiotics and marking a pivotal event in the genetic evolution of antibiotic resistance in *S. aureus* (Hiramatsu, 2001).

Virulence Strategies

S. aureus can thrive in the human host because of its arsenal of virulence factors, molecular determinants that allow the pathogen to overcome host defenses, establish infection and disseminate (fig. 4). These factors are not just proteins but a broader range of molecules that contribute to immune evasion, tissue colonization and damage. Characterizing these virulence elements is key to identifying new targets to combat bacterial infections (Cross, 2008).

Virulence factors of *S. aureus* are broadly classified into secreted and surface associated proteins. One of the earliest steps in *S. aureus* pathogenesis is adhesion to host tissues, a process mediated by bacterial surface molecules often referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). These include cell wall anchored proteins such as clumping factor B which binds to host molecules like fibrinogen (Fbg), cytokeratin and loricrin and plays a key role in colonization (Lacey et al., 2016). Wall teichoic acid, a component of the *S. aureus* cell envelope, binds to nasal epithelial cells via specific scavenger receptors and promotes asymptomatic carriage and persistent colonization (Baur et al., 2014).

Secreted factors are toxins and enzymes with a variety of destructive and modulatory functions while surface bound proteins primarily facilitate adhesion and immune modulation (Lowy, 1998). Among the most well known secreted elements are cytotoxins such as alpha, beta, gamma and delta hemolysins which contribute to erythrocyte lysis and iron acquisition, both essential for pathogen survival and proliferation. Enzymes such as collagenase, hyaluronidase and lipases degrade host tissue components and facilitate invasion and nutrient acquisition during early stages of infection (Dinges, Orwin et al., 2000). In addition to tissue invasion and nutrient extraction *S. aureus* produces a range of potent exotoxins that drive disease pathology. These include toxic shock syndrome toxin-1 (TSST-1), exfoliative toxins (ETA and ETB) and a range of staphylococcal enterotoxins (SEA to SEI) and numerous leukocidins. All of these reflect the diversity and flexibility of this pathogen's virulence strategy (Dinges, Orwin et al., 2000).

To establish infection and persist in the host, *S. aureus* must evade or subvert the innate immune system, which includes physical barriers like the skin and mucosal surfaces, as well as cellular components such as neutrophils and the complement cascade. The bacterium secretes proteases capable of inactivating or degrading elements of the host defense, including complement proteins and neutrophil-derived factors (Pietrocola et al., 2017). Additionally, *S. aureus* manipulates the host's own enzymatic systems by producing proteins that activate zymogens, which in turn modulate host immune responses to favor bacterial survival.

The disruption of epithelial integrity is particularly significant in the context of infection. Under normal conditions, epithelial cells produce antimicrobial peptides, such as defensins and cathelicidins, which directly inhibit bacterial growth. Defensins are not only abundant in epithelial tissues but also stored within neutrophil granules, where they contribute to the destruction of phagocytosed pathogens (Zhao and Lu, 2014). Similarly, cathelicidins, processed into potent antimicrobial peptides, exhibit strong activity against *S. aureus* (Boman, 2003; Sørensen et al., 1997; Travis et al., 2000). Despite this, *S. aureus* employs numerous strategies to resist the actions of these peptides, reinforcing its resilience in the face of host immunity.

Ultimately, the ability of *S. aureus* to express and coordinate such a diverse collection of virulence determinants underlies its versatility as a pathogen, and understanding these mechanisms is essential for the development of innovative antimicrobial interventions.

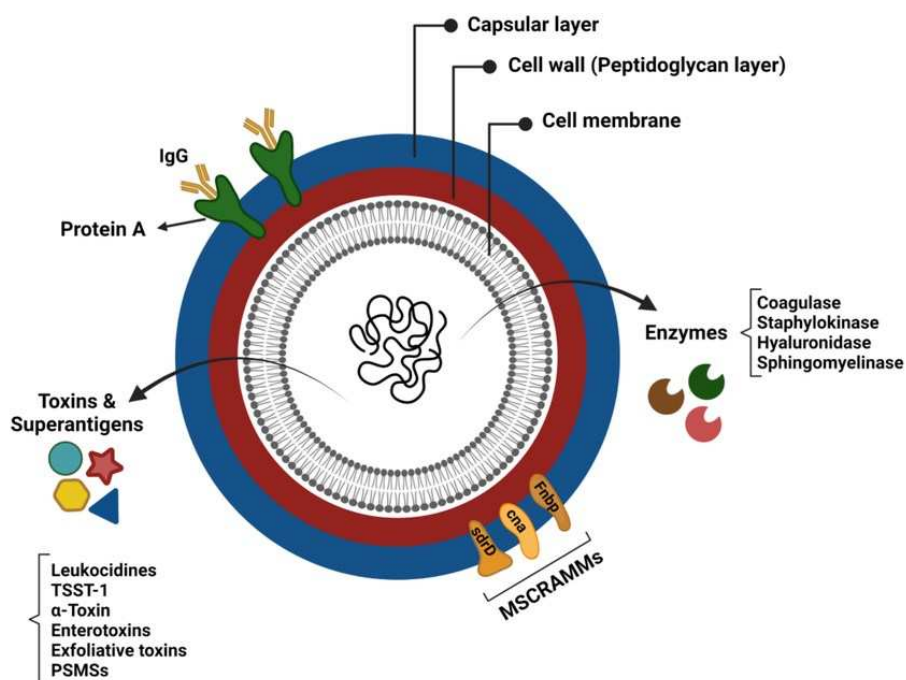


Figure 4. Virulence factors of *S. aureus*. The schematic illustrates key surface-associated and secreted virulence factors that contribute to bacterial adhesion, immune evasion, and tissue damage. Surface proteins mediate attachment to host cells, while secreted toxins such as hemolysins and leukocidins disrupt host defenses. Additional factors include enzymes that degrade host tissues and molecules involved in biofilm formation, all of which facilitate infection and pathogenesis (Shaghayegh et al., 2022).

Cell-wall Anchored Proteins (CWA)

The process of *S. aureus* colonization starts with the bacterium attaching to specific body sites such as the nasal epithelium, skin and mucosal regions like perineal, inguinal, vaginal and colonic areas. Once attached to the host's surface it can invade deeper tissues and internal organs and release toxins and tissue degrading enzymes. This initial attachment phase is crucial for the pathogen to establish infection. To facilitate this *S. aureus* produces

a range of surface proteins called adhesins which anchor the bacterium to host structures (Speziale, Pietrocola et al., 2009).

Among these surface components a specific subset called cell wall-anchored (CWA) proteins have been studied in detail for their functional diversity. These proteins can be grouped into different categories based on their structure and biological roles (Geoghegan and Foster, 2017). *S. aureus* can produce up to 24 different CWA proteins but the specific composition can vary significantly between different strains (McCarthy and Lindsay, 2010). Their expression is also highly dependent on environmental conditions and the bacterial growth phase (McAleese, Walsh et al., 2001). CWA proteins are synthesised with a signal peptide at the N-terminal end which ensures they are targeted to the bacterial secretion machinery. This signal is cleaved during translocation across the cytoplasmic membrane by signal peptidase I (SpsB) allowing the protein to exit the cytoplasm (Cregg, Wilding et al., 1996). At the opposite end the C-terminal region contains a conserved sorting signal that mediates covalent attachment to the bacterial cell wall peptidoglycan (Geoghegan and Foster, 2017). This motif includes three important features: the LPxTG amino acid sequence (where "x" denotes any amino acid), a hydrophobic region necessary for membrane retention and enzyme recognition and a positively charged tail. These features are necessary for the action of the enzyme Sortase A (SrtA) which anchors the protein to the cell wall (fig. 5). Once exported CWA proteins are integrated into the peptidoglycan matrix via transpeptidation and transglycosylation processes (Mazmanian, Ton-That et al., 2001).

Sixteen of these proteins can be released from the cell wall under certain circumstances through the cleavage of the pentaglycine bridges. This release process is mediated by the endopeptidase LytM which plays a role in remodeling the cell envelope and possibly modulating host interactions (Becker, Frankel et al., 2014).

A notable example of CWA proteins is Protein A (SpA). While it is usually anchored to the bacterial surface SpA can also be secreted in a soluble, unprocessed form directly into the surrounding medium (O'Halloran, Wynne et al., 2015). SpA contributes to the virulence of *S. aureus* due to its high-affinity binding to the Fc region of mammalian IgG. This protein acts as a B-cell superantigen meaning it can interact with both the Fc and VH regions of immunoglobulins leading to widespread immunological effects in the host (Palmqvist, Foster et al., 2002; Magizi et al., 2019).

CWA proteins in *S. aureus* are typically categorized into four distinct classes based on the structural motifs they contain. The first group includes proteins with tandemly arranged three-helix bundle domains, such as Protein A (SpA). A second class is characterized by the presence of G5 and E repeat motifs, exemplified by proteins like SasG. The third and most functionally significant group is known as the MSCRAMM family (Microbial Surface Components Recognizing Adhesive Matrix Molecules). These proteins are distinguished by the presence of multiple immunoglobulin-like fold domains arranged in tandem, which play key roles in host matrix recognition. Finally, the fourth category consists of proteins containing NEAT (NEAr iron Transporter) motifs. This group is crucial for bacterial survival in

iron-limited environments like the human host, with the Iron-regulated Surface Determinant (Isd) system being a prominent example (Gruszka, Wojdyla et al., 2012).

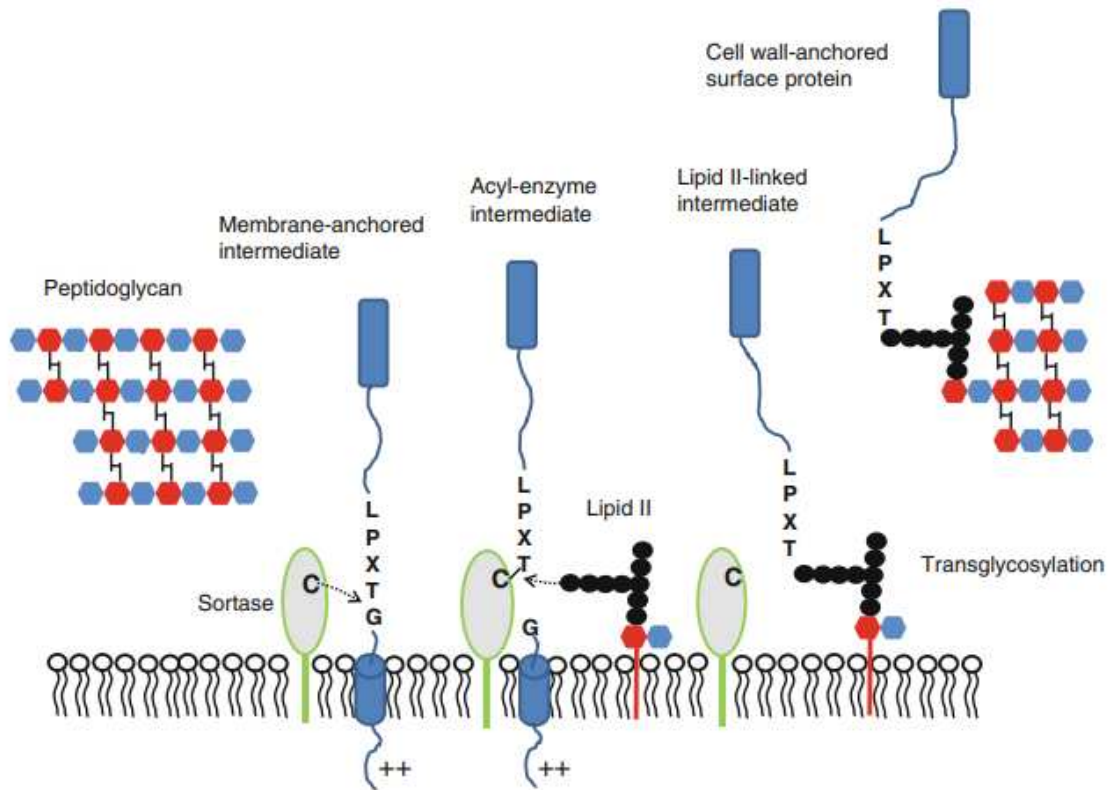


Figure 5. Schematic representation of sortase-mediated anchoring to the cell wall. The figure shows the cytoplasmic membrane leaflet and the peptidoglycan layers (red hexagons: N-acetyl muramic acid; blue hexagons: N-acetyl glucosamine). Sortase A, a membrane enzyme, recognizes the LPXTG motif on secreted protein precursors held at the membrane. Its active cysteine cleaves between Thr and Gly, forming a thioester intermediate. The glycine in lipid II's cross-bridge attacks this intermediate, linking the protein to lipid II. Penicillin-binding proteins then incorporate the protein into the growing peptidoglycan, displaying cell wall-anchored proteins on the surface (Geoghegan and Foster, 2017).

MSCRAMM Family

MSCRAMM is a family of proteins with structural similarities and a common mechanism for ligand binding: Dock, Lock and Latch (DLL). This mechanism is mediated by two adjacent IgG-like folded domains in the N-terminal A region (Foster et al. 2014). Different structural x-ray and biophysical studies have shown that the A regions are composed of three separately folded subdomains: N1, N2 and N3, with N2 and N3 comprising the IgG-like folds (fig. 5) (Geoghegan and Foster 2017). The main MSCRAMM proteins and their functions are shown in table 1. These proteins mediate strong interactions with host matrix components and serve as key mediators of bacterial adhesion, immune evasion, and colonization. The functional diversity and structural complexity of MSCRAMMs make them central to the pathogenicity of *S. aureus*.

Table 1. The main MSCRAMM protein groups in *Staphylococcus aureus* bind host matrix molecules through specific mechanisms, aiding bacterial adhesion and infection (Foster et al., 2014).

MSCRAMMs	Ligand-binding mechanism	Function
Clumping factor A (ClfA)	- Fibrinogen γ-chain carboxyl terminus: DLL - Complement factor I	- Adhesion to immobilised fibrinogen; immune evasion by binding soluble fibrinogen - Immune evasion; degradation of C3b
Clumping factor B (ClfB)	Fibrinogen α-chain repeat 5, keratin 10 and loricrin; DLL	Adhesion to desquamated epithelial cells; nasal colonisation
Serine-aspartate repeat protein C (SdrC)	B-neurexin; DLL- Desquamated epithelial cells	Unknown
Serine-aspartate repeat protein D (SdrD)	Desquamated epithelial cells	Nasal colonisation?
Serine-aspartate repeat protein E (SdrE)	Complement factor H	Immune evasion; degradation of C3b
Bone sialoprotein-binding protein (isoform of SdrE)	Fibrinogen α-chain; DLL	Adhesion to ECM
Fibronectin-binding proteins A (FnBPA)	- FnBPA A domain binds the C terminus of fibrinogen γ-chain and elastin; DLL - Fibronectin	Adhesion to ECM
Fibronectin-binding proteins B (FnBPB)	- FnBPB A domain binds the C terminus of fibrinogen γ-chain, elastin and also fibronectin but not by DLL - FnBPB C terminal repeats binds fibronectin; β-zinc finger	Adhesion to ECM; invasion
Collagen adhesin (CNA)	- Collagen triple helix; collagen hug - Complement protein C1q	Adhesion to collagen-rich tissue

Fibronectin-Binding Protein Family: FnBPA and FnBPB

The fibronectin-binding proteins A and B (FnBPA and FnBPB) of *S. aureus* are part of the MSCRAMM family and are two of the 24 known CWA proteins. These multi-functional adhesins can bind to fibronectin (Fn), fibrinogen (Fbg), and elastin (El) and are involved in bacterial adhesion and virulence (Roche et al., 2004; Wann et al., 2000). FnBPA and FnBPB have significant sequence homology and a modular domain architecture (fig. 6).

Both proteins have an N-terminal signal peptide for secretion and a C-terminal LPETG motif for anchoring to the bacterial cell wall via sortase A. The exposed N-terminal A domains are responsible for binding to Fbg and El (Wann et al., 2000; Keane et al., 2007). Notably these A domains are similar in structure to the Fbg-binding region of clumping factor A (ClfA) and are predicted to have three subdomains, N1, N2 and N3 (Deivanayagam et al., 2002).

Sequence variation in the N2 and N3 regions has led to 7 isoforms of FnBPA and FnBPB. Distal to the A domains are multiple tandem fibronectin-binding repeats (FnBRs) which facilitate strong binding to the type I modules of Fn through a tandem β -zipper mechanism (Schwarz-Linek et al., 2003). FnBPA and FnBPB are highly similar in structure although FnBPB lacks one of the shorter FnBR motifs. Experimental studies have shown that individual FnBRs – FnBPA-1, -4, -5, -9, -10 and -11 – bind to fibronectin with nanomolar affinity (Meenan et al., 2007). FnBPs are key to the pathogenesis of *S. aureus*. Analysis of clinical isolates shows that at least one *fnbp* gene is present in most isolates, suggesting their role in persistent infections (Nashev et al., 2004; Peacock et al., 2000). They are particularly associated with chronic infections of medical implants (Arrecubieta et al., 2006). In FnBPA, the Fbg and Fn binding regions are involved in disease progression and the FnBRs in long term bacterial persistence (Que et al., 2005). The interaction between FnBPA and Fn also facilitates host cell invasion and endothelial activation and triggers a pro-inflammatory response (Peacock et al., 1999; Heying et al., 2007). FnBPA also activates platelets, a key step in thrombus formation (Fitzgerald et al., 2006).

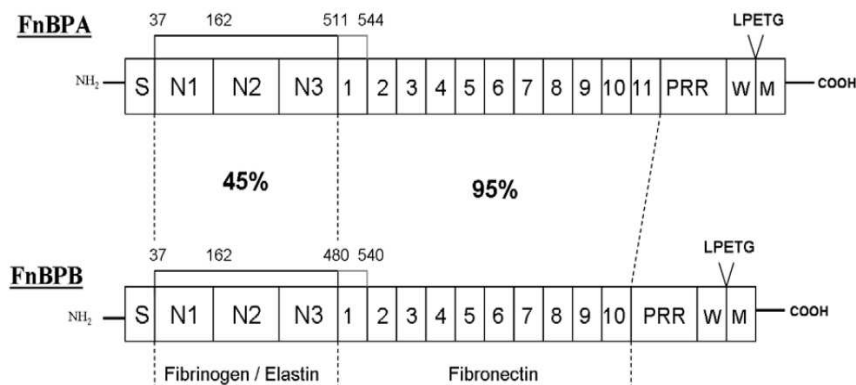


Figure 6. Structural organization of FnBPA and FnBPB from *S. aureus* 8325-4. Both proteins have an N-terminal signal sequence (S) followed by an A domain (subdomains N1, N2, N3) that binds fibrinogen and elastin. This is followed by fibronectin-binding repeats. The A domains span residues 37–511 in FnBPA and 37–480 in FnBPB. At the C-terminus are proline-rich repeats (PRR), wall (W) and membrane (M) domains, and the sortase recognition motif LPETG. Amino acid identity percentages between their binding domains are shown (Burke et al., 2010).

Fibronectin-binding protein A

Fibronectin-binding protein A (FnBPA) of *S. aureus* has dual adhesive activity, binding both fibrinogen (Fbg) and fibronectin (Fn), which is key to tissue colonization and disease progression. The N-terminal A domain of FnBPA recognizes the C-terminal segment of the γ -chain of Fbg (like ClfA) via a tight “dock-lock-latch” interaction (Wann et al., 2000). Meanwhile, the C-terminal fibronectin-binding repeats (FnBRs) engage the N-terminal type I modules of fibronectin in a ‘tandem β -zipper’ fashion to support bacterial invasion (Meenan et al., 2007; Herman-Bausier et al, 2015)

Importantly, these two binding functions are not separate. A 127-amino-acid hinge region that bridges the Fbg-binding A domain and the fibronectin-binding repeats is both necessary and sufficient to mediate FnBPA’s dual adhesion and promote endothelial cell invasion, and even experimental endocarditis in animal models (Piroth et al., 2008)

In this way, FnBPA works through a coordinated mechanism: its A domain docks Fbg to anchor *S. aureus* to damaged tissue surfaces, while its tandem fibronectin repeats engage fibronectin bridges to host integrin receptors to invade tissue. The interplay between Fbg and fibronectin binding is both structurally and functionally synergistic, essential for the pathogen’s virulence in endovascular environments.

Additionally, FnBPA acts as a covalent substrate for host transglutaminase-mediated cross-linking to both fibrin(ogen) and Fn. Matsuka *et al.* showed that recombinant FnBPA supports the formation of ϵ -(γ -glutamyl)-lysine isopeptide bonds when treated with activated coagulation Factor XIIIa, with its natural ligands fibrin(ogen) or Fn. In vitro studies showed that Factor XIIIa catalyzes cross-linking using reactive glutamine and lysine residues in FnBPA, forming high-molecular-weight heteropolymers that incorporate either fibrin(ogen) or fibronectin.

In a subsequent study, Severina *et al.* found Glutamine 103 (Gln103) in FnBPA to be the primary acyl-donor site for the efficient attachment of these extracellular ligands, in particular fibrin(ogen) (fig. 7). This residue is directly involved in the covalent linkage, so it’s critical for *S. aureus* to anchor itself to clot matrices and fibronectin networks. The resulting covalent assemblies probably provide mechanical stability, allowing *S. aureus* to adhere to clot surfaces or host tissues under shear stress. Plus, this linkage may help *S. aureus* survive in vivo by cementing the bacteria to fibrin-rich sites, such as thrombin or injured endothelium, and allowing firm colonization even in the presence of host proteases.

In summary, this virulence factor can be cross-linked by Factor XIIIa to Fbg and Fn, forming covalent attachments that probably help bacterial persistence and virulence in vascular and tissue environments.

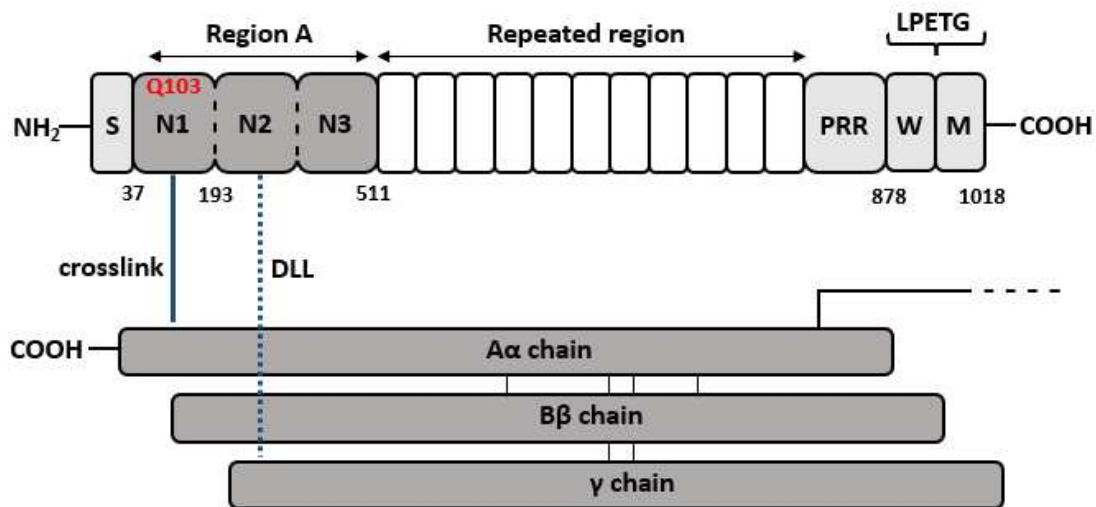


Figure 7. Structure of *S. aureus* FnBPA and interaction with Fbg. The figure is illustrating the N-terminal A region (subdomains N1, N2, N3) responsible for binding Fbg, followed by multiple fibronectin-binding repeats at C-terminus. The figure shows Fbg interacting with the A region, emphasizing crosslink formation through glutamine 103 in the N1 subdomain and the non-covalent interaction through the dock, lock, and latch (DLL) mechanism, specifically involving N2N3 subdomain. The C-terminal region includes proline-rich repeats, wall-anchoring, and membrane-spanning domains, completing the protein's structural organization.

Staphylococcal modulators of host protease activity

Among the secreted virulence factors of *S. aureus*, a subset manipulate the host protease systems. Coagulase, von Willebrand Factor Binding Protein (vWbp), staphylokinase (SAK) and α -toxin (fig. 7) are examples. These factors modulate coagulation cascades and immune responses and facilitate bacterial dissemination and immune evasion (Pietrocola et al., 2017). Coagulase has an N-terminal D1D2 domain necessary for prothrombin activation, a flexible linker and a C-terminal region with Fbg-binding repeats. This protein inserts its N-terminal residues into prothrombin's activation pocket, triggering a conformational change that activates the enzyme (Friedrich et al., 2003). SAK is a 136-amino acid bacteriophage-encoded protein expressed in lysogenic strains (Peetermans et al., 2016). Without intrinsic proteolytic activity, SAK forms a 1:1 complex with plasmin, changes the substrate specificity and activates plasminogen (Collen and Lijnen, 2005; Peetermans et al., 2016). SAK-bound bacteria gets localized fibrinolytic activity which helps in tissue penetration and immune evasion. It can also degrade IgG and C3b, opsonization (Rooijackers et al., 2005) and is involved in biofilm modulation (Kwiecinski et al., 2016). α -Toxin, a pore-forming cytotoxin not only lyses host cells but also interacts with ADAM10, cleaves E-cadherin and disrupts epithelial barriers (Craven et al., 2009; Inoshima et al., 2011). This helps bacterial translocation across epithelial layers during infection.

Von Willebrand Factor Binding Protein (vWbp)

Von Willebrand factor binding protein (vWbp) is a secreted virulence factor of *S. aureus* with a molecular weight of approximately 66 kDa and 482 amino acids. Initially identified for its ability to bind human von Willebrand factor (vWF), vWbp is now known to be a multifunctional effector involved in several host interactions (Bjerketorp et al., 2002) (fig. 8).

Genomically, the *vwb* gene is located between *clfA* and *emp*. ClfA encodes clumping factor A, a major Fbg binding adhesin in *S. aureus* (McDevitt et al., 1994; Ni Eidhin et al., 1998), while *emp* encodes a protein that binds to various extracellular matrix components (Hussein et al., 2001). In fully sequenced strains like N325 and Mu50, these three genes are oriented in the same direction (Kuroda et al., 2001). This clustering of virulence genes is in line with the general pattern where virulence determinants are often found in distinct blocks (Hacker et al., 1997).

Functionally, vWbp is co-expressed with ClfA and cooperates with it. Under flow conditions, vWbp can bridge ClfA and vWF, forming a strong ternary complex (vWbp-ClfA-vWF). This complex is stabilized through interactions that likely involve the Dock, Lock and Latch (DLL) binding mechanism. The result is a very stable bond with high mechanical resistance, which could be a target for inhibitory molecules, including synthetic peptides that disrupt the ClfA–vWF interface (Viljoen et al., 2021).

In vivo studies in mice have shown that vWbp is involved in the pathogenesis of sepsis, endocarditis and abscess formation (Panizzi et al., 2011; McAdow et al., 2011). This is mainly due to its interactions with coagulation factors and host matrix components during early stages of infection.

At the molecular level, the N-terminal region of vWbp binds to prothrombin (ProT), Fbg and coagulation factor XIII (FXIII) and the C-terminal region to fibronectin (Fn) (Thomer et al., 2013; Kroh et al., 2009). Sequence analysis shows that vWbp shares homology with the D1D2 domain of coagulase (Coa) but lacks the linker and C-terminal repeat regions. Instead vWbp has specialized binding motifs for vWF and Fbg (Bjerketorp et al., 2002; Cheng et al., 2010).

vWbp is involved in the formation of fibrin-based aggregates when *S. aureus* is incubated in plasma. Along with Coa and ClfA, vWbp facilitates bacterial clumping by activating ProT to cleave Fbg and ClfA helps bacterial aggregation into fibrin networks (McAdow et al., 2011; Walker et al., 2013). These fibrin structures act as shields for the bacteria from immune cells like neutrophils. Furthermore, the formation of these fibrin barriers has been shown to enhance the virulence of bloodstream infections in mice (Walker et al., 2013).

Mechanistically, vWbp induces a conformational change in ProT upon binding, converting it into active thrombin without proteolytic cleavage. This thrombin then catalyzes the formation of insoluble fibrin and the activation of FXIII which crosslinks fibrin monomers

into polymers (Thomer et al., 2013; Kroh et al., 2009). Importantly, vWbp neutralizing antibodies can inhibit this process (Bjerketorp et al., 2004).

In normal physiology the coagulation cascade is a series of tightly regulated steps that converge on the activation of factor X (FX) which along with cofactors and phospholipid surfaces converts ProT to thrombin. Thrombin then generates fibrin and activates FXIII and ultimately leads to the assembly of a stable cross linked fibrin clot (Hall, 2010; Kumar et al., 2010). While this cascade involves enzymatic cleavage events vWbp bypasses the conventional route by directly binding and activating ProT via a conformational change.

So, while both the natural and vWbp mediated pathways lead to fibrin formation and clot stabilization, the mechanisms are fundamentally different. vWbp's strategy of molecular mimicry and host protein hijacking is a clever adaptation that allows *S. aureus* to establish and sustain infections in hostile host environments.

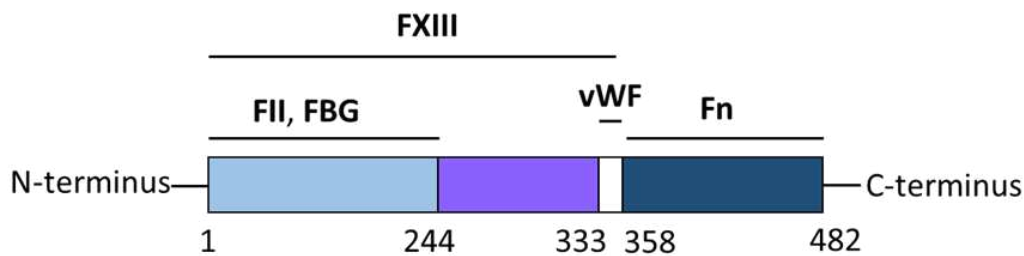


Figure 8. Structure of *S. aureus* von Willebrand factor-binding protein (vWbp) showing its key domains and binding sites. The N-terminal domain binds and activates prothrombin and also interacts with FXIII, while central and C-terminal regions interact with von Willebrand factor (vWF), fibrinogen, fibronectin (Fn). These interactions facilitate clot formation and bacterial adhesion.

Host protein mediators of *S. aureus* adhesion and invasion

S. aureus colonization of human tissues is largely determined by host-derived components, located particularly in the extracellular matrix (ECM) or plasma. Structural proteins like fibronectin, Fbg, collagen, vitronectin and laminin act as binding substrates in skin, nasal mucosa and wounded tissue, allowing initial bacterial adhesion (Bhattacharya et al., 2024). Fibrinogen (Fbg) is a key player in mediating adhesion to damaged endothelium and indwelling catheters, acting as a bridge between bacteria and host surfaces. Soluble plasma proteins like prothrombin and complement factors are also hijacked: *S. aureus* binds prothrombin to induce local fibrin deposition, while interactions with complement components help with immune evasion. On epithelial and endothelial cells, host receptors like integrins (e.g. $\alpha_5\beta_1$) and ICAM-1 mediate bacterial uptake after ECM bridging interactions (Josse et al., 2017; Liesenborghs et al., 2018). Atopic dermatitis, an inflammatory condition, upregulates ECM exposure and cytokines that enhance bacterial binding and colonization (Krishna et al., 2012). In short, *S. aureus* colonization is determined by the availability and

exposure of host proteins that allow adhesion, then evade immune defenses and lay the groundwork for deeper invasion and biofilm formation.

Fibronectin

Fibronectin (Fn) is a big glycoprotein in vertebrates that links cells to the extracellular matrix (ECM). Functionally it's a bridge between cell surfaces and the surrounding matrix. Fn is involved in many physiological processes such as cell adhesion, migration, proliferation and differentiation and interacts with many endogenous ligands (Mosher, 1989; Carsons, 1989; Hynes, 1990; Yamada and Clark, 1996). This protein is produced by many cell types and is essential during vertebrate embryogenesis. In fact, knockout of the Fn gene has shown that embryos without this protein cannot develop to term (George et al., 1993). Fn is a big multidomain protein that is present in body fluids and ECMs across many tissue types. Because of its wide distribution and structure Fn is a target for pathogenic microbes, and often bacterial species have adhesins that recognize and bind to Fn and facilitate colonization and invasion of host tissues (Speziale et al., 2019) (fig. 9).

Structure of fibronectin

Fibronectin (Fn) is a dimer of two nearly identical (of about 250 kDa) subunits, linked together near the C-terminus by two disulfide bonds (fig. 9). Each Fn monomer has a modular structure composed of multiple repeating elements that also called modules, domains or repeats, which are classified into three types: type I, type II and type III. The bulk of the Fn polypeptide sequence is composed of these repeat domains, with each monomer having 12 type I repeats, 2 type II repeats and approximately 15-17 type III repeats. These domains are not only different in size but also in structure. Type I modules are 40 amino acids long and have two disulfide bridges, type II repeats are 60 amino acids long and have two intrachain disulfide bonds, while type III domains are 90 amino acids long and have no disulfide linkages. Interestingly, Fn-like repeats have been found in many other proteins, so it's likely that exon shuffling was involved in the evolution of Fn's domain architecture (Pankov and Yamada, 2002; Patel et al., 1987).

Although Fn is from a single gene, it has multiple isoforms. This diversity is due to alternative splicing, where a single pre-mRNA transcript gives rise to around 20 different Fn variants in humans (Kosmehl et al., 1996). The protein also has carbohydrate moieties, which account for about 4-9% of its total mass. These glycosylation patterns are both N-linked and O-linked and are mainly found in type III domains and the collagen-binding region. Carbohydrates are thought to protect against proteolytic degradation and modulate Fn's binding to different partners. Fn is present in soluble form in plasma (around 300 µg/ml) and other body fluids. It's also a major component of the insoluble extracellular matrix. Two main forms are recognized based on solubility: plasma fibronectin (pFn) and cellular fibronectin (cFn). pFn

is mainly produced by hepatocytes in the liver and has a simpler splicing pattern that excludes the EDA and EDB domains, but includes V0 or V+ variants. cFn has a wider range of isoforms generated by cell- and species-specific alternative splicing events. This variability is crucial in determining Fn's adhesive and ligand-binding properties and reflects the different requirements of tissues during development and in mature states (Pankov and Yamada, 2002).

Also, Fn can be studied through its proteolytic fragments. When digested, Fn is cleaved only at specific regions – likely those that are exposed and flexible. Importantly, many of Fn's binding domains remain active after cleavage so you can map specific ligand-binding activities to specific fragments of the protein (Mosher, 1989; Hynes, 1990).

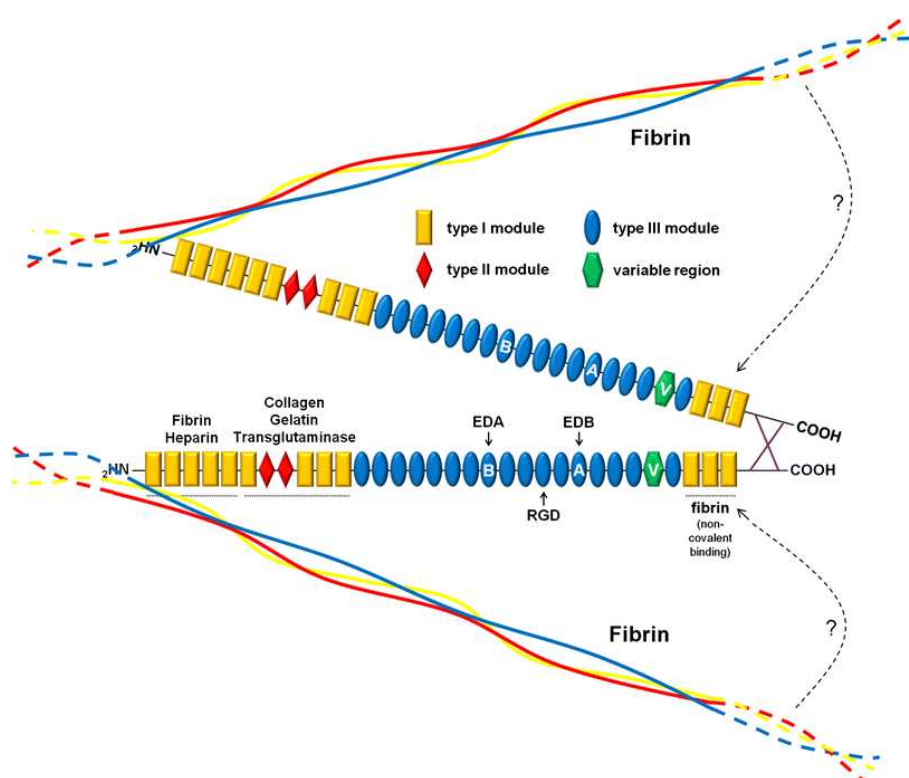


Figure 9. Structure of fibronectin. Fibronectin is a dimer of two 250 kDa subunits, each composed of 12 type I, 2 type II, and 15–17 type III modules. Alternative splicing within type III regions produces EDA and EDB variants, present in cellular fibronectin (cFn) but absent in plasma fibronectin (pFn). The N-terminal 27 kDa region can covalently bind fibrin, promoting fibrin fiber aggregation and offering binding sites for platelets and other circulating cells at vascular injury sites. Dotted lines indicate ligand-binding regions (Wang and Ni, 2016).

Physiological role of fibronectin

Fibronectin (Fn) binds to many integrins, heterodimeric transmembrane receptors that connect the extracellular matrix (ECM) to the cytoskeleton inside the cell. The most well characterized Fn-binding integrin is $\alpha 5 \beta 1$ which binds to the RGD (Arg-Gly-Asp) motif in the type III¹⁰ repeat of Fn. But Fn has other sequences that are recognized by other integrins.

For example, integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$ bind to the LDV and REDV motifs respectively: both are in alternatively spliced regions of the Fn molecule. There are multiple integrin binding sites across type III repeats and spliced regions so Fn's interactions with integrins are structurally diverse and tightly regulated (Plow et al., 2000; Liao et al., 2002).

In addition to its integrin mediated functions, Fn also binds to several other biological molecules such as heparin, collagen, gelatin and fibrin. Much of what we know about these interactions has been studied using specific proteolytic fragments of Fn. Fn has two primary heparin binding domains, one near the N-terminus and a larger one in the C-terminus. In some cell types these domains enhance cellular adhesion by binding to heparan sulfate proteoglycans.

Fn's interaction with collagen, particularly denatured collagen (gelatin) is mediated by the I⁶⁻⁹ and II^{1,2} repeats which together form the collagen binding domain. This binding suggests that Fn may be involved in the recognition and clearance of structurally altered or damaged collagen in tissues.

Fibrin is another important ligand for Fn. Two distinct binding sites facilitate this interaction, the most functionally significant of which is in the N-terminus and comprises type I repeats 4 and 5. This binding may have important physiological implications including promoting cell adhesion and migration into fibrin rich areas such as clots. Fn-fibrin interactions may also support macrophage activity during wound healing and inflammation by allowing cellular access and clearance in fibrinous tissue environments (Hynes, 1990; Yamada and Clark, 1996; Mostafavi-Pour et al., 2001).

Role of fibronectin in infective diseases

fibronectin (Fn) is a major target for a wide range of bacteria, both Gram-positive and Gram-negative. Among the Gram-positive bacteria, *S. aureus* has been studied the most; but other genera such as *Streptococcus*, *Enterococcus*, *Mycobacterium*, *Clostridium*, and *Listeria monocytogenes* also have multiple surface proteins that bind to Fn. On the Gram-negative side, pathogens like *Escherichia coli*, *Campylobacter jejuni*, *Salmonella enterica* serovar Typhimurium, and *Borrelia burgdorferi* have developed similar mechanisms to interact with Fn.

These interactions often involve bacterial receptors that recognize specific Fn domains, particularly the N-terminal segment and the central cell-binding domain. While adhesion is the primary function of these interactions, many bacterial adhesins play more complex roles, attaching and invading and persisting within host tissues (Speziale et al., 2019).

In mammals, the extracellular matrix (ECM) is a highly organized network of secreted proteins that fills the intercellular space. Collectively, these ECM proteins form what is called the "matrisome" (Naba et al., 2016). The matrisome can be further divided into the "core matrisome," which includes about 300 proteins such as collagens, proteoglycans, and

glycoproteins (Ricard-Blum, 2011; Iozzo and Murdoch, 1996), and the “matrisome-associated proteins,” a more functionally diverse group that includes molecules like annexins, enzymes, complement factors, and growth factors (Hynes and Naba, 2012). Given the importance of the ECM in host tissue structure and integrity, it’s no surprise that it’s a prime target for bacterial colonization. To attach, many bacteria produce specific surface proteins called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). These adhesins can bind to several ECM components including collagen, Fbg, laminin and Fn. In *S. aureus*, the best-characterized Fn-binding proteins are FnBPA and FnBPB (Foster et al., 2014; Foster, 2019). These proteins often have multiple functional domains to interact with various host ligands. For example, the A domain of FnBPB can bind Fbg (Burke et al., 2014), elastin (Roche et al., 2004), plasminogen, and histones (Pietrocola et al., 2016) and the C-terminal repetitive region can specifically bind Fn (Jönsson et al., 1991).

FnBPA and FnBPB engage ligands through a variation of the classical “dock, lock, and latch” (DLL) mechanism. The A domain has three subdomains: N1, N2 and N3. When a ligand binds between N2 and N3, a conformational change occurs that redirects a C-terminal extension of N3. This extension folds over the ligand and stabilizes the complex through a new β -strand that complements the β -sheet in N2 (Speziale et al., 2019; Stemberk et al., 2014).

Moreover, *S. aureus* can use adhesin-bound Fn bridges to engage host $\alpha 5 \beta 1$ integrins to enter non-professional phagocytic cells (Sinha et al., 1999; Edwards et al., 2010). Besides FnBPs, *S. aureus* makes other Fn-binding molecules, such as Ebh (extracellular matrix-binding protein homologue), a 1.1 MDa protein, and Eap (extracellular adherence protein), a 50–60 kDa protein (Cheng et al., 2014; Jönsson et al., 1995).

Fibrinogen

Fibrinogen (Fbg) is a high molecular weight protein in human blood plasma, made mainly by the liver. It normally runs at 1.5 to 4 g/L and goes up during inflammation or injury (Mosesson, 2005; Weisel & Litvinov, 2017). Structurally, Fbg is made of 3 pairs of chains (A α , B β , and γ) linked together to form a symmetrical, long molecule (Doolittle, 2011). When an injury happens, the enzyme thrombin converts Fbg into fibrin by removing small parts of the molecule. These changes allow fibrin molecules to join together and form a blood clot (Weisel, 2005).

Fbg does more than just clot blood. It helps cells stick to damaged tissue, supports immune cell activity and healing and inflammation (Adams et al., 2009). Changes in its structure than can be due to genetics or different forms of the molecule, can affect how clots form or dissolve and bleeding or thrombosis risk (Mishra et al., 2021). Because of its role in clotting, inflammation and tissue repair, Fbg is a key player in vascular health and disease.

Structure of fibrinogen

Human Fbg is a 340kDa plasma glycoprotein synthesized by hepatocytes and secreted as a disulfide linked hexamer composed of two symmetrical trimers, each of A α , B β and γ chains (Mosesson, 2005; Doolittle, 1984). Each chain has a signal peptide for targeting to the endoplasmic reticulum, where sequential assembly occurs via dimerization of partial chains followed by formation of full length half molecules which are then covalently linked to produce the mature hexamer (Weisel & Litvinov, 2017; Medved & Weisel, 2009). The structure is stabilized by 17 interchain and 12 intrachain disulfide bonds, with highly conserved intrachain disulfide loops playing a critical role in chain folding and secretion efficiency (Mosesson, 2005).

Fbg has a trinodal structure with a central E domain containing the N terminal ends of all six chains (including fibrinopeptides A and B) flanked by two distal D domains at the C terminal ends of the B β and γ chains (fig. 10). These are connected by triple coiled-coil segments that extend the molecule to approximately 45nm in length (Spraggon et al., 1997; Kollman et al., 2009). The flexible α C regions, which are C terminal extensions of the A α chains, loop back towards the central E domain and radiate outwards. These regions are involved in lateral protofibril aggregation, mechanical stiffness of fibrin clots and modulation of clot properties under physiological conditions (Tsurupa et al., 2009; Weisel, 2004).

Calcium ions are essential for maintaining Fbg's structure and function. There are high and low affinity calcium binding sites within the γ and β nodules. For example the high affinity γ 1 site is important for the structural stability of the D domain and facilitates knob-hole interactions during fibrin polymerization (Veklich et al., 1993). These calcium binding sites also influence the kinetics of protofibril growth and lateral aggregation of fibrin fibers (Weisel & Litvinov, 2017). Post translational modifications also contribute to the molecular diversity and function of Fbg. Glycosylation occurs at specific asparagine residues (B β 364 and γ 52) while other modifications such as hydroxylation, sulfation and phosphorylation occur during Golgi processing (Kollman et al., 2009). The α C regions along with the γ' splice variant of the γ chain influence fibrin structure, clot stability and fibrinolysis (Tsurupa & Medved, 2001).

Overall the complex molecular architecture of Fbg including domain organization, coiled-coil connectors, disulfide network, calcium binding sites and flexible terminal regions indicates its physiological functions in coagulation, clot stabilization and tissue repair (Mosesson, 2005; Weisel, 2004; Weisel & Litvinov, 2017).

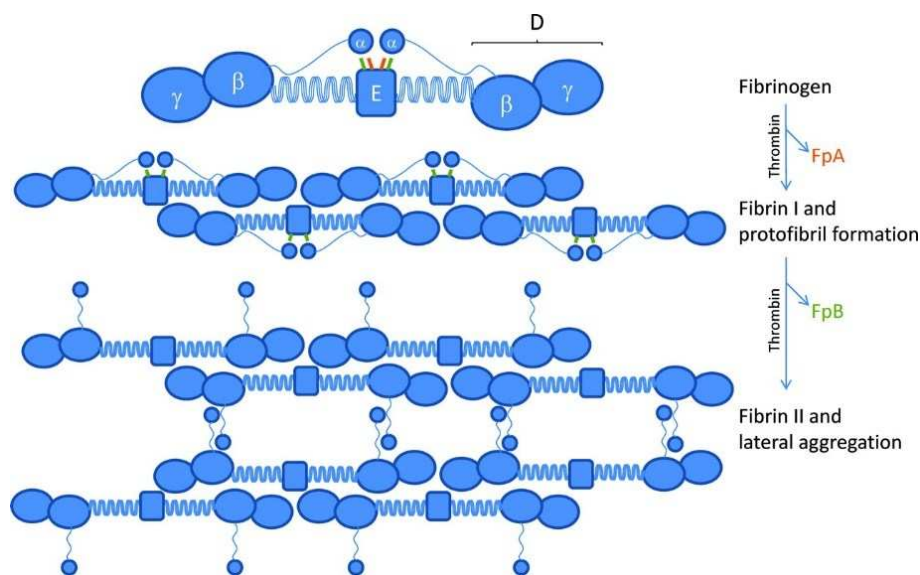


Figure 10. Fibrinogen and fibrin formation. Fibrinogen is a symmetrical dimer made of 2 α , 2 β , and 2 γ chains. The central E-region contains all six N-termini, while the D-regions contain the β - and γ -chain C-termini, connected via coiled coils. The α -chain C-termini fold back to interact with the E-region. During fibrin formation, thrombin first cleaves FpA (orange) to form fibrin I, initiating protofibril formation. Cleavage of FpB (green) follows, releasing α C-domains that promote lateral aggregation into mature fibrin (Undas and Ariens, 2011).

Physiological role of fibrinogen

Fbg and its activated form fibrin orchestrate many physiological functions across hemostasis, fibrinolysis, wound healing, inflammation and innate immunity. In primary hemostasis, Fbg binds to activated platelet integrin α IIb β ₃ (GPIIb/IIIa), crosslinking platelets to form the platelet plug (Marguerie et al 1985; Plow et al 1984). During secondary hemostasis, thrombin generated converts Fbg to insoluble fibrin monomers which polymerize and are stabilized by factor XIIIa to form a durable fibrin network that seals the damage (Chapin & Hajjar 2015).

Fibrin bound to platelets also initiates “outside-in” signaling via α IIb β ₃ that activates the actin-myosin cytoskeleton, leading to clot retraction. This compacts the fibrin mesh, brings wound edges together and enhances mechanical strength while reducing permeability. As repair proceeds the fibrin scaffold is targeted by the fibrinolytic system. Fibrin’s cryptic lysine rich domains concentrate plasminogen and tissue plasminogen activator (tPA) and augment plasmin formation. Plasmin then degrades fibrin into soluble fragments such as D-dimer (Chapin & Hajjar 2015).

This proteolytic activity is tightly regulated: α ₂-antiplasmin is covalently crosslinked into fibrin by factor XIIIa to inhibit plasmin locally; plasminogen activator inhibitor-1 (PAI-1) limits tPA activity; and thrombin-activatable fibrinolysis inhibitor (TAFI) removes exposed lysine residues on fibrin to reduce plasminogen binding (Chapin & Hajjar 2015). This ensures that

fibrinolysis is restricted to sites that need clot resolution and not elsewhere. Fibrin also forms a provisional extracellular matrix that supports wound healing. It binds growth factors like fibroblast growth factor-2 (FGF-2) and facilitates endothelial migration and angiogenesis in granulation tissue (Sahni & Francis 2006). Fibrin also mediates leukocyte recruitment and activation by binding to the integrin $\alpha M\beta_2$ (Mac-1) on neutrophils and macrophages, promoting adhesion, chemotaxis and phagocytosis – functions that are disrupted in Mac-1 deficient mice that show impaired inflammatory responses (Wright et al 1988; Altieri et al 1988). Fibrin matrices can also polarize macrophages to a pro-resolving phenotype and physically trap pathogens to enhance microbe clearance (Wright et al 1988).

Mechanically fibrin networks exhibit strain-stiffening and viscoelastic behavior and adapt to hemodynamic stresses and serve as a scaffold for bioengineered tissue constructs (Weisel & Litvinov 2017). That's why fibrin is widely used in regenerative medicine due to its dynamic structural properties and biocompatibility.

Fbg and fibrin coordinate platelet aggregation, clot stabilization and retraction, spatially regulated fibrinolysis, matrix formation for tissue repair, immune cell modulation, inflammation resolution, mechanical support and microbial containment. Disturbances in any of these pathways (whether via altered Fbg levels, disrupted integrin signaling, impaired fibrinolytic regulation or aberrant fibrin mechanics) can lead to hematological disorders including bleeding, thrombosis, chronic inflammation and defective wound healing.

Role of fibrinogen in infective diseases

Fibrinogen has multiple roles in host defense against infectious diseases by participating in pathogen recognition, immune modulation and formation of protective barriers, but can also be exploited to the benefit of human pathogens. In bacterial infections, Fbg is a key substrate exploited by pathogens like *S. aureus* to establish infection and evade immune clearance. *S. aureus* expresses several surface proteins including clumping factors (ClfA and ClfB) and fibronectin-binding proteins that bind Fbg with high affinity. These interactions promote bacterial adherence to host tissues and allow the formation of fibrin rich abscesses which shield bacteria from phagocytosis and antimicrobial agents (Foster et al. 2014). The fibrin meshwork around bacteria acts as a physical barrier, facilitating immune evasion and persistent infection (McAdow et al. 2012).

Beyond *S. aureus*, other Gram positive bacteria like *Streptococcus pyogenes* also use Fbg binding proteins to colonize and invade host tissues. For example, the M protein of *S. pyogenes* binds to Fbg to inhibit complement activation and neutrophil recruitment, modulating inflammation and immune defense (Frick et al, 2018). *Enterococcus faecalis* can also bind to Fbg, forming biofilms on medical devices which complicates treatment due to increased resistance (Dunne 2002).

Fbg also affects leukocyte function during bacterial infections. Fbg fragments generated during clotting recruit and activate neutrophils and macrophages via integrin receptors like Mac-1 (CD11b/CD18) enhancing phagocytosis and cytokine production (Wright et al. 1988; Altieri et al. 1990). But excessive Fbg deposition can cause tissue damage and exacerbated inflammation, so it has a dual role in host defense and pathogenesis (Ricciardi et al. 2018). In viral infections, Fbg's role is less direct but still important. Elevated Fbg levels are common during acute viral infections as part of the acute phase response and contribute to hypercoagulability and thrombotic complications seen in diseases like COVID-19 (Tang et al. 2020). Fbg may also bind to viral proteins or form fibrin matrices that affect immune cell trafficking and viral clearance, although mechanisms are not fully understood (Mesquita et al. 2021).

Fungal pathogens like *Candida albicans* interact with Fbg during invasion. *Candida* adhesins bind to Fbg, allowing adherence to host tissues and medical devices, forming biofilms that protect fungi from antifungal drugs and immune responses (Tobudic et al. 2012). Fbg's role in fungal infections is similar to its role in bacterial biofilm mediated infections.

Overall, Fbg is both a host defense mediator and a microbial target. Pathogens use Fbg to adhere, invade and form protective niches while the host uses Fbg to recruit immune cells and contain infection through clotting. The balance between these opposing roles determines infection outcomes and offers therapeutic targets to disrupt pathogen-Fbg interactions or modulate Fbg mediated immunity.

Fibrinogen and fibronectin role in the human vascular endothelium

The vascular endothelium is a layer of flat endothelial cells lining the tunica intima of blood vessels, attached to a thin basement membrane made up of type IV collagen, laminins, nidogen and perlecan. This layer controls permeability, leukocyte trafficking and matrix interactions. Below the intima lies the tunica media with smooth muscle and elastin, and the tunica adventitia with connective tissue and vasa vasorum (Kruegel et al., 2010). The vascular endothelium forms a selective barrier that regulates blood-tissue exchange, hemostasis and inflammatory responses (Yuan et al., 2010.) Below this cellular layer lies a basement membrane: a structured extracellular matrix composed of type IV collagen, laminins (laminin-411 and -511), nidogen, perlecan and under certain conditions von Willebrand factor (Tilling et al., 2002) Endothelial cells adhere to this basement membrane via integrin-rich focal adhesions; for example integrin $\alpha_5\beta_1$ binds fibronectin, $\alpha_6\beta_1$ binds laminin and others such as $\alpha_1\beta_1$ and $\alpha_2\beta_1$ bind collagen (Yuan et al., 2010).

Fn is a high-molecular-weight glycoprotein present in both soluble plasma and insoluble matrix forms. In healthy, quiescent adult vessels, fibronectin deposition in the basement membrane is minimal. However, during angiogenesis, wound healing, atherosclerosis, or

inflammatory remodeling, fibronectin expression is significantly upregulated. Endothelial cells synthesize and deposit fibronectin in fibrillar networks via integrin $\alpha_5\beta_1$ -mediated tension-dependent assembly, a critical step for endothelial migration, proliferation, and tubulogenesis (Hamidi et al., 2017). In fact, polymerized fibronectin is essential for 3D vascular morphogenesis; inhibition of fibronectin fibrillogenesis or blockade of $\alpha_5\beta_1$ or $\alpha_v\beta_3$ integrins impairs capillary formation in vitro (Hielscher et al., 2016). Developmental studies in mouse retina further highlight the autocrine role of endothelial-derived fibronectin containing alternatively spliced EIIIA/EIIIB domains in vessel assembly and integrity (Turner et al., 2017).

Fbg is a soluble plasma glycoprotein and not a constitutive basement membrane component. It circulates in the blood and upon tissue injury or inflammation is converted to fibrin by thrombin and forms provisional matrices. These provisional fibrin(ogen) matrices serve as a scaffold for endothelial cell adhesion, migration and recruitment of additional extracellular matrix proteins including fibronectin. Human endothelial cells seeded on Fbg promote endogenous fibronectin secretion and matrix deposition involving integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ necessary for cytoskeletal organization and stable adhesion. (Pieters et al., 2019)

Fbg and its degradation products also modulate endothelial barrier function. Native Fbg has little effect but Fbg fragments (especially those with exposed γ C domains) can increase vascular permeability by engaging endothelial receptors such as ICAM-1 or integrins (e.g. $\alpha_{IIb}\beta_3$) and contribute to microvascular leakage during reperfusion or inflammation (Yuan et al., 2010). Fbg deposition at injured endothelium promotes platelet adhesion via ICAM-1 bridging and influences thrombosis in post-ischemic vessels (Steffen et al., 1999).

So, under normal conditions fibronectin is absent from the mature basement membrane but becomes essential during vascular remodeling, while Fbg is a plasma-borne provisional scaffold that supports wound associated endothelial responses. Their interplay (Fbg initiating matrix assembly and fibronectin providing structural integrity) ensures dynamic remodeling of the endothelium during development and pathology.

Human transglutaminase enzymes

Human transglutaminases are a family of Ca^{2+} -dependent enzymes that form ϵ -(γ -glutamyl)-lysine isopeptide bonds between glutamine and lysine residues, resulting in protein cross-linking and resistance to proteolysis or glutamine deamidation. In humans, nine active transglutaminases are recognized: Factor XIIIa (FXIIIa), tissue transglutaminase 2 (TG2), and TGs 1-7 and one inactive erythrocyte protein EPB42 (Lorand & Graham, 2003; Iismaa et al., 2009) (Table 2).

FXIIIa is unique in its role in hemostasis: it circulates as a heterotetramer (A_2B_2) and upon thrombin activation and Ca^{2+} -induced dissociation of B subunits, the catalytic A subunits cross-link fibrin to stabilize clots (Lorand & Graham, 2003; Iismaa et al., 2009). FXIII-A is also

expressed in monocytes, macrophages and platelets where it supports tissue repair, extracellular matrix (ECM) remodeling and immune modulation (Lorand & Graham, 2003).

TG2 (encoded by *TGM2*) is the most widely expressed isoform and is multifunctional: besides transamidation it has GTPase, protein disulfide isomerase and kinase activities. Localized in cytosol, nucleus, mitochondria and ECM, TG2 regulates cell adhesion, apoptosis, ECM stabilization and wound healing (Park et al., 2010; Verderio et al., 2004; Haroon et al., 1999). It is the main autoantigen in celiac disease where it deamidates gliadin peptides, making them more immunogenic (Molberg et al., 1998; Sollid & Jabri, 2005). Dysregulated TG2 has been linked to fibrotic disorders, neurodegeneration and cancer (Iismaa et al., 2009). The other transglutaminases are tissue restricted but biologically important. TG1, TG3 and TG5 are highly expressed in epidermis and are involved in skin barrier formation by protein cross-linking; mutations in *TGM1* and *TGM5* cause autosomal recessive congenital ichthyoses (Iismaa et al., 2009). TG4 is prostate specific and involved in semen coagulation. TG6 and TG7 are poorly characterized but seem to be important in neurological and testicular physiology (Iismaa et al., 2009).

Table 2. Summary of key human transglutaminase enzymes. Below are indicated their genes, main functions, and tissue localization, including TG2 and Factor XIII involved in ECM remodeling and coagulation (Komaromi et al., 2011; Lorand and Graham, 2003).

Enzyme	Gene Name	Common Name / Alias	Main Functions	Tissue/Context
TG1	<i>TGM1</i>	Epidermal TG	Crosslinks proteins in the cornified envelope of the skin	Epidermis (keratinocytes)
TG2	<i>TGM2</i>	Tissue TG	Crosslinks ECM and intracellular proteins, cell adhesion, apoptosis, signal transduction; involved in celiac disease	Ubiquitous (high in liver, heart, gut)
TG3	<i>TGM3</i>	Epidermal TG	Forms crosslinks in hair follicles and skin	Epidermis, hair follicles
TG4	<i>TGM4</i>	Prostatic TG	Involved in semen coagulation and fertility	Prostate
TG5	<i>TGM5</i>	Epidermal TG	Important in skin barrier formation; mutations cause skin disorders	Skin
TG6	<i>TGM6</i>	Neural TG	Implicated in neurological function; autoantibodies linked to gluten ataxia	Brain, nervous system
TG7	<i>TGM7</i>	(Less studied)	Possible role in epidermal differentiation	Skin (expression less well defined)
Factor XIII-A	<i>F13A1</i>	FXIII-A (plasma)	Crosslinks fibrin during clot formation; stabilizes blood clots	Circulating blood, platelets, monocytes
Factor XIII-B	<i>F13B</i>	FXIII-B subunit	Carrier subunit that stabilizes FXIII-A in plasma	Plasma

FXIII: structure and physiological role

Factor XIII (FXIII), also known as fibrin-stabilizing factor, is a zymogen of the transglutaminase family that plays a key role in hemostasis, tissue repair and extracellular matrix (ECM) remodelling. In plasma FXIII circulates as a heterotetramer of two catalytic A subunits and two non-enzymatic B subunits (FXIII-A₂B₂). The A subunits (*F13A1*) have an N-terminal activation peptide, a β -sandwich domain, a catalytic core with the conserved Cys–His–Asp triad and two β -barrel domains; the B subunits (*F13B*) are carrier proteins and regulate the enzyme's solubility and plasma stability (Lorand & Graham, 2003; Schröder et al., 2016).

Upon vascular injury thrombin cleaves the activation peptide of the A subunits in a calcium dependent process that causes B subunit dissociation and yields the active dimeric enzyme FXIIIa (Schröder et al., 2016) (fig. 11). FXIIIa crosslinks fibrin molecules through ϵ -(γ -glutamyl)-lysine isopeptide bonds, reinforcing the fibrin network and making it more resistant to proteolytic degradation. This is essential for stabilizing the hemostatic plug and for incorporating antifibrinolytic proteins such as α_2 -antiplasmin into the fibrin clot so it doesn't lyse prematurely (Alshehri et. al, 2021). Beyond clot stabilisation FXIIIa is involved in multiple physiological processes. It mediates wound healing by crosslinking fibronectin and vitronectin in the provisional matrix, enhancing platelet adhesion, leukocyte recruitment and angiogenesis via integrins and VEGFR-2 signalling (Kleber et al., 2022; Schröder et al., 2016). In bone tissue FXIIIa crosslinks ECM proteins such as collagen VI and osteopontin and influences osteoblast differentiation and mineralisation (Al-Jallad, 2011). Cellular FXIIIa expressed in monocytes, macrophages and platelets is activated non-proteolytically and is involved in immune defence by anchoring pathogens in fibrin clots and modulating macrophage signalling pathways relevant to vascular inflammation and atherosclerosis (Schröder et al., 2016). FXIII is also essential for pregnancy as it stabilises the utero-placental interface and its deficiency is associated with recurrent miscarriage (Inbal et al., 2003).

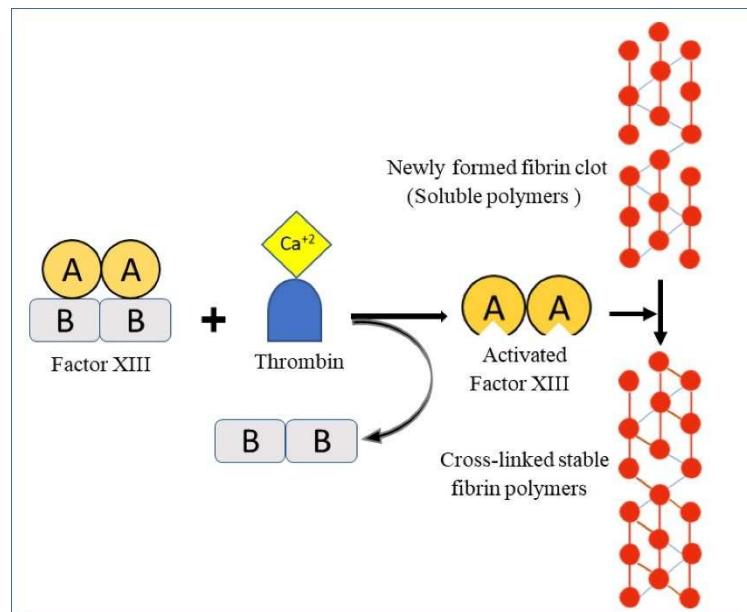


Figure 11. FXIII activation. Thrombin activates Factor XIII by causing dissociation of its B subunits, exposing the catalytic sites of the A subunits. The activated A subunits then form covalent bonds between fibrin chains, stabilizing the clot structure (Aljabry, 2023).

FXIII role in infective diseases

Factor XIII (FXIII) goes beyond hemostasis and could be an essential player in host defense against infection, or could also be exploited by bacterial pathogens to their own benefit. During bacterial invasion, activated FXIII (FXIIIa) forms covalent ϵ -(γ -glutamyl)-lysine bridges between fibrin and pathogen surface proteins, trapping microbes within clots to reduce dissemination and enhance clearance by immune cells (Dickneite et al. 2015). In vivo models have shown that bacterial entrapment within fibrin, facilitated by FXIIIa, limits systemic spread during sepsis, so it's a protective barrier (Kearney et al., 2022; Dickneite et al. 2015).

Among pathogens, *S. aureus* (especially methicillin-resistant strains) has developed sophisticated interactions with FXIIIa. Secreted coagulases, including von Willebrand factor-binding protein (vWbp), activate FXIII non-proteolytically at the bacterial surface. This triggers FXIIIa-mediated cross-linking of fibronectin-binding proteins (e.g., FnBPA) to fibrin, trapping bacteria within clots (Thomer et al., 2013; Motta C et al., 2023). These covalent complexes resist shear forces and ionic stripping, so bacteria can evade the immune system and persist in fibrin-rich niches (Motta C et al., 2023). Interestingly, similar FXIIIa-mediated cross-linking is seen with *Streptococcus pyogenes* and Gram-negative bacteria like *E. coli*, so it's a broad-spectrum microbial sequestration mechanism (Loof et al., 2014; Wang et al.; 2010).

FXIII also enhances innate immunity through macrophage functions. FXIIIa-modified fibrin structures increase macrophage adhesion and phagocytic efficiency against immobilized bacteria. Furthermore, complement components (e.g., C3) are cross-linked by FXIIIa to fibrin, strengthening the clot matrix and potentially modulating complement-mediated opsonization (Ichinose 2012).

FXIII deficiency is associated with impaired infection control. Lower FXIII levels are seen in septic patients and correlate with worse outcomes. Experimental administration of FXIII in animal sepsis models preserves microcirculatory perfusion and reduces bacterial dissemination (Birnbaum et al., 2006). While most of the research is on bacterial infections, FXIII also seems to play a role in viral and fungal infections by maintaining vascular integrity and preventing bleeding. (Lichter et al., 2021).

TG2: structure and physiological role

Human tissue transglutaminase 2 (TG2), a 687 amino acid (~70-78 kDa) protein, has a characteristic 4-domain structure consisting of an N-terminal β -sandwich, a catalytic core containing the active-site Cys-277-His-335-Asp-358 triad, and two C-terminal β -barrel domains (Lee and Park, 2017; Begg et al., 2006) (fig. 12). In the “closed” conformation, stabilized by bound GTP or GDP, TG2 has GTPase signaling activity, whereas binding to calcium shifts it to an “open” conformation that exposes the catalytic triad and enables calcium-dependent transamidation (cross-linking of glutamine and lysine residues) or deamidation of glutamines for isopeptide bond formation (Lee and Park, 2017). Additionally, redox regulation via disulfide bonding between Cys-370 and Cys-371 prevents extracellular TG2 activation despite millimolar Ca^{2+} levels; thioredoxin-mediated reduction reverses this inhibition (Plugis et al, 2017; Stamnaes et al., 2010)

Mechanistically, TG2 catalyzes acyl transfer by nucleophilic attack of the thiol of Cys-277 on the γ -carboxamide of a glutamine residue, forming a thioester intermediate that either reacts with a lysine ϵ -amino group (cross-linking) or is hydrolyzed to glutamate (deamidation) (van Geel et al., 2012). This enzymatic versatility underlies TG2's multifunctionality: beyond cross-linking, it acts as a kinase, protein disulfide isomerase (PDI), scaffolding protein, and G-protein in various cellular compartments (Nurminskaya and Belkin, 2012). TG2 is expressed everywhere (cytosol, nucleus, mitochondria, plasma membrane, extracellular matrix) and is involved in cell adhesion, ECM stabilization, wound healing, apoptosis, differentiation, angiogenesis, bone formation, insulin secretion, and stress responses (Nurminskaya and Belkin, 2012). For example, TG2-mediated serotonylation of G-proteins in β -cells supports glucose-stimulated insulin secretion, and its cross-linking and scaffolding roles modulate vascular stiffness and cell signaling via interactions with integrins and fibronectin (Wang et al., 2021; Nurminskaya and Belkin, 2012). Dysregulated TG2 activity is involved in celiac disease, fibrosis, cancer progression,

neurodegeneration, and diabetes, so it's a key player in physiological and pathological processes (Lee and Park, 2017).

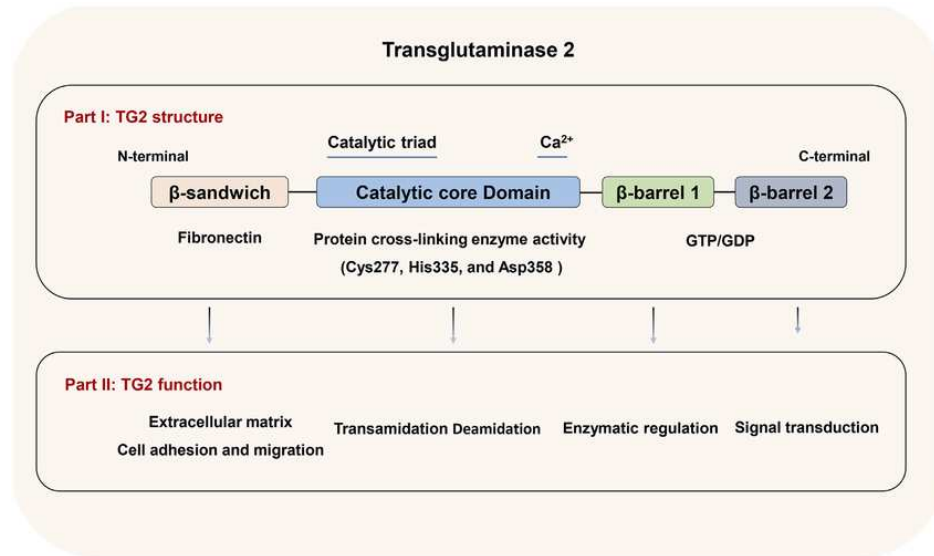


Figure 12. TG2 structure and functions. TG2 consists of four main domains: an N-terminal β -sandwich domain, a catalytic core containing the active site triad (Cys-His-Asp), and two C-terminal β -barrel domains involved in GTP/GDP binding. TG2 functions in a calcium-dependent manner to catalyze protein crosslinking (transamidation), while its GTP-bound form regulates signaling pathways. It plays key roles in extracellular matrix stabilization, cell adhesion, apoptosis, and inflammation, and is notably implicated in celiac disease and fibrosis (Li et al., 2024).

TG2 role in infective diseases

Tissue transglutaminase 2 (TG2) has a role in infectious disease in many ways. It can covalently crosslink microbial surface proteins to host extracellular matrix components. TG2, along with FXIIIa, can incorporate *S. aureus* fibronectin-binding protein A (FnBPA) into Fbg forming high molecular weight heteropolymers. TG2 crosslinking targets the N1 domain of FnBPA and the α -chain of Fbg in vitro (Motta et al., 2023). This likely traps bacteria in the bloodstream and tissues and enhances virulence and immune evasion.

Beyond *S. aureus*, TG2 modulates host-pathogen interactions in many microbial contexts. In *Porphyromonas gingivalis*, TG2 is hijacked to form fibronectin-bacterial protein complexes that facilitate adhesion, with TG2 knockdown reducing bacterial attachment by ~90% (Boisvert et al., 2014). In intracellular pathogens like *Mycobacterium tuberculosis*, TG2's transamidase activity inhibits phagosome maturation, impairs autophagy and enhances bacterial survival; pharmacologic TG2 inhibition reduces mycobacterial replication (Palucci et al., 2020). In fungal infections like *Candida albicans*, TG2 is activated in immune cells and hepatocytes during systemic infection, suggesting a role in sepsis-related tissue remodeling (Su et al., 2021). Viral pathogens also use TG2: respiratory syncytial virus triggers NF- κ B-dependent extracellular TG2 activation, intensifying lung inflammation through matrix crosslinking (Rayavara et al, 2022)

In summary, TG2's enzymatic activity allows bacterial and fungal adhesion and persistence, impairs host antimicrobial processes and exacerbates viral-induced tissue pathology. Specifically the TG2-FnBPA-Fbg crosslinking is a new and clinically relevant mechanism of *S. aureus* pathogenesis, making TG2 a target for therapy.

Serine proteases family

Serine proteases (also known as Peptidase S1 family) are a big family of enzymes with a conserved catalytic triad (His-Asp-Ser) and include both coagulation factors and immune effectors. In humans, about 1/3 of proteases are serine proteases, with 178 family members involved in critical physiological processes like coagulation, inflammation, apoptosis and tissue remodeling (Patel, 2017; Heutinck et al., 2010). Key immune linked serine proteases include neutrophil elastase, proteinase 3, cathepsin G, granzymes, tryptase and chymase which coordinate pathogen clearance and immune signaling (Patel, 2017). Neutrophil elastase for example degrades bacterial surface proteins like *E. coli* OmpA, aiding in microbial killing (Belaouaj et al., 2000). Serine proteases also play key roles in infectious diseases by modulating host defense and pathogen virulence. They participate in immune responses by degrading microbial proteins and activating signaling pathways, while some pathogens exploit host serine proteases or produce their own to enhance infection and evade immunity (Patel, 2017).

Among coagulation associated serine proteases, factor II or prothrombin is the zymogen converted to active thrombin by factor Xa in the prothrombinase complex. Prothrombinase complex includes factor Xa, factor Va, calcium ions and a negatively charged surface found on activated platelets. Factor Xa cuts prothrombin at two specific sites and turns it into active thrombin. Thrombin then converts soluble Fbg into insoluble fibrin strands to form a blood clot, activates platelets to enhance aggregation and triggers positive feedback loops by activating factors V, VIII and XI to amplify the coagulation cascade. This tightly regulated activation ensures rapid yet localised clot formation in response to vascular injury while preventing excessive thrombosis (Davie et al., 1991).

Prothrombin: structure and physiological role

Prothrombin or factor II (ProT) is a ~72 kDa multidomain vitamin K-dependent zymogen with an N-terminal γ -carboxyglutamic acid (Gla) domain followed by two kringle domains and a C-terminal serine-protease module (fig. 13). High-resolution structural and fluorescence studies show that ProT converts between open and closed conformers with kringle-1 to protease domain distance of ~54 Å (open) and ≤ 34 Å (closed) (Ruben et al., 2022). Activation by the membrane-anchored prothrombinase complex (fXa/fVa/Ca²⁺/phospholipids) involves ordered cleavages at Arg-271 and Arg-320. Initial cleavage at Arg-271 (exposed in open conformers) releases Fragment 1•2 and generates prethrombin-2 which reorients to

allow subsequent Arg-320 cleavage to yield thrombin (Brass, 2003; Ruben et al, 2002). Alternatively meizothrombin forms first through Arg-320 cleavage in the closed conformer; cryo-EM structures (4.1 Å) show fVa's A2-domain acting as a “lid” that sequesters Arg-271 and directs Arg-320 into fXa's active site, guiding activation via the meizothrombin pathway (Di Cera et al., 2022; Ruben et al., 2022). Physiologically, ProT is synthesized in hepatocytes in a vitamin K-dependent process that carboxylates the Gla domain for Ca²⁺-mediated membrane binding and achieves plasma concentrations of ~1-1.5 μM in healthy humans. Upon vascular injury, ProT is rapidly converted to thrombin which cleaves Fbg, activates factor V, VIII, XI, XIII and platelets and engages protease-activated receptors (PARs) to drive coagulation, clot stabilization and inflammatory signaling (Bristol et al., 1996; Davie et al., 1991). Genetic ablation of ProT in mice leads to ~50% embryonic lethality by embryonic day 10.5 due to vascular defects; survivors die neonatally from hemorrhage (Xue et al., 1998). Adult conditional ProT knockouts exhibit spontaneous bleeding in heart and brain and impaired pathogen clearance, underscoring its critical roles in hemostasis and immunity (Mullins et al. 2009). Overall, prothrombin's structural flexibility, precise control of cleavage site exposure and assembly into the prothrombinase complex coordinates a finely tuned mechanism for thrombin generation essential for physiological hemostasis, vascular integrity and inflammatory responses.

Prothrombin role in infective diseases

In bacterial infections, prothrombin is hijacked by pathogens like *S. aureus* to form pathogen-specific thrombin complexes (“staphylothrombin”) that overcome host defenses.

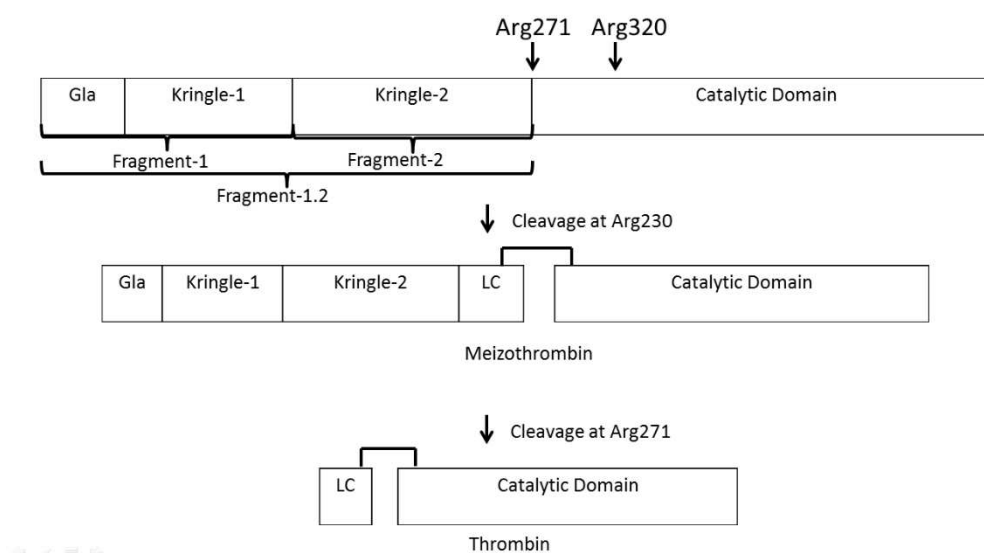


Figure 13. Schematic of physiological prothrombin activation.

Prothrombin is activated on membrane surfaces by the prothrombinase complex via proteolytic cleavage at Arg271 and Arg320. The molecule contains an N-terminal Gla domain for calcium-dependent membrane binding, followed by two kringle domains, which mediate protein–protein interactions and a C-terminal catalytic domain. Cleavage first at Arg320 forms the meizothrombin intermediate, which retains partial activity. Subsequent cleavage at Arg271 yields fully active thrombin. This ordered activation ensures spatial and temporal regulation of coagulation (protopedia.org).

S. aureus secretes two coagulases, staphylocoagulase (Coa) and von Willebrand factor-binding protein (vWbp), which bind prothrombin non-proteolytically and activate it to cleave Fbg into fibrin, generating fibrin scaffolds that help bacteria aggregate and evade the immune system (Cheng et al., 2010; Vanassche et al., 2012). Histologically, prothrombin and fibrin co-localize with coagulases within abscess pseudocapsules in mouse models, showing that this fibrin shield physically protects bacterial communities from immune attack (Cheng et al., 2010). In bloodstream infections, staphylothrombin-mediated fibrin deposition and immunoglobulin interactions facilitate platelet aggregation under shear, promoting vascular colonization (Vanassche et al., 2012). *S. aureus* also produces secreted proteases like aureolysin that activate prothrombin and further drive staphylocoagulation, reinforcing fibrin-mediated immune evasion (Thomer et al., 2016). Beyond bacterial disease, other pathogens exploit prothrombin activation: microbial proteases from fungi (*Candida albicans*) and bacteria (*Pseudomonas aeruginosa*, *Serratia marcescens*) can directly cleave prothrombin to generate thrombin in vitro, possibly contributing to disseminated intravascular coagulation during sepsis (Kaminishi et al., 1994). Viral infections also disrupt prothrombin-dependent pathways: severe viral illnesses (e.g., hemorrhagic fevers, influenza) have been linked to coagulopathy through dysregulated thrombin generation, leading to thrombotic and bleeding complications, though the mechanisms are not yet fully understood (Goeijenbier et al., 2012). In summary, pathogens hijack prothrombin to form fibrin barriers in bacterial infection, while broader coagulation disruptions in fungal and viral disease highlight prothrombin's key role in infection-driven immunothrombosis and sepsis pathology.

Host-pathogen interaction models

Understanding the complex interactions between *S. aureus* and the human host is crucial to understanding its pathogenesis and developing treatments. To this end, various in vitro and in vivo models have been used to mimic infection and study host-pathogen dynamics.

In vitro models often use human cell lines like keratinocytes, endothelial cells and immune cells to study adhesion, invasion and immune evasion. For example, human vascular endothelial cell cultures have been used to study *S. aureus* adhesion mediated by fibronectin-binding proteins (FnBPs) and elucidate key steps in bloodstream infections (Shinji et al., 2011). Three-dimensional tissue cultures and organoids are emerging tools that better mimic tissue architecture and cellular heterogeneity and provide more physiologically relevant insights than traditional monolayer cultures (Reddersen et al., 2019).

In vivo models are essential for studying the systemic progression of *S. aureus* infections and the host immune response. Mouse models including skin abscess, bacteremia and implant-associated infection models are widely used due to their genetic tractability and availability of immunological reagents (Kim et al., 2014). Despite interspecies differences, mouse models have given us insights into bacterial virulence factors, host defense pathways and

vaccine candidates. Alternative models like zebrafish larvae and *Galleria mellonella* (wax moth larvae) offer the advantages of optical transparency and cost-effectiveness for real-time infection imaging and high-throughput screening (Rasheed et al., 2021; Alnezary et al, 2023).

But there are limitations: in vitro models lack systemic immune complexity and mouse models don't fully mimic human immune responses particularly in coagulopathy and toxin susceptibility. Therefore, a combination of models is necessary to capture the multifaceted nature of *S. aureus* infections. Advances in humanized mouse models will help bridge the gap and accelerate translation (Wang and Parker, 2025).

Murine models of *S. aureus* infections

Murine models are vital to understand *S. aureus* pathogenesis, immune responses and treatment strategies, where a controlled investigation of specific virulence mechanisms and host immune pathway is required.

The skin and soft tissue infections (SSTI) models, such as the dermonecrosis model, where *S. aureus* is injected subcutaneously, replicates abscess formation and neutrophil recruitment and is a platform for studying leukocidin function and immune evasion (Cho et al., 2012). The intravenous inoculation model mimics bloodstream infections and allows for examination of complications such as infective endocarditis and osteomyelitis (Cheng et al., 2009). These models have shown the roles of bacterial adhesins, toxins and immune-modulatory proteins in systemic disease.

Also implant-associated infection models, often using catheters or orthopedic implants, have been crucial in dissecting biofilm-mediated persistence and testing anti-biofilm therapies (Riccardi et al., 2018). However, there are limitations, particularly in replicating human-specific factors such as coagulopathy and superantigen sensitivity due to interspecies immune differences. But despite this, murine models are still vital for *S. aureus* research and essential for preclinical testing of vaccines, monoclonal antibodies and small molecule inhibitors (Mestand and Hughes, 2004).

Aim of the study

Staphylococcus aureus is an opportunistic, gram-positive bacteria that colonizes around 20-30% of the human population. It can cause a wide range of infections from mild skin and soft tissue infections to life threatening conditions like sepsis, pneumonia, osteomyelitis and toxic shock syndrome. The emergence and global spread of methicillin-resistant (MRSA) and vancomycin-resistant (VRSA) strains is a major public health issue, complicating treatment and driving the need for new therapeutic strategies (Stefani et al., 2012). So, a lot of research has been focused on identifying new drug targets and understanding the molecular mechanisms of *S. aureus* virulence and host colonization.

S. aureus secretes various proteins and peptides that interfere with the host innate immune response. One of these secreted factors is the von Willebrand factor-binding protein (vWbp), a 66kDa protein. This virulence factor is considered to be a multifunctional protein: apart from von Willebrand Factor (vWF), it is able to bind to FXIII, prothrombin (ProT), and Fn (Thomer et al., 2013; Kroh et al., 2009). Furthermore, vWbp binds and non-proteolytically activates host prothrombin, converting it to thrombin, which then cleaves fibrinogen (Fbg) into fibrin, forming fibrin cables. This is the key to *S. aureus* pathogenicity as shown in animal models (Panizzi et al., 2011). vWbp-activated FXIII, a transglutaminase, catalyzes the formation of ϵ -(γ -glutamyl)-lysine isopeptide bonds by cross-linking lysine and glutamine residues, stabilizing fibrin clots by forming γ -chain dimers and α -chain multimers (Thomer et al., 2013; Kroh et al., 2009).

Apart from secreted factors, CWA proteins are crucial for *S. aureus* mechanisms of adhesion to host tissues. Fibronectin Binding Protein A (FnBPA), a major *S. aureus* surface-anchored adhesin, is a key factor for bacterial adhesion to host extracellular matrix proteins, tissue colonization, immune evasion and biofilm formation. Fibronectin-binding protein A (FnBPA) interacts with host extracellular matrix components, mainly fibrinogen (Fbg) and fibronectin (Fn). Fbg is a key plasma glycoprotein involved in clot formation, and can act as a scaffold for bacterial adhesion. In particular, FnBPA binding to Fbg through can facilitate strong attachment to the host, allowing bacterial colonization at wound sites and damaged tissues (Wann et al., 2000). On the other hand, Fn is a major glycoprotein of the extracellular matrix and also a critical ligand for FnBPA, allowing *S. aureus* to adhere to tissues and is involved in biofilm formation (Meenan et al., 2007; Herman-Bausier et al, 2015). Ultimately, both Fbg and Fn binding, can contribute to *S. aureus* pathogenesis by mediating bacterial adherence and persistence in the host environment.

Previous studies have shown that FnBPA can be crosslinked to both Fbg and Fn by physiologically-generated FXIII (Matsuka et al., 2003), but vWbp coagulase activity was not investigated yet. For this reason, this study focuses on the involvement of vWbp in FnBPA crosslinking to host factors, in order to investigate the molecular interactions between *S. aureus* virulence factors and host transglutaminases that facilitate bacterial stable attachment to the tissues.

First of all, the expression dynamics and post-secretion localization of vWbp during *S. aureus* growth was investigated. Then, the study continued further on by exploring whether vWbp-activated FXIII catalyzes covalent cross-linking between fibrin(ogen) and FnBPA. Also, other MSCRAMMs proteins sharing the same FnBPA architecture were tested to see if there was a conserved mechanism. Furthermore, the human host expresses several tissue transglutaminases beyond Factor XIII, including TG2, which is widely distributed in various tissues and the extracellular matrix. To broaden the scope of the study, TG2 was also tested for its ability to mediate crosslinking between FnBPA and Fbg, exploring additional host factors that may contribute to *S. aureus* adhesion and persistence. Apart from Fbg, since also Fn was described in literature as a suitable crosslinking substrate for FnBPA, its involvement with the vWbp-activated FXIII crosslinking was also investigated.

At this point, it remained to be elucidated the involvement of specific reactive residues inside FnBPA sequence in the mechanism of bacterially-induced crosslinking. Previously, Severina et al. identified glutamine 103 (Q103) in the as a key reactive residue involved in transglutamination processes. Based on this finding, recombinant FnBPA isoforms were produced with a substitution of this residue to alanine (Q103A). After validating the reactivity of Q103 in vWbp-activated FXIII crosslinking, further investigation with the whole bacterial cells were carried on to confirm the model. To do this, an *S. aureus* strain expressing mutant FnBPA was constructed with site-directed mutagenesis and its capacity to be incorporated into fibrin(ogen) matrices was evaluated.

Finally, it was necessary to assess the biological relevance of FnBPA-fibrin(ogen) cross-linking in vivo using murine infection models, comparing wild-type and mutant strains to determine the impact on bacterial virulence and persistence.

This comprehensive study aims to elucidate a novel pathogenic mechanism where host transglutaminases, activated by bacterial coagulase activity, can covalently link *S. aureus* to the fibrin(ogen) and fibronectin. This interaction could promote immune evasion and facilitates long-term tissue colonization. Understanding this novel molecular mechanism may reveal promising therapeutic targets to disrupt bacterial persistence, and could be particularly useful for the treatment of the increasing antibiotic-resistant *S. aureus* infections.

Material and Methods

Bacterial strains and culture conditions

All bacterial strains used in this study are listed in table 3. *S. aureus* cells were grown to stationary or exponential phase in Brain Heart Infusion (BHI) or Tryptic Soy Broth (TSB) (VWR International Srl, Milan, Italy) at 37 °C with shaking. Vector pFnBA4 was transferred to *S. aureus* SH10004X that were made electrocompetent as previously described (Lofblom et al., 2007; Monk et al., 2012) grown in BHI agar and BHI broth containing 10 µg/ml chloramphenicol (Merck, Darmstadt, Germany).

E. coli BL21 (DE3) (Invitrogen, Carlsbad, CA, USA), TOP10 (Thermo Scientific, MA, USA) and IMO8B were grown in Luria agar and Luria broth (VWR International) containing 100 µg/ml ampicillin (Merck) at 37 °C with shaking. BL21 cells were transformed with vector pQE30 (Qiagen Aarhus, Denmark), while TOP10 and IMO8B were transformed with vector pFnBA4 derivatives.

Where needed, bacterial cells were harvested from cultures by centrifugation (10 minutes, 4000 rpm), washed in PBS (Phosphate Buffered Saline, 10 mM Na₂HPO₄, 130 mM NaCl, pH 7.4) and resuspended at the suitable density by spectrophotometer measurements at A_{600nm} (Jasco, V-630).

Table 3. All bacterial strains used in this study.

Bacterial strain	Relevant properties	Reference
<i>S. aureus</i>		
LAC wt	Community-associated MRSA of USA300 lineage	Diep et al., 2006
LAC <i>spa</i>	Strain derivative of LAC* deficient in protein A; constructed by transduction of <i>spa</i> ::Kanamycin resistance by phage 85 into strain LAC*	O'Halloran et al., 2015
LAC <i>srtA</i>	Deficient in sortase A; constructed by the transduction of <i>srtA</i> ::Erythromycin resistance from Newman <i>srtA</i> using bacteriophage 85	Pietrocola et al., 2019
SH10004X	SH1000 is a <i>S. aureus</i> strain 8325-4 derivative with a restored <i>rsbU</i> gene. This is a quadruple deleted SH1000 strain ($\Delta clfA$, $\Delta clfB$, $\Delta fnbA$ and $\Delta fnbB$)	Greene et al. 1995, Ní Eidhin et al., 1998
SH10004X::pFnBA4	SH10004X derivative carrying pFnBA4 overexpressing <i>fnbA</i> ; ampicillin and chloramphenicol resistance	O'Neill et al, 2008; Greene et al, 1995
SH10004X::pFnBA4 Q103A	SH10004X::pFnBA4 carrying pFnBA4 overexpressing <i>fnbA</i> with the Q103A mutation; ampicillin and chloramphenicol resistance	This study
<i>E. coli</i>		
BL21 (DE3)	Cloning host	Invitrogen
XL1Blue	Cloning host	Agilent
TOP10	Cloning host	Thermo Scientific
IMO8B	Plasmid propagation for transformation into <i>S. aureus</i> (SA08B Ω PN25- <i>hsdS</i>)	Monk et al., 2015

Plasmid and DNA Manipulation

The DNA fragments encoding the wild type N1 (residues 37-193), N1N2N3 (residues 37-511) and N2N3 (193-511) regions of FnBPA were PCR amplified from the genome of *S. aureus* strain 8325-4 and cloned as previously reported (Motta C et al., 2023; Pietrocola et al., 2016)

The DNA fragment encoding FnBPA N1 Q103A (residues 37–193) was purchased from Eurofins Genomics. This DNA fragment (insert) was amplified with the following forward (TCA CGG ATC CGC ATC AGA ACA AAA GAC AAC) and reverse (ACG TCG ACC TAC GTT TCC ACT TTC GCG TTA C) primers, digested alongside the vector (pQE30) with BamHI and Sall restriction enzymes and ligated with T4 DNA ligase (Thermo Scientific).

The N1N2N3 Q103A mutation was obtained through a fusion PCR with templates N1 Q103A and N1N2N3 with the following forward (GTTACATCTGTACCCGTTTCCACTTTCGCGTTAC) and reverse (GTAACGCGAAAGTGGAACGGGTACAGATGTAAC) primers, thus generating the insert. The DNA fragment encoding for FnBPA N1N3N3 was amplified from the construct obtained in previous work (see above). The insert and the vector (pQE30) were digested and ligated as reported above.

DNA purification after digestion steps or PCR was carried out using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Plasmid DNA was isolated through the NucleoSpin Plasmid kit (mini prep or midi prep) (Macherey-Nagel), according to the manufacturer's instructions, and transformed into the suitable cloning host. Transformant colonies were selected and screened by colony PCR. DNA purification was carried out using the NucleoSpin Gel and PCR Clean-up kit (MachereyNagel GmbH & Co. KG, Düren, Germany)

All constructs were verified by sequencing using primers pQEfor (5'-GTATCACGAGGCCCTTTCGTCT) and pQErev (5'- CATTACTGGATCTATCAACAGGAG).

Site-directed mutagenesis for mutant pFnBA4 production

The production of a mutant pFnBA4 Q103A plasmid through an established site-directed mutagenesis protocol was carried out at the Institute of Microbiology and Infections, University of Birmingham. Plasmid pFnBA4 Q103A was constructed using site-direct mutagenesis overlapping primers in two rounds of PCR amplification. The first round generated an intermediate codon (GAA), obtained with the following forward (ctcaagacaatagcggagatGaaagacaagtagatttaac) and reverse (gttaaatctacttgtctttCatctccgctattgtcttgag) primers, while with the second round isolated the alanine codon (GCA) with caatctcaagacaatagcggagatAaaagacaagtagatttaacac (forward) and gtgttaaatctacttgtctttTatctccgctattgtcttgagattg (reverse) primers. A dual polymerase approach, 0.5 U of Phusion (NEB) and 0.25 U of Phire hotstart II DNA polymerase (Thermo Fisher), previously described (Monk and Stinear, 2021) was adapted for PCR amplifications

completed under the thermocycling conditions of denaturation 98°C for 2 minutes, 18 cycles of 98°C for 30 s, 55°C for 30 s, and 72°C for 15 s per kb with a final extension of 72°C, 10 minutes.

The final PCR product was treated with DpnI enzyme (NEB) for 1 h at 37°C before using it to transform TOP10 cells. Whole plasmid sequencing (PlasmidsNG, UK) was carried out to confirm Q103A substitution.

Expression and purification of recombinant proteins

All recombinant *S. aureus* FnBPA mutant or wild type regions were expressed using the pQE30 plasmid (which provided His₆ N-terminal affinity tags) in *E. coli* BL21 (DE3). Overnight starter cultures were diluted 1:40 in LB containing ampicillin (100 µg/ml) and incubated with shaking until reached the exponential phase ($OD_{600nm} = 0.4 - 0.6$). Recombinant protein expression was induced by addition of 1mM (final concentration) Isopropyl 1-Thio-β-D-Galactopyranoside (IPTG) (Merck) to the culture, then grown overnight at 28°C in shaking. The next day, bacterial cells were harvested by centrifugation (4000 rpm for 20 minutes) and (eventually) frozen at -80°C. Then the cells were treated with a lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) containing lysozyme 0,2 µg/mL, phenyl-methanesulfonyl-fluoride (PMSF) 1mM, protease-free DNase I 20 µg/mL and MgCl 1mM (Merck). After this, the cells were sonicated for 12 cycles (70% amplitude, 12 cycles of 30 seconds on/off with 1 minute 30 seconds intervals). The cell debris was removed by centrifugation (13000 rpm, at 10°C, 20') and the supernatant applied to a 1 mL Ni²⁺-affinity chromatography (HiTrap chelating column) (GE Healthcare, Buckinghamshire, UK) (Binding buffer: 50 mM Na₂H₂PO₄, 300 mM NaCl, pH = 7,4 and elution buffer: 50 mM Na₂H₂PO₄, 300 mM NaCl, 500 mM Imidazole, pH = 7). The obtained protein was dialyzed overnight, at 4°C in shaking against PBS (1L). Protein purity was confirmed by SDS-PAGE followed by Bio-Safe Coomassie staining (BioRad, Hercules, CA, USA). The concentration of the purified proteins was determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

Other recombinant proteins FnBPA repeated region (RR) (Provenza et al., 2010), FnBPB N1N2N3 (Burke et al., 2011), ClfA N1N2N3 (O' Connell et al., 1998), ClfB N1N2N3 (Walsh et al., 2008), and vWbp (Thomer et al., 2013) were expressed with His₆ N-terminal affinity tags and purified as reported above.

Proteins, reagents and antibodies

BSA (bovine serum albumin) and skim milk were purchased from Merck. Dansyl-ε-aminocaproyl-QQIV was synthesized by GenScript (Rijswijk, Netherlands) and dansylcadaverine was purchased from Merck. Human prothrombin, human factor XIII and Fbg were purchased by Prolytix (VT USA). Fn was purified from human plasma with a combination of gel filtration and affinity chromatography (Speziale et al., 2008). The N29

fragment of Fn was isolated following the protocol reported by Borsi et al. Ultrapure peptidoglycan (PPG) and lipoteichoic acid (LTA) from *S. aureus* were purchased by InvivoGen (CA, USA). Transglutaminase-2 (TG2) was obtained from Zedira GmbH (Darmstadt, Germany).

Human IgG was isolated from the sera of patients diagnosed with infective endocarditis, as previously described (Rindi et al., 2006). All procedures involving human samples were conducted in accordance with institutional and national ethical standards. The study protocol was approved by the Ethics Committee of the University of Pavia, and written informed consent was obtained from all participants.

Polyclonal antibodies against *S. aureus* surface proteins (vWbp, FnBPA, FnBPB, ClfA, and ClfB) or the whole *S. aureus* cell were generated in mice through standard immunization protocols using purified recombinant proteins as antigens. Polyclonal anti-Fbg antibody was also produced following standard immunization procedures. Animal experimentation was conducted in accordance with institutional and national regulations for the care and use of laboratory animals. The protocol received prior approval from the Ethics Committee of the University of Pavia and is reported in compliance with the ARRIVE guidelines (<https://arriveguidelines.org>).

Monoclonal rabbit anti- α chain antibodies were purchased from Bioss Antibodies (MT, USA). Rabbit anti-mouse, rabbit anti-human, goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Dako Cytomation (Glostrup, Denmark). Avidin-peroxidase was purchased from Merck. OPD tablets (*o*-phenylenediamine dihydrochloride) were purchased by Thermo Scientific.

Incorporation of dansylcadaverine and dansyl- ϵ -aminocaproyl-QQIV into FnBPA by vWbp-activated FXIII

Fluorescent probes are tools for studying transglutaminase activity. They are small molecules or peptides with a fluorescent tag that can be attached to target proteins by transglutaminases during the crosslinking reaction. Once attached, the fluorescence makes it easy to detect the labeled proteins.

To assess the presence of reactive sites in FnBPA by the activity of vWbp-activated FXIII, the synthetic probes dansylcadaverine and dansyl- ϵ -aminocaproyl-QQIV were used. In particular, dansylcadaverine was utilized to probe reactive glutamines, and the peptide dansyl-PGGQQIV was used to probe reactive lysines.

Reaction mixtures were prepared containing 2 μ M FnBPA, 200 nM ProT, and 200 nM vWbp. These were incubated with 500 μ g/mL Factor XIII in the presence of either 2 mM dansylcadaverine or 2 mM dansyl-PGGQQIV. Reactions were carried out at 37 °C for 20

minutes in a buffer containing 20 mM Tris-buffered saline (TBS), pH 7.4, with 150 mM NaCl, 5 mM dithiothreitol (DTT), and 5 mM CaCl₂.

To halt the reaction, a stopping buffer with 2% SDS and 10% β-mercaptoethanol (Merck) was added. As negative controls, identical mixtures were incubated with 2 mM EDTA instead of calcium. All samples were then separated by SDS-PAGE, and the resulting gels were initially visualized under UV light to detect fluorescence, then followed by protein staining with Bio-Safe Coomassie.

Enzyme-linked immunosorbent assays (ELISA)

Von Willebrand factor binding protein (vWbp) expression in different stages of bacterial growth

To construct the growth curve, a strain of *S. aureus* lacking protein A (LAC *spa*) was used. The lack of protein A avoids aspecific signals during antibody detection. Overnight bacterial cultures grown in BHI medium were diluted to a starting OD₆₀₀ of 0.05 in the same medium. Every 60 minutes, the OD₆₀₀ was monitored using a spectrophotometer until reaching a plateau indicating the start of the stationary phase (after about 5-6 hours). After each step of incubation, the bacterial cells were removed by centrifugation, and the supernatants were filtered using 0.22 μm membranes. Then, 100 μl of each sample was added to wells of a microtiter plate and left overnight at 4 °C to allow the proteins to bind to the plastic.

To monitor how vWbp is expressed during different stages of bacterial growth, an ELISA was carried out with the immobilized bacterial supernatants. The next day, the wells were washed three times with PBS containing 0.5% Tween 20, then blocked with 2% (v/v) BSA in PBS for one hour at room temperature. To detect vWbp, the wells were incubated for an hour with a mouse polyclonal anti-vWbp antibody (1:2000 in 1%, v/v BSA), followed by a 45-minute incubation with an HRP-linked anti-mouse IgG (1:1000 in 1%, v/v BSA). After a final wash, OPD substrate was added, and absorbance at 490 nm was measured using a Bio-Rad plate reader.

Detection of vWbp-specific IgG in infective endocarditis patients

Human IgG was isolated from the sera of patients diagnosed with infective endocarditis, as previously described (Rindi et al., 2006). Reactivity of IgGs from endocarditis sera against vWbp was tested by coating microtiter wells with recombinant vWbp (1 μg/well), incubating overnight at 4°C. The next day, after blocking with BSA, wells were incubated for one hour with IgG (1 μg/well) from patients and healthy donors. IgG binding was revealed by adding an HRP-conjugated polyclonal rabbit anti-human IgG (1:1000) and absorbance was read at 490 nm in an ELISA plate reader (Bio-Rad).

Rebinding of vWbp to the bacterial surface

Before starting the assay, vWbp was previously biotinylated using a standard protocol with the Fast Type A Biotin Conjugation Kit from Abcam (Cambridge, MA, USA). After labeling, the sample underwent dialysis, and protein concentration was determined with the BCA protein assay (Pierce).

S. aureus LAC wt cells were initially grown overnight in BHI, shaking at 37°C. The next day, the overnight bacterial cultures were diluted to a starting OD₆₀₀ of 0.05 in the same medium. Bacterial growth was monitored at the spectrophotometer to reach exponential phase. After that, both overnight (stationary) and exponential cultures were washed three times in PBS by centrifugation (4000rpm, 10'). Washed bacterial cultures were then diluted in PBS to reach an OD_{600nm} of about 1.0.

To measure rebinding of vWbp to the bacterial surface, microtiter wells were coated overnight at 37 °C (with an open lid to dry the plate) with 100 µl of *S. aureus* LAC wt suspensions (OD_{600nm} = 1.0) in PBS from both stationary and exponential phases. After blocking the wells with BSA, the plates were incubated for 1 h with increasing concentrations of biotinylated vWbp serially diluted (from 0 to 10 µg/well). After several washings, the wells were treated with avidin-peroxidase (1:1000) and the absorbance determined.

The same assay was carried out by comparing *S. aureus* LAC *srtA* and wt (in the stationary phase of growth). *S. aureus* LAC *srtA* is a strain deleted for sortase A gene, a crucial enzyme for anchoring CWA proteins in the bacterial cell wall.

Saturation binding curves of FnBPA N1N2N3 wild type and Q103A mutant to fibrinogen

To test whether the binding affinity to Fbg of the wild type and mutant form of the region A (N1N2N3) of FnBPA is maintained, saturation of binding curves were constructed. Microtiter wells were coated with 1 µg/well of each of the recombinant protein and immobilized overnight at 4°C. The wells were washed three times with (PBST) and treated for 1 h at room temperature with 2% BSA. After that, 1:2 serial dilutions of Fbg in PBS (starting from 4 µM) were added to the wells and incubated again for 1h at room temperature. The binding of Fbg to FnBPA was assessed by incubating the wells with a mouse polyclonal anti-Fbg IgG (1:1000), followed by an HRP-conjugated anti-mouse IgG (1:1000). After thorough washing, OPD was added to the wells, and the absorbance at 490 nm was measured.

Quantitative analysis of FnBPA-Fibrin(ogen) cross-linking mediated by vWbp-activated FXII

To verify and quantify the results from the Western blot analysis, an ELISA assay was carried out to assess the crosslinking of N1N2N3 wild type or Q103A. Microtiter wells were coated with 3 µg/mL of Fbg in PBS and incubated overnight at 4 °C. Following a blocking step with BSA, the wells were incubated with a mixture prepared in 25 mM Tris-HCl (TBS, pH 7.5), containing 200 nM ProT, 200 nM vWbp, 15 µg/mL FXIII, and 300 nM of each FnBPA variant. Either 2 mM CaCl₂ or 2 mM EDTA was included in the buffer. The presence of calcium ions allows the reactions, while EDTA inhibits the formation of covalent crosslinks. To allow fibrin formation and FnBPA-fibrin(ogen) crosslink generation, microtiter wells were incubated at 37 °C for 1 hour. Binding was detected using a mouse polyclonal anti-FnBPA IgG (1:1000), followed by an HRP-conjugated anti-mouse IgG (1:1000), absorbance values were measured as described previously.

The same assay was performed with the N1 subdomain (wild type or mutant Q103A) instead of the whole region A.

Quantitative analysis of FnBPA-Fibrinogen cross-linking mediated by TG2

To validate and quantify the Western blotting results, the crosslinking of FnBPA N1N2N3 wild type and N1N2N3 Q103A was assessed using an ELISA assay. Microtiter wells were coated with 3 µg/mL Fbg in PBS and incubated overnight at 4 °C. Following BSA treatment, the wells were treated with mixtures prepared in 25 mM Tris-HCl buffer (pH 7.5) containing 50 nM TG2 and 300 nM of each FnBPA variant, supplemented with either 10 mM CaCl₂ or 2 mM EDTA. The plates were incubated at 37 °C for 1 hour, after which binding was detected using a mouse polyclonal anti-FnBPA IgG (1:1000), followed by an HRP-conjugated anti-mouse IgG (1:1000). Absorbance values were measured as described previously.

The same assay was performed with the N1 subdomain (wild type or mutant Q103A).

Adhesion of *S. aureus* SH10004X::pFnBA4 wild type and Q103A mutant to fibrinogen

To test if the mutant *S. aureus* strain retains the same binding interaction to Fbg as the wild type strain, another ELISA assay was performed.

S. aureus SH10004X::pFnBA4 wild-type or mutant strains were cultured overnight in BHI supplemented with chloramphenicol (10 µg/ml). The following day, bacteria were washed three times by centrifugation, and diluted in PBS to an OD₆₀₀ of 1.

Microtiter wells were coated with 1:2 serial dilution of Fbg in PBS, starting from 1µg/well, and incubated overnight at 4 °C. After blocking with BSA, the wells were treated with 100 µL of each diluted bacterial solution (OD = 1) and incubated at 37 °C for 1 hour to allow bacterial adhesion. Binding was detected using a mouse polyclonal anti-*S. aureus* IgG (1:1000), followed by incubation with an HRP-conjugated anti-mouse IgG (1:1000). Absorbance values were measured as described previously.

Cross-Linking of *S. aureus* SH10004X::pFnBA4 Wild Type and Q103A to fibrin(ogen) mediated by vWbp-activated FXIII

To test whether the Q103A mutation is able to inhibit the bacterial incorporation into the fibrin mesh through the covalent interaction between fibrin(ogen) and FnBPA, another ELISA assay was performed.

S. aureus SH10004X::pFnBA4 wild type or mutant strains were grown overnight in BHI medium supplemented with chloramphenicol. The following day, bacteria were washed three times by centrifugation, and diluted in PBS to an OD₆₀₀ of 1.

Microtiter wells were coated with 3 µg/mL Fbg in PBS and incubated overnight at 4 °C. Following BSA treatment, the wells were treated with mixtures prepared in 25 mM Tris-HCl buffer (TBS, pH 7.5) containing 200 nM ProT, 200 nM vWbp, 15 µg/mL FXIII, and 100 µL of each diluted bacterial solution, supplemented with either 2 mM CaCl₂ or 2 mM EDTA. The wells were incubated at 37 °C for 1 hour to allow crosslink formation. Binding was detected using a mouse polyclonal anti-*S. aureus* IgG (1:1000), followed by incubation with an HRP-conjugated anti-mouse IgG (1:1000). The absorbance values were then measured as outlined previously.

Dot Blot and Western Blot assays

Rebinding of vWbp to peptidoglycan and lipoteichoic acid

To assess the binding of vWbp to peptidoglycan (PPG) or lipoteichoic acid (LTA), 5 µg of each compound was dotted onto a PVDF membrane (Bio-Rad). The membrane was blocked overnight at 4 °C with 5% (w/v) skim milk in PBST. After blocking, the membrane was incubated for 1 hour at 22 °C with vWbp (2 µg/mL). Detection was carried out using a mouse polyclonal anti-vWbp antibody (1:5000) in 2% (w/v) skim milk for 1 hour at 22 °C, followed by a 45-minute incubation with an HRP-conjugated rabbit anti-mouse IgG (1:1000), also diluted in 2% (w/v) skim milk. Dot blots were developed using the Westar Supernova detection kit (Cyanagen Srl, Bologna, Italy), and images were acquired with an ImageQuant™ LAS 4000 mini biomolecular imager (GE Healthcare). Signal intensities were quantified using ImageJ and plotted using GraphPad Prism.

Cross-linking of recombinant *S. aureus* CWA proteins to fibrin(ogen) by vWbp-activated FXIII

To evaluate FnBPA (N1, N2N3, N1N2N3 subdomains), FnBPB, ClfA and ClfB cross-linking to fibrin(ogen) in the presence of vWbp-activated FXIII, Western blot assays were performed. Regarding FnBPA N1N2N3 or N1 subdomain, the following experimental conditions were carried out also with the mutant recombinant proteins N1 Q103A or N1N2N3 Q103A, to perform a comparative analysis. Mixtures containing 200 nM ProT, 200 nM vWbp, 15 µg/ml FXIII, 5 µM Fbg and 2.5 µM of each bacterial protein were incubated at 37 °C in 25 mM Tris-HCl buffer, pH 7.5 containing 5 mM CaCl₂ for the indicated times (0', 2', 5', 15', 60', 120') to allow crosslinking reactions. Reactions were stopped by addition to the mixtures of a buffer containing 62.5 mM Tris-HCl, pH 6.8, 4 M urea, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue. Samples were heated at 95 °C for 10 min, separated by 4.8-10% polyacrylamide gels SDS-PAGE and electroblotted onto a PVDF membrane. After overnight blocking with skim milk and washings in PBS, the membranes were immunostained with the appropriate mouse antibody against each the bacterial protein (1:10 000, in 2% w/v skim milk). The membranes were treated with an HRP-conjugated rabbit anti-mouse IgG (1:10 000, in 2% w/v skim milk), and the blots developed as reported above.

The same experimental conditions were applied for the detection of the presence of fibrin(ogen) α-chain in the FnBPA-fibrin(ogen) heteropolymers. After blocking, PVDF membranes were probed with a monoclonal rabbit anti-α chain (1:5000) primary antibody and a goat anti-rabbit HRP-conjugated secondary antibody (1:10 000).

Cross-linking of *S. aureus* FnBPA subdomains to fibrinogen by TG2

To evaluate crosslinking formation between FnBPA and Fbg promoted by the catalytic action of TG2, each recombinant FnBPA subdomain (N1N2N3, N2N3 or N1) was tested by Western Blotting. Regarding FnBPA N1N2N3 or N1 subdomain, the following experimental conditions were carried out also with the mutant recombinant proteins N1 Q103A or N1N2N3 Q103A, to perform a comparative analysis. Each FnBPA recombinant protein diluted to a final concentration of 2.5 µM was incubated with 5 µM Fbg in the presence of 50 nM TG2 in 25 mM Tris-HCl buffer, pH 7.5 containing 5 mM CaCl₂ for increasing time intervals. Reactions were stopped by addition to the mixtures of a buffer containing 62.5 mM Tris-HCl, pH 6.8, 4 M urea, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue. Samples were heated at 95 °C for 10 min, separated by 4.8–10% polyacrylamide gels SDS-PAGE and electroblotted onto a PVDF membrane. After overnight blocking with 5% skim milk, membranes were washed and immunostained with a FnBPA mouse polyclonal antibody diluted 1:5000. The bound antibody was detected by treatment of the membrane with an HRP-conjugated rabbit anti-mouse IgG (1:10 000) and the image developed as reported above.

Cross-linking of *S. aureus* FnBPA domains to Fibronectin by vWbp-activated FXIII

To evaluate crosslinking formation between Fn and FnBPA promoted by vWbp-activated FXIII, the Region A of FnBPA (N1N2N3) or the C-terminal repeated region (FnBPA RR) were tested by Western Blotting. Each FnBPA recombinant protein was diluted to a final concentration of 2.5 μ M and was incubated with 1.25 μ M Fn in the presence of 200 nM ProT, 200 nM vWbp and 15 μ g/ml FXIII in 25 mM Tris-HCl buffer, pH 7.5 containing 5 mM CaCl₂ for increasing time intervals to allow crosslinking reactions (0', 15', 30', 60, 120', 240'). Reactions were stopped by addition to the mixtures of a buffer containing 62.5 mM Tris-HCl, pH 6.8, 4 M urea, 2% SDS, 10% glycerol, 5% β -mercaptoethanol and 0.01% bromophenol blue. Samples were heated at 95 °C for 10 min, separated by 4-15% polyacrylamide gels SDS-PAGE and electroblotted onto a PVDF membrane. After overnight blocking with 5% skim milk, membranes were washed and immunostained with a FnBPA mouse polyclonal antibody diluted 1:5000. The bound antibody was detected by treatment of the membrane with an HRP-conjugated rabbit anti-mouse IgG (1:10 000) and the image developed as reported above.

Cross-linking of *S. aureus* FnBPA repeated region to N29 fragment of Fibronectin by vWbp-activated FXIII

To evaluate crosslinking formation between Fn and FnBPA RR promoted by vWbp-activated FXIII a Western Blotting was performed. FnBPA RR was diluted to a final concentration of 2.5 μ M and was incubated with 1.25 μ M N29 in the presence of 200 nM ProT, 200 nM vWbp and 15 μ g/ml FXIII in 25 mM Tris-HCl buffer, pH 7.5 containing 5 mM CaCl₂ for increasing time intervals to allow crosslinking reactions (0', 2', 5', 15', 60', 120'). Reactions were stopped by addition to the mixtures of a buffer containing 62.5 mM Tris-HCl, pH 6.8, 4 M urea, 2% SDS, 10% glycerol, 5% β -mercaptoethanol and 0.01% bromophenol blue. Samples were heated at 95 °C for 10 min, separated by 4-15% polyacrylamide gels SDS-PAGE and electroblotted onto a PVDF membrane. After overnight blocking with 5% skim milk, membranes were washed and immunostained with a FnBPA mouse polyclonal antibody diluted 1:5000. The bound antibody was detected by treatment of the membrane with an HRP-conjugated rabbit anti-mouse IgG (1:10 000) and the image developed as reported above.

Murine infection models of *S. aureus*-induced dermonecrosis

To assess the in vivo impact of the Q103A mutation in FnBPA on *S. aureus* pathogenicity, a murine dermonecrosis model was employed. A mouse model of necrotic dermatitis mimics several aspects of human staphylococcal skin infections. The experiments were carried out adapting a well-established model from Lentini et al.

C57BL/6 mice (Charles River Laboratories, Calco, Italy) were born and developed normally in the animal facilities of the Department of Human Pathology of the University of Messina. All mice used in the present study were housed under specific pathogen-free conditions in individually ventilated cages at these animal facilities.

Female C57BL/6 mice of 6–8 weeks old were shaved on the back injected intradermally with 50 μ L of 10⁷ CFU of *S. aureus* SH10004X strains expressing either wild-type or Q103A-mutated FnBPA in sterile PBS. Inoculation was carried out using a 30-gauge U-100 insulin syringe (BD Biosciences). Infections were carried out under anesthesia induced by intraperitoneal injection of tiletamine/zolazepam (40 mg/kg) and xylazine (10 mg/kg), in accordance with institutional and national ethical guidelines.

Infected mice were monitored for up to a week. In each experiment, the actual number of injected bacteria was determined by colony counting. Measurements of total lesion areas were made by analyzing digital photographs (Nikon Coolpix S9600) at 3, 5, and 7 d postinfection, using the ImageJ image processing software program (<https://imagej.nih.gov/ij/>) and a millimeter ruler as a reference. At day 7 post-infection, lesion areas were measured and skin biopsies were collected from the site of infection for CFU quantification. Skin biopsy specimens were collected using an 8-mm punch biopsy device (Acuderm), weighted, and placed in gentleMACS M Tubes (Miltenyi Biotec) for homogenization in the gentleMACS dissociation system (Miltenyi Biotec). Bacterial load was determined by homogenizing tissue in sterile PBS, followed by serial dilution and plating on TSA plates.

Lesion size (mm²) and CFU/biopsy data were analyzed using the Mann–Whitney U test. Results showed that mice infected with the wild-type strain developed significantly larger necrotic lesions and higher bacterial burden compared to those infected with the Q103A mutant ($p < 0.01$).

Multiple sequence analysis

Sequences encoding for FnBPA and FnBPB from several *S. aureus* clonal complexes were obtained from KEGG Genomes database with the following entries: T00557 (subsp. Newman - CC8, NWMN_2397/ NWMN_2397); T00324 (subsp. 8325 - CC8, SAOUHSC_02803/ SAOUHSC_02802); T00086 (subsp. MW2 – CC1, MW2041/MW2040); T02702 (subsp. CA347

– CC45, CA347_2575/ CA347_2574); T00182 (subsp. MRSA252 – CC30, SAR2580) ; T00051 (subsp. N315 – CC5, SA2291/SA2290); T02059 (subsp. HO 5096 0412 - CC22, SAEMRSA15_23990) ; T02071 (subsp. ST398 – CC398, SAPIG2553) Sequence alignment of the DNA sequences was performed in ClustalOmega Multiple Sequence Alignment (MSA) tool.

Statistical methods

Analyses of *in vitro* data were carried out with Prism 4.0 (GraphPad). A minimum of three biological replicates (three independent experiments) were conducted for each experiment. The two-tailed Student's t-test was employed to compare two groups. P values < 0.05 were considered statistically significant, and the following symbols are used, *, $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Concerning the mic infection model, lesion size (mm²) and CFU/biopsy of *in vivo* data were analyzed using the Mann–Whitney U test.

Results

vWbp is predominantly expressed during exponential growth and serves as an immunogenic target in *S. aureus* infective endocarditis

vWbp is a secreted protein by *S. aureus* (Bjerketorp et al., 2002). Bacterial protein expression can change depending on the protein itself, but also on the environmental conditions. The temporal patterns of expression are usually biologically significant (Oogai et al., 2011).

To assess in which phase of the bacterial growth the protein is mainly expressed, a mutant LAC strain lacking the *spa* gene (which encodes protein A) was used. The lack of protein A avoids aspecific signals during antibody detection. *S. aureus* LAC *spa* was cultured in BHI medium, and samples were taken at various growth stages. At each time point, culture supernatants were collected, immobilized to microtiter wells for ELISA, and probed with a polyclonal anti-vWbp antibody to measure protein levels. As illustrated in fig. 14A, vWbp expression is peaking during the exponential growth phase (mainly between 2 and 4 hours after inoculation, with the highest levels detected around 3 hours).

S. aureus has become a leading cause of infective endocarditis, particularly in healthcare settings and among patients with prosthetic devices or indwelling catheters (Elgharably et al. 2024; Pietrocola et al., 2022) Furthermore, vWbp has been implicated in the early stages of infective endocarditis in animal models (Mancini et al., 2018; Panizzi et al.; 2011; McAdow et al., 2011). For this reason, the IgG level against vWbp in previously characterized sera from patients with *S. aureus* infective endocarditis were examined (Rindi et al., 2006), to determine wherever after secretion vWbp can be immunologically active. Although the antibody response varied among individuals, all patient sera showed significantly stronger reactivity to vWbp compared to sera from healthy controls. These findings support the idea that vWbp is produced during infection and is immunogenic *in vivo* (fig. 14B).

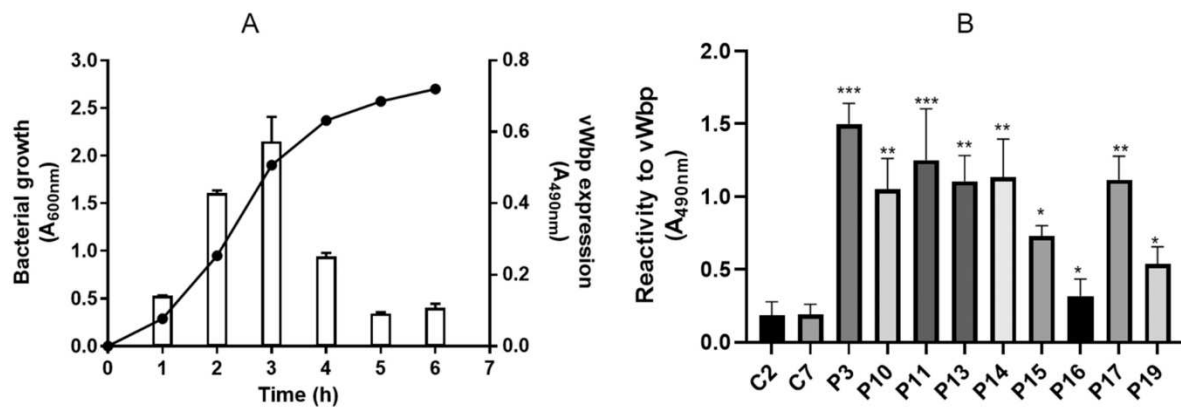


Figure 14. Expression of vWbp during bacterial growth and its detection in patient sera. (A) To monitor the expression of vWbp over time, culture supernatants were collected at specific intervals during bacterial growth, filtered, and applied to microtiter wells. The presence of vWbp was then detected using a primary anti-vWbp IgG antibody, followed by an HRP-conjugated secondary antibody (right Y-axis). In parallel, the bacterial growth curve was plotted (left Y-axis) to correlate protein expression with bacterial growth. (B) To assess the immune response to vWbp in humans, microtiter plates coated with purified vWbp were incubated with IgG antibodies isolated from the sera of patients diagnosed with *S. aureus* infective endocarditis (labeled as P). For comparison, sera from healthy individuals (C2, C7) were included as controls. The bound antibodies were detected with an HRP-conjugated secondary IgG. A significant increase in vWbp-specific IgG binding was observed in patient samples compared to controls. Statistical significance is indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001. Data represent the mean $\pm$ standard deviation of experiments performed in triplicate.

Secreted vWbp rebinds to the cell surface through both peptidoglycan and lipoteichoic acid

In general, bacterial proteins are divided into secreted and CWA, but there are a number of cases documented in literature where a secreted bacterial protein can rebind to the cell wall (Pietrocola et al., 2016; Sibbald et al., 2006).

To evaluate whether vWbp can re-associate with the bacterial surface after secretion, *S. aureus* LAC cells from both exponential and stationary growth phases were immobilized to ELISA microtiter wells and incubated with increasing concentrations of biotin-labeled vWbp. Binding of the protein to bacterial cells was subsequently detected using avidin-conjugated peroxidase. vWbp displayed binding to the surface of bacterial cells from both growth phases, indicating that the cell wall components responsible for this interaction are present throughout bacterial growth and not expressed only during a specific phase (fig. 15A).

To further investigate the nature of the molecules interacting with vWbp during the re-association process, vWbp rebinding was compared between *S. aureus* LAC wt and LAC *srtA* (an isogenic *srtA* mutant lacking sortase A). Sortase A is a membrane-anchored transpeptidase enzyme of *S. aureus* and also widely conserved among Gram-positive

bacteria. It catalyzes the covalent attachment of surface proteins to the bacterial cell wall by recognizing and cleaving a conserved LPXTG motif within the substrate proteins (Mazmanian et al., 2001). A bacterial strain lacking this enzyme has a largely lower variety of molecules and virulence factors expressed on the surface. When vWbp rebinding was tested with both wild type and deleted strain, no significant differences in vWbp binding were observed between the two, suggesting that the interaction is not dependent on sortase A-anchored CWA proteins (fig. 15B).

S. aureus cell wall presents a diverse array of surface-exposed molecules, including peptidoglycan (PPG), lipoteichoic acid (LTA), but also sortase B-anchored proteins (such as IsdC), and various lipoproteins (Geoghegan and Foster, 2017; Rohde, 2019). To evaluate the potential involvement of PPG and LTA in vWbp rebinding, highly purified preparations of both components were immobilized onto a PVDF membrane and incubated with biotinylated vWbp. vWbp demonstrated ability to bind both PGN and LTA, with a stronger interaction observed for PPG (fig. 15C). However, considering the molecular complexity of the *S. aureus* cell wall, the possibility that vWbp interacts with additional, yet unidentified, surface components cannot be totally excluded.

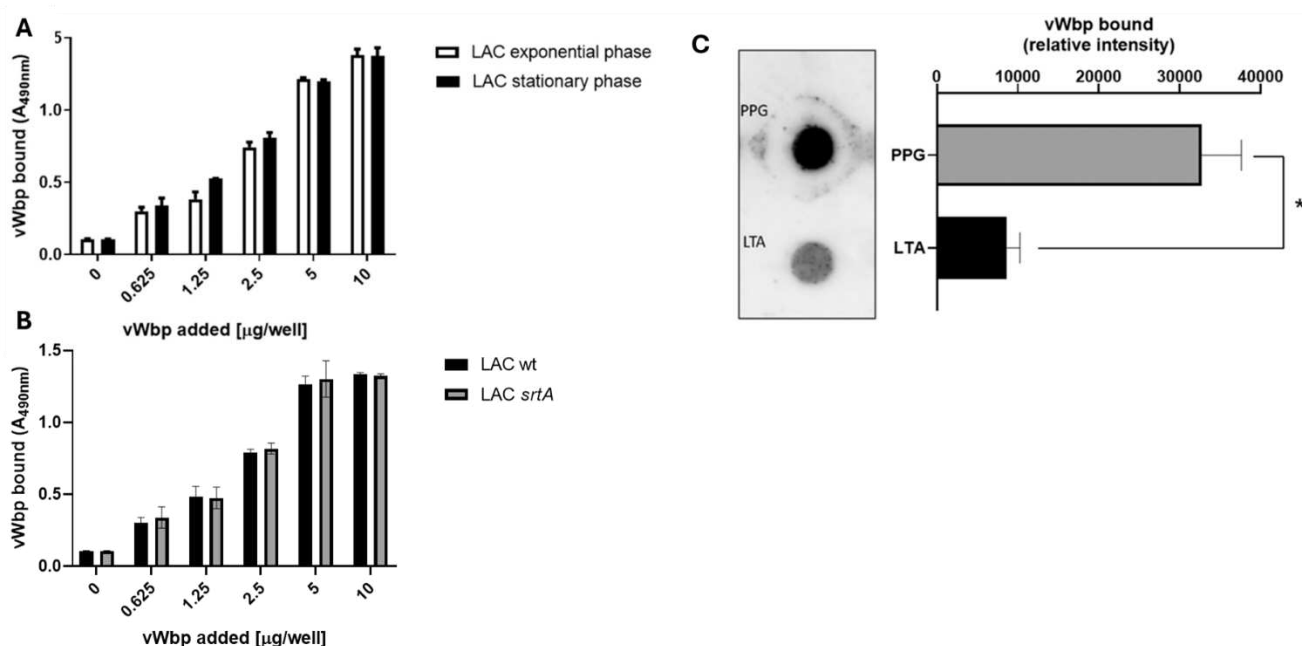


Figure 15. vWbp re-associates to the bacterial cell surface via peptidoglycan and lipoteichoic acid. (A) Binding of increasing concentrations of biotinylated vWbp to *S. aureus* LAC wt cells from exponential and stationary phases immobilized onto microtiter wells was determined by addition of avidin-peroxidase. (B) Binding of increasing concentrations of biotinylated vWbp to *S. aureus* LAC wt or LAC *srtA* cells immobilized onto microtiter wells was determined by addition of avidin-peroxidase. Data are expressed as means $\pm$ S.D. of triplicate tests. (C) Binding of biotinylated vWbp to PPG or LTA dotted onto PVDF membrane was detected incubating the membrane with avidin-peroxidase. Densitometric analysis of vWbp bound to PPG or LTA is shown on the right. Statistically significant difference is reported (*P < 0.05).

vWbp-activated FXIII mediates dansylcadaverine incorporation into FnBPA via reactive glutamine

A previous study by *Severina et al.* demonstrated that thrombin-activated FXIII mediates covalent cross-linking between full-length FnBPA and Fbg. Building on these findings, the presence of reactive glutamine or lysine residues within the N1N2N3 (A region) region of FnBPA was investigated by assessing the ability of vWbp-activated FXIII to incorporate either an amine-donor probe (dansylcadaverine) or an amine-acceptor probe (dansyl- ϵ -aminocaproyl-QQIV peptide) into the recombinant protein. In particular, dansylcadaverine was utilized to probe reactive glutamines, and the peptide dansyl-PGGQQIV was used to probe reactive lysines.

Reaction products were analyzed by SDS-PAGE, followed by visualization under ultraviolet (UV) light (fig. 16A) and then Coomassie Blue staining (fig. 16B). The UV light resulting image revealed incorporation of dansylcadaverine into FnBPA (lane 1), but no signal was observed for the other probe (lane 2), suggesting that the Region A contains reactive glutamine residues but lacks accessible lysine residues for vWbp-activated FXIII crosslinking.

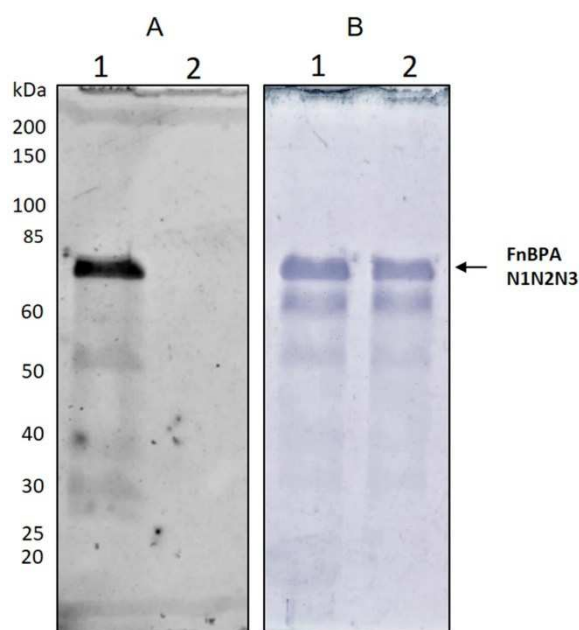


Figure 16. Incorporation of dansyl cadaverine into FnBPA N1N2N3 by action of vWbp-activated FXIII. Mixtures incubated with dansyl cadaverine (lane1) or dansyl- ϵ -aminocaproyl-QQIV peptide (lane 2) were separated by SDS-PAGE and the gel was examined under ultraviolet light (**A**) and then stained with Bio-Safe Coomassie staining (**B**). The figures are representative of three independent experiments. Molecular masses of standard proteins are indicated on the left side of the panel. Arrows show the positions of FnBPA N1N2N3 domain.

vWbp-activated FXIII mediates crosslinking between FnBPA Region A and fibrin(ogen) α -chain

To further characterize the formation of covalent crosslinks between the Region A of FnBPA and fibrin(ogen), FnBPA N1N2N3 was incubated with vWbp-activated FXIII for increasing time intervals. The incubation of the reaction mixtures at 37°C up to 2 hours allows crosslinking formation, which can increase over time. Reaction mixtures were then analyzed by SDS-PAGE under reducing conditions, followed by Western blotting using an anti-FnBPA antibody. Under these conditions, the generation of high molecular weight complexes containing FnBPA was observed (fig. 17A). No such complexes were detected in reactions lacking Fbg (lane 8, fig. 17A), indicating the requirement of both substrates for crosslink formation. Additionally, the anti-FnBPA showed no reactivity with samples containing Fbg alone, confirming the specificity of the antibody (lane 1, fig. 17A).

Fbg is a large plasma glycoprotein composed of three pairs of polypeptide chains: A α , B β , and γ , forming a symmetrical hexamer (Kollman et al., 2009). Further analysis using an antibody specific to the Fbg α -chain revealed that the high molecular weight complexes consisted of covalently linked FnBPA with the α -chain of fibrin(ogen) (Fig. 17B).

Region A is a conserved structural domain found in multiple *S. aureus* MSCRAMMs (including FnBPA, FnBPB, ClfA, and ClfB). Despite differences in primary sequence among these proteins, Region A across different *S. aureus* virulence factors exhibits significant structural and functional homology (Foster, 2019). For this reason, the possibility of the formation of heterocomplexes between other bacterial adhesins and fibrin(ogen) was investigated. The specificity of the observed crosslinking with FnBPA only was supported by additional experiments in which the region A (N1N2N3) of FnBPB, ClfA, or ClfB were individually incubated with Fbg in the presence of vWbp-activated FXIII. In these reactions, no high molecular weight complexes were observed, as assessed by SDS-PAGE and immunoblotting with the respective antibodies (fig. 17C, 17D, and 17E), indicating that covalent complex formation through the action of vWbp-activated FXIII is specific to FnBPA.

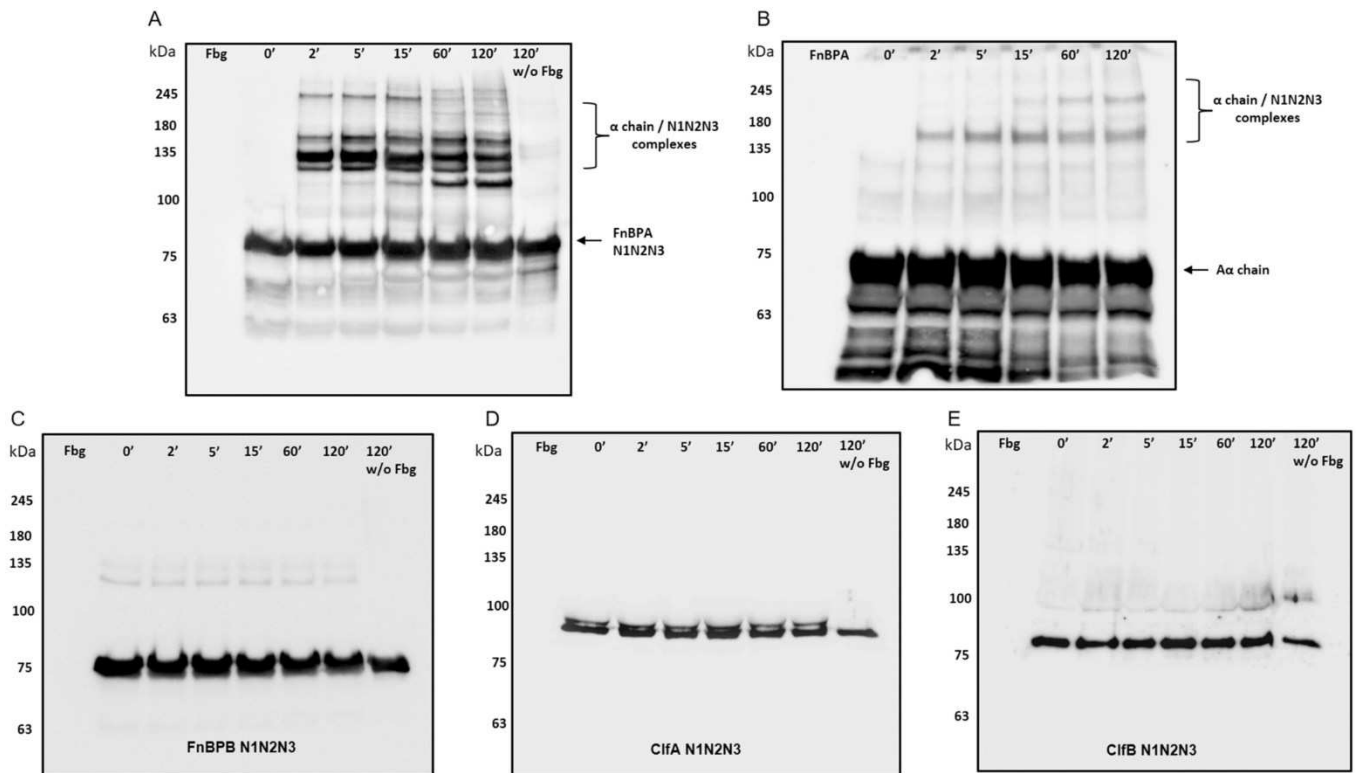


Figure 17. vWbp-activated FXIII elicits cross-links formation between region A of FnBPA and α -chain of fibrin(ogen). Panel (A) shows cross-linking of recombinant FnBPA N1N2N3 domain to Fbg by action of vWbp-activated FXIII. After indicated time intervals mixtures were separated by SDS-PAGE under reducing conditions and transferred to a PVDF membrane. The membrane was probed with anti-FnBPA IgG followed by secondary HRP-conjugated IgG. (B) Cross-links formation between FnBPA N1N2N3 domain and Fbg by vWbp-activated FXIII was analyzed by incubating the membrane with anti- α chain Fbg IgG followed by secondary HRP-conjugated IgG. Arrows show the positions of the α chain or α chain/FnBPA N1N2N3 complexes. (C-E) Cross-linking formation of the recombinant A domain of FnBPB (C), ClfA (D) or ClfB (E) with Fbg by vWbp-activated FXIII were analyzed incubating the PVDF membranes with mouse FnBPB, ClfA or ClfB IgG, respectively, followed by secondary HRP-conjugated IgG. The figures are representative of three independent experiments. Molecular masses of standard proteins are indicated on the left side of each panel. Incubation times (0–120 min) on the top of each panel are also specified.

FnBPA is crosslinked to fibrin(ogen) through the N1 subdomain in Region A

The subdomains (N1, N2, and N3) of Region A in *S. aureus* are arranged sequentially. In particular, the N2 and N3 subdomains function cooperatively to mediate high-affinity non-covalent binding to Fbg through the DLL mechanism. In contrast, the N1 subdomain does not participate in non-covalent Fbg binding (Wann et al., 2000).

To identify the region of FnBPA N1N2N3 involved in covalent crosslink to fibrin(ogen), the recombinant N1 and N2N3 subdomains were individually incubated with Fbg in the presence of vWbp-activated FXIII for increasing time. Reaction products were analyzed by SDS-PAGE followed by Western blotting using an anti-FnBPA antibody. No covalent complexes were observed in mixtures containing the N2N3 fragment and Fbg (fig. 18A). In contrast, incubation of the N1 subdomain with Fbg resulted in the appearance of high molecular weight heterocomplexes (fig. 18B). The predominant complex observed, approximately 110 kDa, is consistent with a covalent association between the 40 kDa N1 subdomain and the 70 kDa Fbg α -chain.

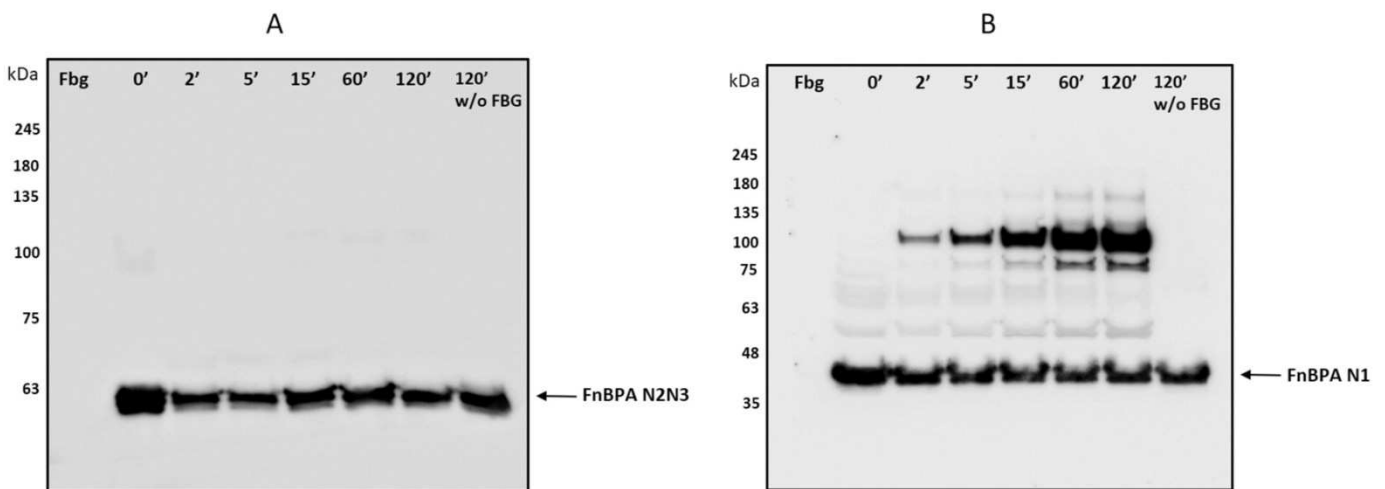


Figure 18. N1 subdomain of region A of FnBPA is involved in cross-link formation with fibrin(ogen). Mixtures containing FnBPA N2N3 (**A**) or FnBPA N1 (**B**) proteins and Fbg were incubated in the presence of vWbp-activated FXIII for the indicated times (top of each panel) and then separated by SDS-PAGE under reducing conditions and transferred to a PVDF membrane. Membranes were probed with FnBPA IgG followed by secondary HRP-conjugated IgG. Arrows show the positions of FnBPA N2N3 domain (**A**) or FnBPA N1 subdomain (**B**). The figures are representative of three independent experiments. Molecular masses of standard proteins are indicated on the left side of each panel.

Covalent crosslinks between FnBPA and Fbg are formed also through the enzymatic activity of TG2

TG2 is the most largely distributed and abundantly expressed member of the transglutaminase enzyme family. Considering its high expression in various tissues (Park et al., 2010; Al-U'datt et al., 2022), the potential role of TG2 in catalyzing covalent crosslinking between FnBPA and Fbg was investigated.

Recombinant FnBPA constructs (including the full N1N2N3 domain, the isolated N1 subdomain, and the N2N3 subdomain) were individually incubated with Fbg in the presence of TG2 over increasing time intervals. Reaction mixtures were analyzed by SDS-PAGE and Western blotting using an anti-FnBPA antibody. As shown in fig. 19A the full-length N1N2N3 domain formed high molecular weight complexes with Fbg, with a pattern similar to that observed in reactions catalyzed by vWbp-activated FXIII. Additionally, TG2 promoted cross-linking between the N1 subdomain and Fbg (fig. 19B), while no covalent complexes were detected in reactions containing only the N2N3 fragment and Fbg (fig. 19C).

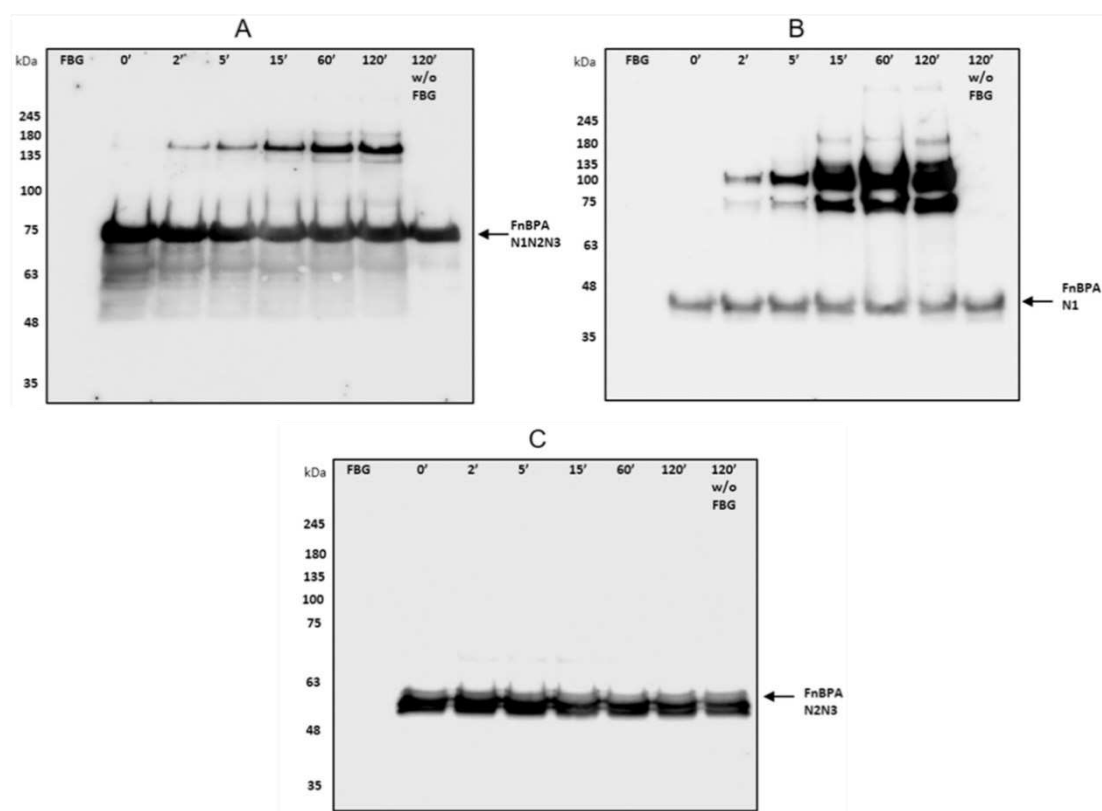


Figure 19. TG2 promotes formation of crosslinks between N1 domain of FnBPA and Fbg. Products of cross-linking reactions between FnBPA N1N2N3 (A), N1 (B) and N2N3 (C) proteins and Fbg generated by action of TG2 were separated by SDS-PAGE under reducing conditions and transferred to a PVDF membrane. Membranes were probed with anti-FnBPA IgG followed by secondary HRP-conjugated IgG. Arrows show the positions of unreactive FnBPA proteins. The figures are representative of three independent experiments. Molecular masses of standard proteins are indicated on the left side of each panel. Incubation times (0–120) of the mixtures are indicated on the top of each panel.

Glutamine103 (Q103) is highly conserved across *S. aureus* clonal complexes

Severina et al. demonstrated that thrombin-activated FXIII catalyses covalent crosslinking between full-length FnBPA and fibrin(ogen) and proposed the Q103 residue as a major crosslinking site. Along this line, this study continued focusing on the importance of this glutamine residue.

To begin, the conservation of the residue in the FnBPA gene sequence in different *S. aureus* clonal complexes was investigated. A bacterial clonal complex is a group of closely related bacterial strains that have descended from a common ancestor and share similar genetic profiles. To this end, a multiple sequence alignment of the *fnbA* gene across several *S. aureus* clonal complexes was performed. It was found that, across all strains analyzed, Q103 is highly conserved in the major lineages. (fig. 20A)

Next, to assess the importance of the Q103 residue, the gene sequences of *fnbB* from several clonal complexes were aligned with the *fnbA* gene of the reference strain (*S. aureus* 8325-4, CC8) and verified that this residue is absent in *fnbB* (Fig. 20B). This result is consistent with previous data (Motta et al., 2023), which shows that FnBPB does not crosslink with fibrin(ogen), in contrast to FnBPA, thereby confirming the critical role of Q103 in covalent crosslink formation.

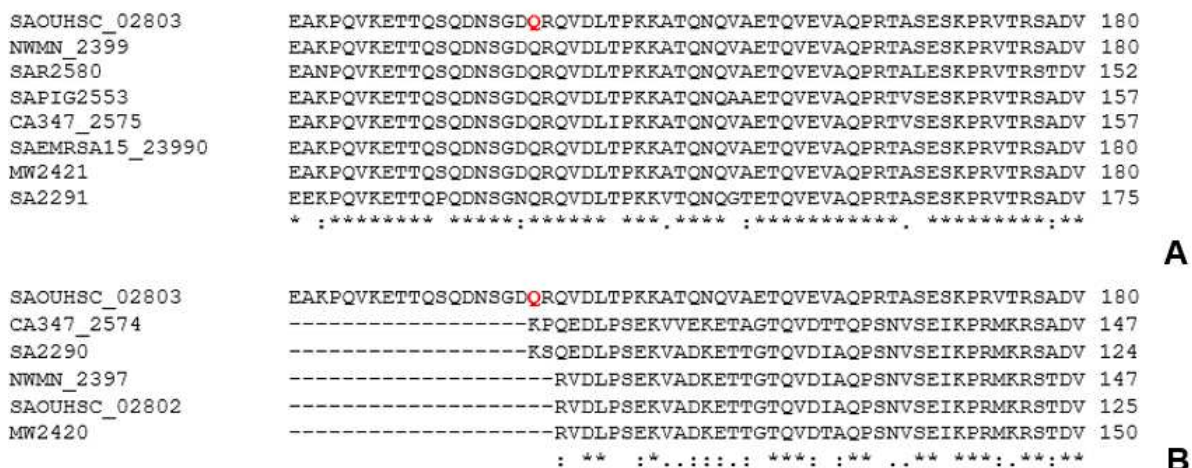


Figure 20. Multiple sequence alignment of *fnbA/fnbB* genes from representative *S. aureus* strains across major clonal complexes. (A) The conservation of the Q103 residue (highlighted) in *fnbA* genes, implicated in bacterially-mediated cross-linking with fibrinogen (Fbg) was determined. (B) Multiple sequence alignment of *fnbB* sequences from corresponding clonal complexes with *fnbA* (subsp.8325, Q103 residue highlighted). Alignments in fig. 20A and 20B were carried out with ClustalOmega. Symbols below the sequences indicate conservation: an asterisk (*) shows identical residues, a colon (:) marks strong similarity, a period (.) indicates weak similarity, and a space means no conservation. These symbols help highlight important conserved regions in the alignment. Gene sequences were retrieved from KEGG GENOME database with the following entries: T00557 (subsp. Newman - CC8, NWMN_2397/ NWMN_2397); T00324 (subsp. 8325 - CC8, SAOUHSC_02803/ SAOUHSC_02802); T00086 (subsp. MW2 – CC1, MW2041/MW2040); T02702 (subsp. CA347 – CC45, CA347_2575/ CA347_2574); T00182 (subsp. MRSA252 – CC30, SAR2580) ; T00051 (subsp. N315 – CC5, SA2291/SA2290); T02059 (subsp. HO 5096 0412 - CC22, SAEMRSA15_23990) ; T02071 (subsp. ST398 – CC398, SAPIG2553)

The Q103A mutation does not alter binding affinity of FnBPA N1N2N3 to Fbg

Given the high degree of Q103 conservation, this study continued with the production of recombinant mutant domains of FnBPA, where Q103 was replaced by an alanine residue (Q103A). Specifically, we produced the N1 subdomain, where Q103 is located, as well as the N1N2N3 domain of FnBPA, which corresponds to the A region of the protein. These mutant FnBPA subdomain were produced in order to investigate the role of the mutation with vWbp-activated FXIII crosslinking to fibrin(ogen).

As reported in the literature, the A region of FnBPA is capable of binding to Fbg through the N2N3 subdomains, whereas the N1 subdomain does not (Wann et al., 2000). To verify that the Q103A mutation does not alter the binding of the N1N2N3 domain to Fbg, saturation binding curves were constructed, by comparing the binding of the mutated protein with that of the wild type (WT). For this purpose, microtiter wells were coated with either the WT or mutated form of the protein, incubated with increasing concentrations of Fbg, and then the complex was revealed with an anti-Fbg antibody. As shown in Fig. 21, the Q103A mutation does not affect the binding of the N1N2N3 region to Fbg.

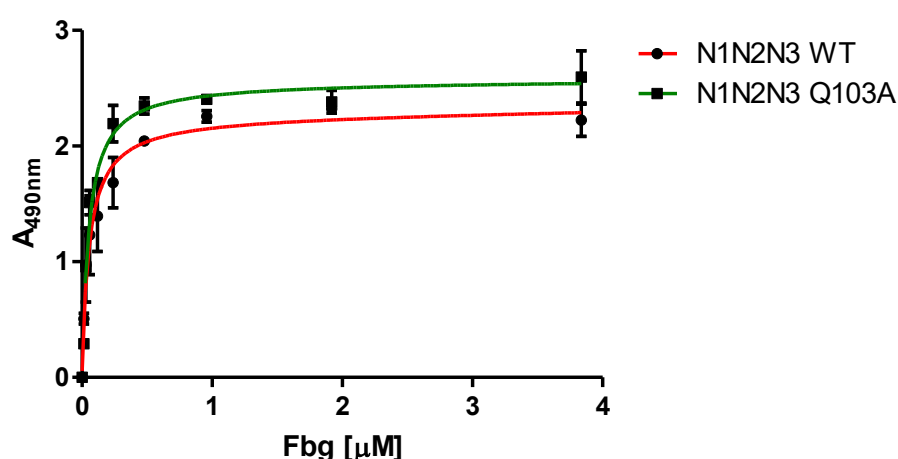


Figure 21. Saturation binding curves of recombinant WT and Q103A-mutated FnBPA N1N2N3 to Fbg. Microtiter plates were coated with each protein variant and incubated with increasing concentrations of Fbg. Detection was performed using anti-Fbg antibodies and HRP-conjugated secondary antibody. Absorbance at 490nm is depicted on y-axis and increasing Fbg concentration on x-axis. Data are expressed as means $\pm$ S.D. of tests performed in triplicate.

The Q103A substitution strongly reduces crosslink formation to fibrin(ogen) by vWbp-activated FXIII

It was previously demonstrated in this work that N1N2N3 region of FnBPA, particularly the N1 subdomain, is capable of covalently binding to fibrin(ogen) through the action of vWbp-activated FXIII, resulting in the formation of high molecular weight heterocomplexes (Motta et al., 2023).

To assess whether the mutated recombinant FnBPA still retains the ability to be crosslinked to fibrin(ogen) by bacterially-activated FXIII, both WT and Q103A mutant forms of the N1N2N3 domain were incubated with vWbp-activated FXIII and fibrin(ogen) for increasing periods of time (0 to 120 minutes) in presence of calcium ions. The reaction products were then subjected to SDS-PAGE under reducing conditions and transferred to a PVDF membrane by Western blotting. The membrane was subsequently incubated with an anti-FnBPA antibody to detect the formation of heterocomplexes with fibrin(ogen). The results demonstrated again that the WT protein successfully covalently binds to fibrin(ogen) via FXIII activation by vWbp, generating high molecular weight complexes, thus confirming the previous findings (fig. 22A). In contrast, when the FnBPA Q103A mutant was incubated in the same reaction, the formation of high molecular weight complexes was completely inhibited (fig. 22B). In mixtures where Fbg was omitted, no high molecular weight complexes were detected, confirming the validity of the assay (lane 8, fig 22A,22B). The specificity of the anti-FnBPA antibody was verified by loading a mixture that excluded all components except Fbg (lane 1, fig 22A, 22B).

To compare the interaction levels of N1N2N3 WT and Q103A mutant proteins with fibrin(ogen) in the presence or absence of activated FXIII, ELISA-based assays were conducted. Mixtures containing vWbp-activated FXIII, vWbp-generated FXIII, and either WT or mutated FnBPA were incubated on fibrin(ogen)-coated microtiter wells in the presence of calcium or EDTA. Calcium was added as a necessary cofactor for FXIII activation, while EDTA, by sequestering calcium, inactivated FXIII. Consequently, protein binding to fibrin(ogen) in the presence of calcium reflects the combined contribution of noncovalent interactions, mediated by the DLL mechanism, and covalent crosslinks catalyzed by FXIII. Conversely, in the presence of EDTA, only noncovalent interactions between FnBPA and fibrin(ogen) are observed. The results show that the binding level of N1N2N3 WT to fibrin(ogen) in the presence of calcium was significantly higher ($^{**}P < 0.01$), approximately 4-fold, compared to the binding observed in the presence of EDTA (fig. 22C). This indicates that FXIII-mediated cross-link formation substantially enhances the incorporation of N1N2N3 WT into fibrin(ogen). In contrast, the Q103A mutant showed no difference in binding to fibrin(ogen) in presence or absence of calcium, confirming that the Q103A mutation prevents vWbp-mediated FXIII crosslink formation (fig. 22C). This result suggests that the binding of the mutant protein is exclusively due to non-covalent interactions. Ultimately, in presence of calcium, the mutant protein showed a decrease of binding, which accounted for only 30% of the reactivity displayed by the wild type FnBPA (fig. 22C, inset).

To confirm these results, we performed additional assays to compare the ability of the recombinant N1 WT subdomain, and the mutated N1 Q103A subdomain to form crosslinks with fibrinogen(ogen) through the action of FXIII activated by vWbp. As shown in fig. 22D, the N1 WT subdomain retains its ability to form high-molecular weight heterocomplexes with fibrin(ogen) through FXIII activation by vWbp. In contrast, the Q103A mutation completely abolishes the formation of these complexes (fig. 22E).

The ELISA assay also confirmed that the substitution of Q103 significantly disrupts the interaction between the N1 subdomain and fibrin(ogen) via FXIII activation by vWbp. Since the N1 subdomain does not interact with Fbg through non-covalent interactions, as the N1N2N3 domain does, the results in Fig. 22F clearly show that the N1 subdomain is able to interact with fibrin(ogen) only in the presence of FXIII activated by vWbp and Ca^{2+} , via crosslinking. In fact, no interaction of the WT protein was observed in the presence of EDTA, nor was there any interaction of the Q103A protein in the presence or absence of calcium. Similarly to what was previously observed, the interaction of N1 WT to fibrin(ogen) in the presence of calcium was significantly higher (** $P < 0.01$), approximately 15-fold, compared to what observed in the presence of EDTA (fig. 22F). Instead, mixtures containing calcium ions or EDTA with N1 Q103A showed almost identical absorbance values (fig. 22F). These data indicate that the Q103A mutation is sufficient to inhibit the formation of covalent complexes. In particular, when the mixtures were incubated with calcium, the binding of N1 WT to fibrin(ogen) was more than 90% higher than that of the mutant protein, further demonstrating the key role of Q103 (fig. 22F, inset).

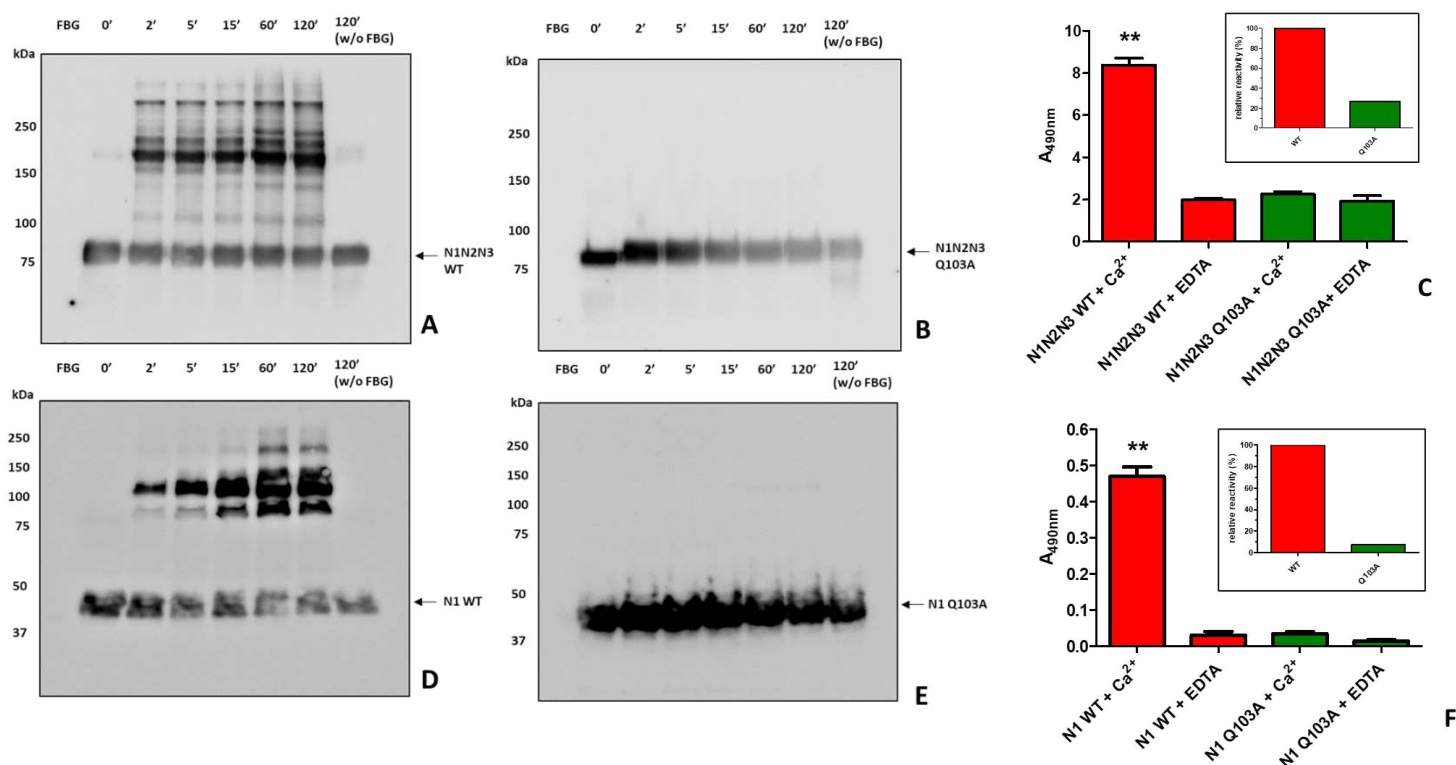


Figure 22. Role of Q103A residue in vWbp-mediated cross-linking of FnBPA to fibrin(ogen). (A–B) Cross-link formation of recombinant N1N2N3 domain of FnBPA WT (A) or Q103A mutant (B) to fibrin(ogen) by vWbp-activated FXIII was analyzed by Western Blotting. Reaction mixtures were incubated for increasing time points (0–120 min) in the presence of calcium, separated by SDS-PAGE under reducing conditions and then transferred to PVDF membranes, then probed with anti-FnBPA IgG and HRP-conjugated secondary antibody. Lane 8 in both panels lacks fibrin(ogen); lane 1 contains only fibrin(ogen) Arrows show the positions of FnBPA WT (A) or FnBPA Q103A (B). Molecular mass markers (kDa) are indicated on the left. Data is representative of three independent experiments. (C) ELISA analysis of fibrin(ogen) crosslinking with WT and Q103A N1N2N3 domains in the presence of Ca^{2+} or EDTA. Microtiter wells were coated with Fbg and then probed with mixtures containing FnBPA WT or Q103A, vWbp, ProT, FXIII and either calcium chloride or EDTA. Binding was measured using anti-FnBPA IgG and HRP-conjugated secondary antibody. Statistically significant difference is reported (** $P < 0.01$). Inset: binding of the mutant protein was reduced to approximately 30% of the reactivity observed for the wild-type FnBPA. Data are expressed as means $\pm$ S.D. of triplicate tests. (D–E) Cross-link formation of recombinant N1 subdomain of FnBPA WT (D) or Q103A (E) to fibrin(ogen) by vWbp-activated FXIII, analyzed as described for panels A–B. Arrows show the positions of FnBPA WT or FnBPA Q103A. (F) ELISA analysis of fibrin(ogen) crosslinking with WT and Q103A N1 domains in the presence of Ca^{2+} or EDTA. Statistical analysis is reported (** $P < 0.01$). Inset: WT signal in presence of Ca^{2+} ions is >90% higher than mutant.

The Q103A substitution strongly reduces crosslink formation to Fbg by TG2

As recently published and previously demonstrated in this work (Motta et al., 2023), the most ubiquitously expressed member of the tissues transglutaminase family, TG2, is able to catalyse the formation of high molecular weight complexes between FnBPA N1 and Fbg.

To determine whether the mutated recombinant FnBPA retains its ability to be crosslinked to Fbg by TG2, the WT and Q103A mutant N1N2N3 domains were incubated with TG2 in presence of calcium ions for up to 120 minutes. Reaction products were subjected to SDS-PAGE under reducing conditions, transferred to a PVDF membrane, and analyzed by Western blotting with an anti-FnBPA antibody to detect heterocomplex formation with Fbg. The results showed that the WT protein was able to generate high molecular weight complexes with Fbg through TG2-mediated cross-linking (fig. 23A), consistent with previous findings. In contrast, the Q103A mutant failed to form these complexes under the same conditions (fig. 23B).

To evaluate the interaction levels of N1N2N3 WT and Q103A mutant proteins with Fbg in the presence or absence of TG2, ELISA-based assays were performed. Mixtures containing TG2, and either WT or mutant FnBPA were incubated on Fbg-coated microtiter plates in the presence of calcium or EDTA. The findings revealed that N1N2N3 WT exhibited significantly higher binding to Fbg in the presence of calcium (approximately five times greater, $**P < 0.01$) compared to binding observed in the presence of EDTA (fig. 23C). This indicates that TG2-mediated cross-link formation plays a major role in enhancing the incorporation of N1N2N3 WT into Fbg. On the other hand, the Q103A mutant showed no significant difference in binding between conditions with or without calcium, demonstrating that the mutation prevents TG2-mediated crosslinking (fig. 23C). Finally, when calcium was present, the Q103A mutant displayed a marked reduction in binding, accounting for only 30% of the binding observed for the wild-type FnBPA (fig. 23C, inset). This suggests that while the mutant retains some binding affinity, the lack of TG2-mediated crosslinking severely limits its interaction with Fbg.

To validate our previous findings, we decided to perform the same experiments with the N1 domain of FnBPA. We incubated N1 WT or N1 Q103A with Fbg in the presence of TG2 and calcium. The resulting samples were analyzed via SDS-PAGE, followed by Western blotting using an anti-FnBPA antibody. As expected, the WT protein formed high molecular weight complexes, consistent with our prior results (fig. 23D). In contrast, the Q103A mutant completely lacked the ability to form such complexes under identical conditions (fig. 23E).

To confront the levels of binding of the wild type and mutated protein, we employed the ELISA-based assay described earlier. We incubated N1 WT or Q103A with TG2 on Fbg-coated microtiter wells in the presence of either calcium chloride or EDTA. Consistent with previous observations, the N1 WT protein demonstrated significantly higher binding to Fbg in the presence of calcium ($***P < 0.001$, approximately 13-fold) compared to EDTA (fig. 23F). In

contrast, mixtures containing the Q103A mutant showed comparable absorbance values regardless the presence of calcium or EDTA, indicating that the Q103A mutation effectively prevents covalent complex formation (fig. 23F). Notably, in calcium-containing mixtures, the binding of N1 WT to fibrin(ogen) was over 90% greater than that of the Q103A mutant, underscoring the critical role of Q103 in this interaction (fig. 23F, inset).

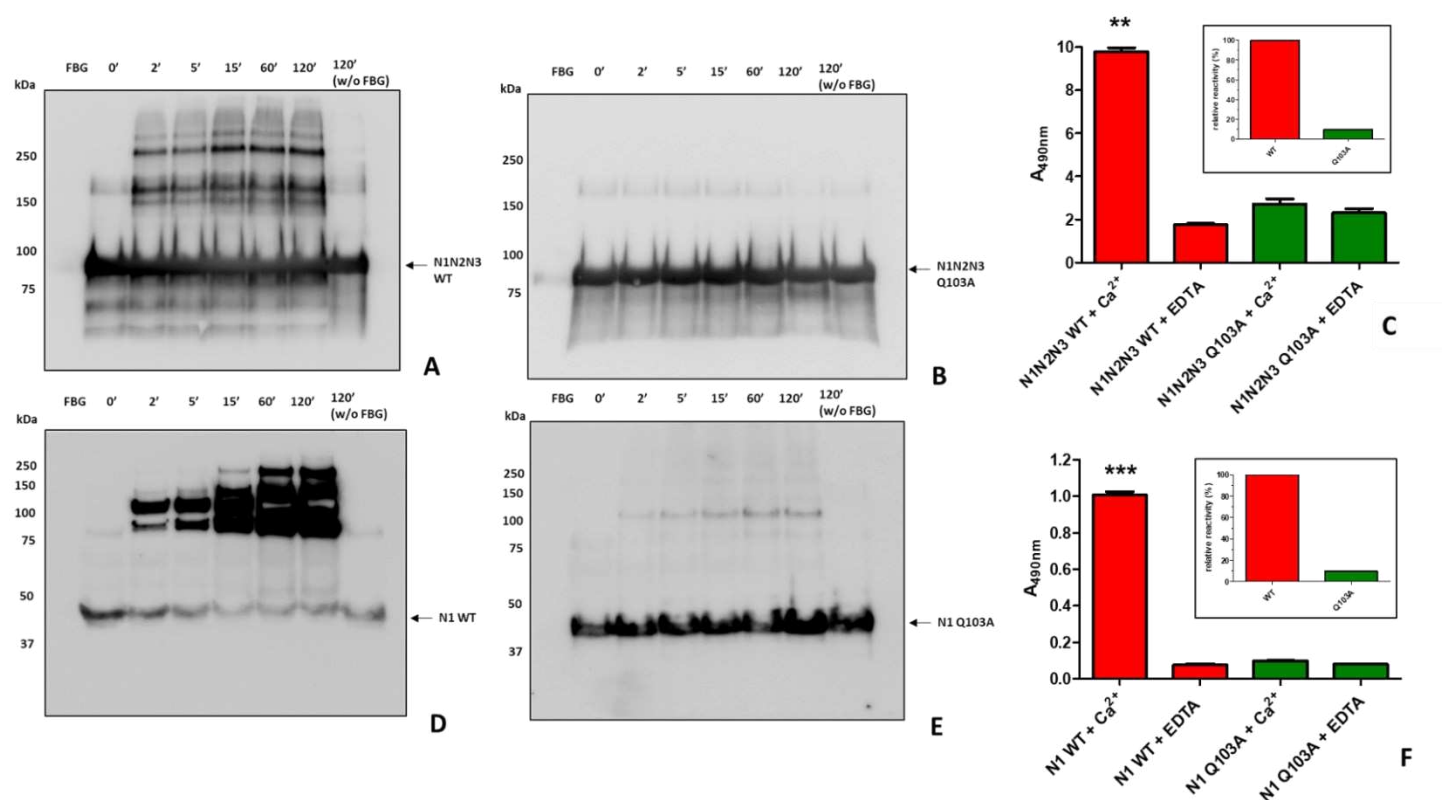


Figure 23. Role of Q103A residue in TG2- mediated cross-linking of FnBPA to fibrinogen.(A–B) Cross-link formation of recombinant N1N2N3 domain of FnBPA WT (A) or Q103A mutant (B) to Fbg by TG2 was analyzed by Western Blotting. Arrows show the position of the wild type N1 (A) or Q103A (B) subdomain of FnBPA. Reaction mixtures were incubated for increasing intervals (0–120 min) in the presence of calcium, separated by SDS-PAGE and transferred to PVDF membranes, then probed with anti-FnBPA IgG and HRP-conjugated secondary antibody. Lane 8 in both panels lacks Fbg; lane 1 contains only Fbg. Molecular mass markers (kDa) are indicated on the left. Data is representative of three independent experiments. (C) ELISA analysis of Fbg crosslinking with WT and Q103A N1N2N3 domains in the presence of Ca^{2+} or EDTA by TG2. Microtiter wells were coated with Fbg and then incubated with mixtures containing FnBPA WT or Q103A, vWbp, ProT, FXIII and either calcium chloride or EDTA. Binding was measured using anti-FnBPA IgG and HRP-conjugated secondary antibody. Statistically significant difference is reported (** $P < 0.01$). Inset: binding of the mutant protein was reduced to about 30% of the interaction of WT FnBPA. Data are expressed as means $\pm$ S.D. of triplicate tests.(D–E) Cross-link formation of recombinant N1 subdomain of FnBPA WT (D) or Q103A (E) to Fbg by TG2, analyzed as described for panels A–B.(F) ELISA analysis of Fbg crosslinking with WT and Q103A N1 domains by the action of TG2 in the presence of Ca^{2+} or EDTA. Statistically significant differences are reported (*** $P < 0.001$). Inset: binding of WT protein was about 90% greater of the FnBPA Q103A.

Adhesion to Fbg by *S. aureus* Q103A mutant is unaltered, but covalent incorporation into fibrin is inhibited

In view of the results obtained with the recombinant mutant proteins, the investigation continued by the generation of an *S. aureus* SH1000 mutant strain deleted for *fnbA/B*, *clfA/B* (SH10004X), harbouring FnBPA containing the Q103A substitution. Plasmid pFnBA4 containing the whole FnBPA protein (Greene et al., 1995) was subjected to site-directed mutagenesis to obtain the substitution. Subsequently, *S. aureus* SH10004X was transformed with the mutated construct, following a previously existing adapted protocol (Löfblom et al., 2007) As *S. aureus* SH10004X is deleted for FnBPA, the final result is a bacterial strain which overexpresses a mutated form of the protein of interest.

To investigate if the amino acid substitution is able to influence the bacterial non-covalent adhesion to Fbg, we decided to test the bacterial interaction confronting the SH10004X strain carrying the wild-type FnBPA and the mutant strain harbouring the Q103A substitution. Demonstrating that non-covalent binding to Fbg is retained despite the Q103A mutation is crucial, as it confirms that the amino acid substitution does not disrupt the overall adhesive function of the bacterial cell. To do this, microtiter wells coated with the wild type or the mutated strain were incubated with serial dilution of Fbg and the binding was revealed with an anti-Fbg antibody. As shown in fig. 24A, SH10004X pFnBA4::Q103A maintained the same interaction to Fbg of the strain carrying the wild-type protein.

Since we demonstrated the ability of the recombinant mutant proteins to inhibit the crosslinking to Fbg, we proceeded with testing the covalent incorporation of the mutant *S. aureus* strain into fibrin by an ELISA assay. Microtiter wells coated with Fbg were incubated with mixtures containing vWbp-activated FXIII and the strain carrying the wild type or the mutated FnBPA, additioned with calcium ions or EDTA. Binding was revealed with an anti-*S. aureus* antibody. Similarly to the previous experiments with the recombinant proteins, if SH10004X wild type was incubated in presence of calcium, the absorbance values were higher compared with those with EDTA, reflecting the covalent incorporation of the bacterial cell in the fibrin mesh (fig. 24B). Instead, the mutant Q103A strain showed the same absorbance levels regardless the presence of calcium or EDTA (fig. 24B). This result demonstrates that this single amino acid substitution is able to inhibit the cross-linking of *S. aureus* into fibrin. This evidence confirms that the covalent anchoring of the whole bacterial cell to the fibrin network depends critically on Q103.

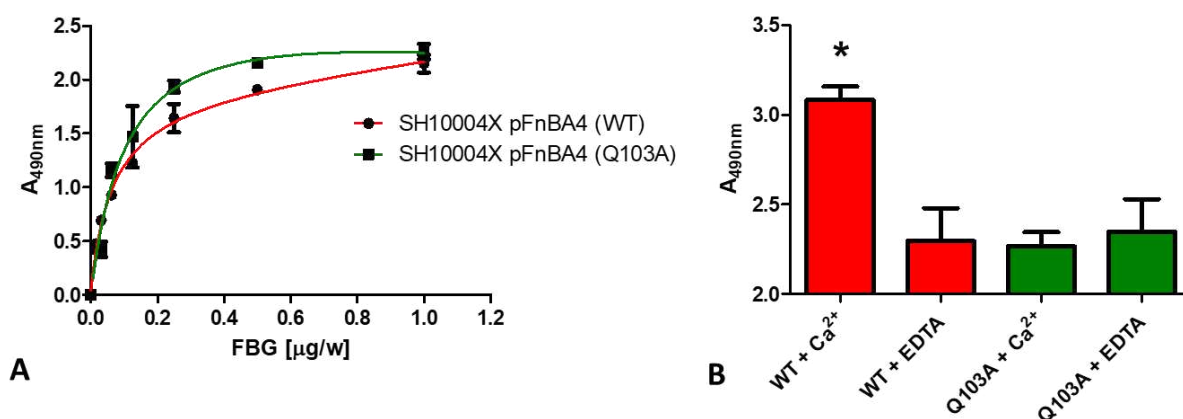


Figure 24. Impact of the Q103A substitution in FnBPA on *S. aureus* SH10004X binding to Fbg and cross-linking to fibrin. (A) Binding of Fbg to *S. aureus* SH10004X strains expressing either wild-type or Q103A-mutated FnBPA. Microtiter wells were coated with diluted bacterial suspensions (OD = 1) and incubated with serial dilutions of Fbg. Binding was detected using an anti-Fbg antibody and a HRP-conjugated secondary antibody. Data are expressed as means $\pm$ S.D. of tests performed in triplicate. (B) vWbp-activated FXIII mediated incorporation of *S. aureus* SH10004X WT or Q103A into fibrin in presence of calcium or EDTA. Microtiter wells were coated with Fbg and incubated with mixtures containing *S. aureus* SH10004X strains expressing either wild-type or Q103A-mutated FnBPA, vWbp, FXIII, ProT and either calcium ions or EDTA. Binding of bacteria to fibrin was then detected using an anti-*S. aureus* antibody and an HRP-conjugated secondary antibody. Statistically significant differences are reported (*P < 0.05). Data are expressed as means $\pm$ S.D. of triplicate tests.

The *S. aureus* Q103A mutant is significantly impaired in its ability to evade host defenses and induce tissue damage

To assess the mutant's virulence, we utilized a well-established mouse model of necrotic dermatitis, which mimics several aspects of human staphylococcal skin infections (Lentini et al., 2021).

In this model, bacteria are injected subcutaneously, and the progression of necrotic ulcers is monitored over time, along with bacterial load assessments in skin biopsies. As shown in fig. 25A and 25C, the lesion area, a reliable marker of infection severity, was significantly smaller in mice infected with the SH10004X Q103A mutant compared to those infected with the wild-type strain. Additionally, bacterial burden was found to be more than 10^5 -fold higher in skin biopsies from mice infected with the wild-type strain (fig. 25B). Taken together, these results demonstrate that the SH10004X Q103A mutation substantially diminishes the virulence of *S. aureus*.

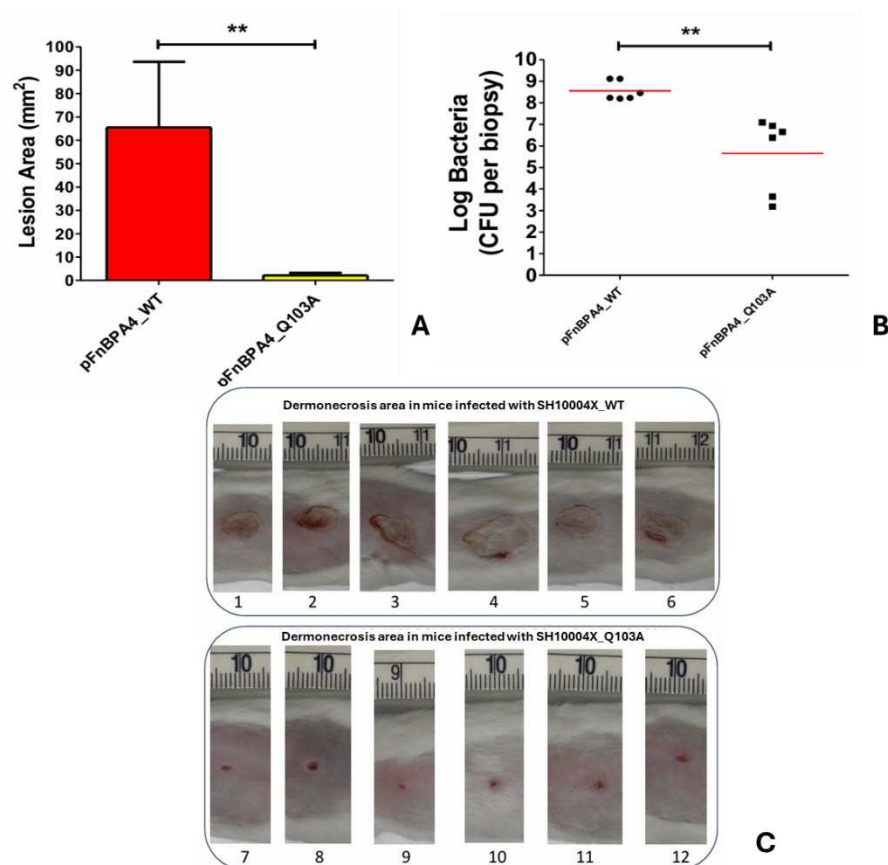


Figure 25. In vivo impact of Q103A mutation in a murine dermonecrosis model. **(A)** Histogram showing lesion areas (mm²) in mice infected with *S. aureus* SH10004X strains expressing either wild-type or Q103A-mutated FnBPA, measured at day 7 post-infection. Bars indicate mean $\pm$ S.D. **(B)** Bacterial load in skin biopsy samples quantified as CFU per biopsy. Each dot represents an individual mouse. **(C)** Representative lesion photographs from infected mice (6 per group), illustrating dermonecrotic injury caused by wild-type versus Q103A mutant strains. Statistical significance was determined using the Mann–Whitney U test ($p < 0.01$).

vWbp-activated FXIII mediates FnBPA crosslinking to fibronectin via the FnBPA repeated region

Matsuka et al. demonstrated that *S. aureus* FnBPA can serve as a substrate for FXIIIa, providing evidence for FXIIIa-mediated covalent crosslinking of FnBPA to Fn and not only fibrin. Based on these findings, the involvement of vWbp-activated FXIII in the crosslinking of FnBPA to Fn was evaluated in the present study.

To do this, the Region A of FnBPA (N1N2N3) or the C-terminal repeated region (FnBPA RR) were tested by Western Blotting. Each FnBPA recombinant protein was incubated with Fn in the presence of vWbp-activated FXIII and CaCl_2 for increasing time intervals to allow crosslinking reactions (up to four hours). Samples were subject to SDS-PAGE and then electroblotted onto a PVDF membrane. After immunostaining with a mouse anti-FnBPA antibody, signal was detected by an HRP-conjugated rabbit anti-mouse IgG. The results showed that the repeated region of FnBPA was able to generate high molecular weight complexes with Fn through vWbp-mediated cross-linking (fig. 26A). In contrast, the N1N2N3 domain failed to form these complexes under the same conditions (fig. 26B). These finding point to an additional mechanism in which bacterially-activated FXIII is able to mediate crosslinking not only to fibrin(ogen), but also to Fn.

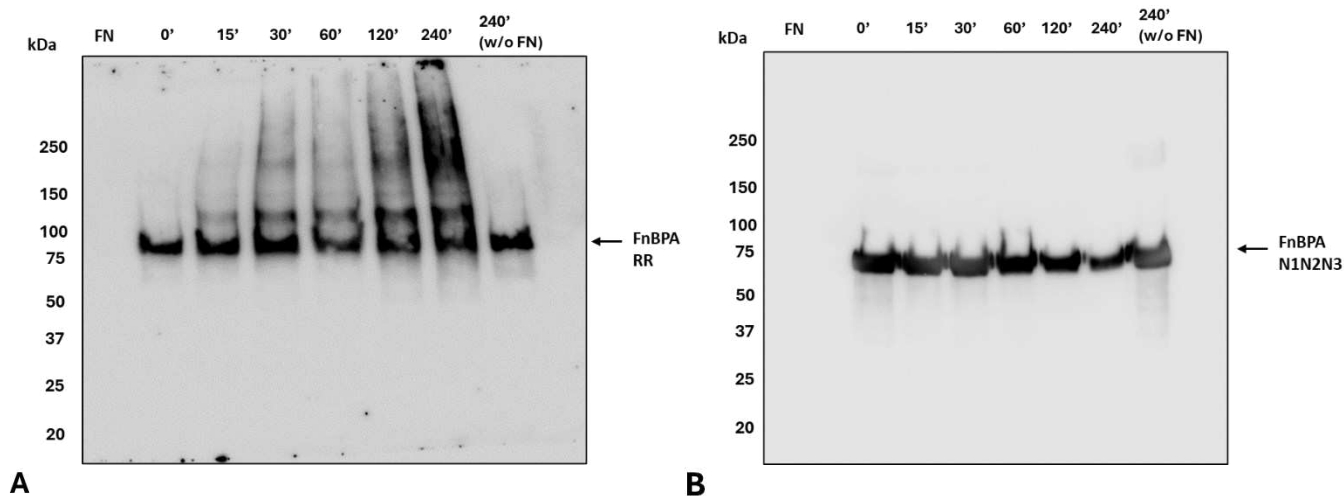


Figure 26: FnBPA RR is involved in cross-link formation with Fn. Mixtures containing FnBPA RR (A) or FnBPA N1N2N3 (B) proteins and Fn were incubated in the presence of vWbp-activated FXIII for the indicated times (top of each panel) and then separated by SDS-PAGE under reducing conditions and transferred to a PVDF membrane. Membranes were probed with FnBPA IgG followed by secondary HRP-conjugated IgG. Arrows show the positions of FnBPA RR domain (A) or FnBPA N1N2N3 (B). The figures are representative of three independent experiments. Molecular masses of standard proteins are indicated on the left side of each panel.

FnBPA is crosslinked to the N29 domain of fibronectin by vWbp-activated FXIII

Building up on previous findings indicating that FnBPA RR can be crosslinked to the full-length Fn molecule, an approach was taken to narrow down the specific region of Fn involved in this interaction. To this end, a Fn domain corresponding to the N-terminal 29 kDa fragment of fibronectin (N29) was employed to assess its capacity to serve as a crosslinking substrat. The N29 is critical for Fn matrix assembly and mediates interactions with several endogenous ligands, including heparin and fibrin (Quade and McDonald, 1988). This region is also involved in the non-covalent interaction with bacterial fibronectin-binding proteins such as FnBPA (Huff et al., 1994).

FkBPA RR recombinant protein was incubated with Fn in the presence of vWbp-activated FXIII and CaCl₂ for increasing time intervals to allow crosslinking reactions (up to two hours). Samples were subjected to SDS-PAGE and then electroblotted onto a PVDF membrane. After immunostaining with a mouse anti-FkBPA antibody, signal was detected by an HRP-conjugated rabbit anti-mouse IgG. The results showed that the repeated region of FkBPA was able to generate high molecular weight complexes with the N29 region of Fn through vWbp-mediated cross-linking (fig. 27), indicating that this domain is directly involved in the bacterially-mediated crosslinking of FkBPA to Fn.

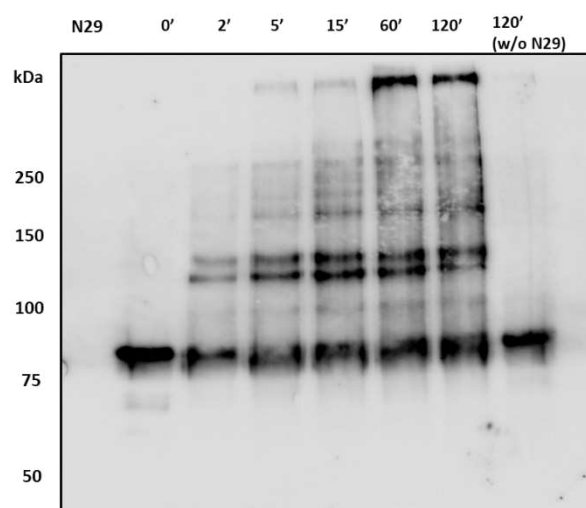


Figure 27: FkBPA RR is involved in cross-link formation with the N29 subdomain of Fn. Mixtures containing FkBPA RR Fn were incubated in the presence of vWbp-activated FXIII for the indicated times (top of each panel) and then separated by SDS-PAGE under reducing conditions and transferred to a PVDF membrane. Membranes were probed with FkBPA IgG followed by secondary HRP-conjugated IgG. The figure is representative of three independent experiments. Molecular masses of standard proteins are indicated on the left side.

Discussion

S. aureus has evolved intricate strategies to colonize host tissues, evade immune clearance, and establish persistent infections. Central to this process is the ability of *S. aureus* to adhere to host extracellular matrix (ECM) proteins, and eventually become incorporated into the fibrin network. Among the staphylococcal surface proteins, FnBPA plays a pivotal role in mediating bacterial adhesion to Fbg and Fn, thereby facilitating tissue colonization, particularly under pathological conditions such as vascular injury or implantation of biomaterials (Wann et al., 2000; Motta et al., 2023; Severina et al., 2023). The present study elucidates a covalent binding mechanism by which FnBPA undergoes cross-linking to host fibrin(ogen) and Fn via the enzymatic activity of host transglutaminases, particularly FXIII and TG2. This covalent cross-linking significantly enhances the stability of the bacterial-host interface and has profound implications for *S. aureus* pathogenesis and host-pathogen interactions.

The enzymatic cross-linking activity of FXIII is activated by thrombin during coagulation, enabling the formation of $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ isopeptide bonds between ECM proteins and bacterial surface components (Matsuka et al., 2003). Early studies have demonstrated that activated FXIII facilitates the covalent incorporation of bacteria such as Group B *Streptococcus* and *Streptococcus pyogenes* into fibrin matrices through cross-linking of bacterial surface proteins to host Fbg (Piliponsky et al., 2022; Loof et al. 2011).

Importantly, this process is not limited to the physiological activation of FXIII via thrombin. In particular, *S. aureus* has evolved a mechanism to induce FXIII activation via secreted coagulases, including vWbp. This virulence factor is able to non-proteolytically activate ProT and, in turn, promote conversion of Fbg to fibrin and activation of FXIII (Thomer et al., 2013; Bjerketorp et al., 2004). In this study, it was demonstrated that the expression of vWbp peaks during the exponential growth phase, and the protein is both secreted and capable of rebinding to the bacterial surface. The anchoring of vWbp to the *S. aureus* surface is mediated by two major components: peptidoglycan (PPG) and lipoteichoic acid (LTA), suggesting the presence of distinct binding regions within the vWbp molecule (Motta et al., 2023). Once associated with the bacterial surface, vWbp could recruit and activate ProT, likely generating catalytically active FXIII in close proximity to bacterial adhesins. This microenvironment supports the formation of covalent bonds between fibrin(ogen) and FnBPA, thereby embedding *S. aureus* cells within the developing fibrin scaffold. In this way, *S. aureus* could attract and exploit host coagulation factors to its own benefit to promote infection.

Molecular characterization of the vWbp-mediated cross-linking process has revealed that the N-terminal Region A of FnBPA (comprising N1N2N3 subdomains) is essential for covalent complex formation with fibrin(ogen). Within this region, the N1 subdomain contains reactive glutamine (Gln) residues capable of serving as acyl donors for transglutaminase activity. Notably, Gln103 within the N1 subdomain was identified as the principal site for

cross-linking, forming isopeptide bonds with the α -chain of Fbg (Motta et al., 2023). These findings are consistent with and expand upon prior work by Severina et al., which proposed Gln103 as a key residue in transglutaminase-mediated cross-linking. In contrast, the N2N3 domains mediate non-covalent binding to the γ -chain of Fbg via the well-characterized Dock, Lock, and Latch (DLL) mechanism (Wann et al., 2000), and do not support covalent complex formation. This bifunctional binding strategy, non-covalent attachment via N2N3 and covalent anchoring via N1, demonstrates a finely tuned evolutionary adaptation that maximizes adhesion stability.

To evaluate the specific contribution of Gln103, different recombinant FnBPA proteins, containing the whole Region A (N1N2N3) or just the first segment (N1), were produced with a Q103A substitution. Biochemical assays confirmed that while the Q103A variant retained its non-covalent binding to Fbg, it completely lost the ability to form covalent cross-links in the presence of both vWbp-activated FXIII and TG2. These results establish Gln103 as indispensable for the covalent cross-linking function of FnBPA, while being dispensable for initial non-covalent adhesion. The functional importance of Gln103 is further supported by sequence conservation analysis, which shows high conservation of this residue across major *S. aureus* clonal complexes. In contrast, other Fbg-binding adhesins such as ClfA, ClfB and FnBPB cannot be crosslinked by the action of vWbp-activated FXIII (Motta et al., 2023). Supporting this evidence, the homologous FnBP protein (FnBPB) in particular lacks a homologous Gln residue and does not undergo cross-linking. This specificity underscores the unique role of FnBPA in transglutaminase-mediated host interaction.

Beyond FXIII, TG2 has emerged as an additional host enzyme capable of catalyzing cross-linking between FnBPA and Fbg. While FXIII is primarily active during coagulation, TG2 is widely expressed and functions in diverse tissues, particularly under conditions of inflammation or tissue remodeling (Al-U'datt et al., 2022). Incubation of recombinant FnBPA with Fbg in the presence of TG2 resulted in rapid formation of high molecular weight complexes, very similar to the cross-linking profile observed with vWbp-activated FXIII (Motta et al., 2023). Like vWbp-activated FXIII, also TG2 demonstrated a reliance on Gln103. Despite their distinct regulatory pathways and tissue distributions, these transglutaminases generate similar bioproducts and act on the same reactive residue, suggesting that *S. aureus* may exploit multiple host enzymes with similar pathways to achieve stable tissue colonization across a range of physiological contexts. Supporting this, evidence from infection models indicates that TG2 is upregulated during bacterial infections and inflammation. For example, TG2 expression increases in macrophages during sepsis, where it participates in NF- κ B-mediated inflammatory responses and enhances macrophage survival (Su et al., 2021). TG2 also facilitates *Chlamydia trachomatis* survival in infected epithelial cells (Maffei et al., 2020), supporting a broader role for this enzyme in microbial persistence.

The covalent incorporation of *S. aureus* into the fibrin matrix through FnBPA-fibrin(ogen) cross-linking has multiple potential consequences for pathogenesis and host immunity. One

hypothesis posits that this process constitutes a host defense mechanism, limiting bacterial dissemination from localized sites of infection. In support of this, FXIII-deficient mice exhibit enhanced susceptibility to systemic infection following GBS challenge, while administration of exogenous FXIII reduces bacterial spreading and promotes containment (Piliponsky et al., 2022). An alternative, and not mutually exclusive, interpretation is that *S. aureus* subverts this host response to facilitate its own survival and persistence. By embedding itself within fibrin-rich environments, such as damaged heart valves, inflamed tissues, or implanted medical devices (Halper 2021; Pereira et al., 2002), the bacterium may evade immune clearance and establish chronic or biofilm-associated infections. The formation of stable FnBPA-fibrin(ogen) heteropolymers provides mechanical stability and may shield bacteria from phagocytosis and antimicrobial agents.

Experiments using *S. aureus* SH10004X strains harboring the Q103A mutation confirmed the *in vivo* relevance of this mechanism. These mutant strains failed to incorporate into fibrin matrices in the presence of vWbp-activated FXIII, highlighting the importance of Gln103 and covalent binding for bacterial entrapment within fibrin. Further *in vivo* evidence for the functional relevance of FnBPA-mediated covalent cross-linking in *S. aureus* pathogenicity was obtained through a mice model of subcutaneous infection. In this experiment, mice were subcutaneously infected with either the SH10004X strain expressing wild-type FnBPA or the Q103A mutant variant lacking the critical glutamine residue required for transglutaminase-mediated cross-linking. The extent of dermonecrotic lesions within the infected skin were assessed over time. Notably, animals infected with the Q103A mutant strain developed significantly smaller areas of dermonecrosis compared to those infected with the wild-type strain. In addition, quantitative culture of skin biopsies revealed a markedly lower number of colony-forming units (CFUs) in mice infected with the Q103A mutant, suggesting reduced bacterial persistence within the tissue. These findings support the hypothesis that covalent anchoring of *S. aureus* to the host fibrin network via Gln103 enhances bacterial survival and tissue damage *in vivo*. The reduced virulence phenotype observed in the Q103A mutant further underscores the importance of transglutaminase-mediated stabilization of the host-pathogen interface in promoting deep tissue colonization and evasion of host defenses. These data extend the role of FnBPA beyond vascular infections, highlighting its contribution to skin and soft tissue infections and reinforcing its potential as a target for therapeutic intervention.

From an evolutionary point of view, the presence of conserved glutamine-rich regions, or compositionally biased regions (CBRs), across multiple pathogenic bacteria underscores the evolutionary utility of specific glutamine residues (Mier and Andrade-Navarro, 2021). In fact, other Gram-positive pathogens, such as *Streptococcus pyogenes* and GBS (Piliponsky et al., 2022; Loof et al. 2011), have similarly evolved mechanisms to exploit FXIII or TG2 for covalent attachment to ECM components. In contrast, Gram-negative organisms such as *Escherichia coli* rely on non-covalent fibrin-like matrices, and do not engage transglutaminases in the same manner (Persson et al., 2003). This evolutionary divergence highlights the potential for targeting transglutaminase-mediated adhesion in therapeutic

strategies. Inhibiting the formation of FnBPA-Fbg covalent complexes could impair *S. aureus* persistence without affecting initial non-covalent adhesion or systemic coagulation. Such anti-adhesive therapies may be especially valuable in biofilm-associated infections, where conventional antibiotics often fail due to reduced penetration and the protective nature of the fibrin matrix.

Additionally, the variety of FnBPA-mediated covalent interactions with host proteins was further explored. In particular, the possibility that Fn, could also serve as a crosslinking substrate in the presence of vWbp-activated FXIII was investigated. Previous studies by Matsuka et al. had suggested that FnBPA could form covalent complexes with Fn by the action of physiologically-activated thrombin, but the specific domain of FnBPA involved in this interaction had not been clearly defined. To address this, we compared the crosslinking ability of the N-terminal Region A of FnBPA (comprising the N1N2N3 domains) and its C-terminal repeated region (RR), using vWbp-activated FXIII. Western blot analysis revealed that only the RR domain of FnBPA was capable of forming high molecular-weight complexes with full-length Fn, whereas the N1N2N3 region did not show any detectable crosslinked products under identical conditions. To identify the specific domain of Fn involved in this interaction, a truncated N-terminal 29 kDa fragment of fibronectin (N29), which includes critical binding sites for heparin, fibrin, and bacterial adhesins (Quade and McDonald, 1988; Huff et al., 1994) was tested as a potential substrate. Notably, the RR domain of FnBPA was also able to form covalent complexes with the N29 fragment in a vWbp-FXIII-dependent manner, suggesting that the N-terminal region of Fn harbors reactive residue(s) necessary for crosslink formation. This newly characterized interaction expands the repertoire of host-pathogen crosslinking substrates and reinforces the importance of the FnBPA RR domain in mediating stable anchorage of *S. aureus* to extracellular matrix components such as Fn.

In addition to mediating covalent cross-linking with Fn, the C-terminal repeated region of FnBPA is also known for its non-covalent interaction with Fn via its tandem repeats that interact with the N-terminal type I modules (in the N29 region) of fibronectin through a tandem β -zipper mechanism (Meenan et al., 2007). Thus, the RR domain serves a dual function: it mediates classical non-covalent Fn adhesion and, under an enzymatic mechanism by bacterially-activated host transglutaminase, supports covalent anchorage. This duality indicated the biochemical versatility of the RR domain in host-pathogen adhesion and highlights its potential as a multifaceted therapeutic target. Further studies are needed to better understand the biochemical mechanisms of FnBPA crosslinking to Fn, including which specific residues are involved and how the interaction is regulated.

In conclusion, this work elucidates a novel mechanism by which *S. aureus* exploits host transglutaminases to form covalent bonds with fibrin(ogen) and Fn via the surface protein FnBPA, through the action of vWbp-activated FXIII and TG2. Central to this process is Gln103 in the N1 subdomain of FnBPA, which serves as a critical glutaminy donor in cross-linking reactions mediated with fibrin(ogen) by both bacterially-activated FXIII and TG2. The covalent interactions not only stabilize bacterial adhesion, but may also represent a

mechanism by which *S. aureus* persists in hostile environments and resists immune clearance.

By dissecting the molecular basis but also *in vivo* relevance of FnBPA cross-linking to host protein, this study provides a deeper understanding of *S. aureus* pathogenicity and identify new avenues for therapeutic intervention.

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Publications

Part of the experimental evidence presented in this thesis is published a manuscript:

- Motta C, Pellegrini A, Camaione S, Geoghegan J, Speziale P, Barbieri G, Pietrocola G. von Willebrand factor-binding protein (vWbp)-activated factor XIII and transglutaminase 2 (TG2) promote cross-linking between FnBPA from *Staphylococcus aureus* and fibrinogen. *Sci Rep.* 2023 Jul 19;13(1):11683. doi: 10.1038/s41598-023-38972-3.

The remaining data was submitted for publication and waiting for review.

The publications listed below were produced throughout the research fellowship of one year preceding the PhD course and the PhD course itself, and are presented in their published form on the following pages.

These articles were the result of collaborative research with different institutes in Italy and abroad, including University of Messina, University of Louvain and University of Birmingham.

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OPEN

von Willebrand factor-binding protein (vWbp)-activated factor XIII and transglutaminase 2 (TG2) promote cross-linking between FnBPA from *Staphylococcus aureus* and fibrinogen

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The secreted von Willebrand factor-binding protein (vWbp) from *Staphylococcus aureus* interacts with the coagulation factors prothrombin and fibrinogen (Fbg), leading to the non-proteolytic transglutaminase activation of Factor XIII (FXIII). In this study we found that vWbp-activated FXIII catalyses the incorporation of amino-donor dansylcadaverine into region A of fibronectin-binding protein A (FnBPA). Incubation of Fbg with recombinant region A of *S. aureus* Fbg-binding proteins FnBPA, FnBPB, ClfA or ClfB in presence of vWbp-activated FXIII resulted in the formation of high molecular heteropolymers with FnBPA only, suggesting a specificity of the cross-linking reaction between fibrin(ogen) and the staphylococcal surface. As previously observed, cross-linking sites were mapped to the α -chain and the N1 subdomain of fibrin(ogen) and region A of FnBPA, respectively. Comparable results were obtained when tissue transglutaminase-2 (TG2) was tested for cross-linking of FnBPA and Fbg. Of note, FnBPA-mediated covalent cross-linking promoted by vWbp-activated FXIII was also observed when bacteria were allowed to attach to fibrin(ogen). Together these findings suggest a novel pathogenetic mechanism by which the transglutaminase action of FXIII and/or TG2 contributes to entrapment and persistence of *S. aureus* in blood and host tissues.

Staphylococcus aureus is a human pathogen well-known for its ability to cause community- and health care-associated infections ranging from mild skin infections to severe diseases including pneumonia, sepsis and toxic shock syndrome¹. Moreover, this bacterium has been identified as the major etiological agent of infective endocarditis, having a propensity to adhere to fibrin-platelet clots on damaged cardiac valves, referred to as vegetations^{2,3}. In addition to becoming increasingly resistant to antibiotics, *S. aureus* is a master in adapting to its host by avoiding almost every facet of the immune system⁴. The high pathogenetic potential of *S. aureus* is due to the expression of a plethora of virulence factors, most of which are surface proteins covalently anchored to the wall peptidoglycan (CWA, cell wall anchored proteins). These proteins, called MSCRAMMs (Microbial Surface Component Recognizing Adhesive Matrix Molecules), specifically recognize host matrix components⁵. The fibronectin (Fn)-binding MSCRAMMs FnBPA and FnBPB (Fn-binding protein A and B) are two structural and functional homologous CWA proteins, among the most intensively studied adhesins of *S. aureus*. FnBPs have been found to bind Fn⁶, fibrinogen (Fbg)^{7,8}, plasminogen⁹, elastin¹⁰ and histones¹¹. Furthermore, FnBPs play an important role in the formation of biofilm¹² and are involved in the invasion of a variety of non-phagocytic cell

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lines^{13–17}. Structurally, FnBPs comprise two distinct domains, the N-terminal and the C-terminal, which are similarly organized in the two proteins. The N-terminal domain comprises the region A, organized in three separately folded subdomains N1, N2 and N3¹⁸. N2 and N3 together form IgG-like folds typical of MSCRAMM proteins, with a potential to bind ligands such as Fbg by the “dock, lock and latch” (DLL) mechanism^{19,20}. The C-terminal domain comprises 10–11 tandemly repeated Fn binding units¹⁸. In addition to CWA proteins, *S. aureus* expresses a multitude of secreted proteins/peptides that compromise innate immune response²¹ and two hemostasis factors, the coagulase (Coa)²² and the von Willebrand factor-binding protein (vWbp)^{14,23,24}. Both Coa and vWbp are able to independently bind and non-proteolytically activate prothrombin (ProT) to thrombin, which in turn cleaves Fbg to fibrin and forms fibrin cables^{25–27}. By effect of this action, both coagulases contribute to the pathogenicity of the microorganism as proved in several animal models^{26,28}. Coa and vWbp have significant structural and functional similarities: in fact, the N-terminal regions of vWbp and Coa are structurally similar, and able to interact with and activate ProT in an analogous mechanism²⁹. Moreover, both the N- and C-terminal moieties of vWbp and Coa bind Fbg, although with different affinities³⁰. Differently from Coa, vWbp interacts with vWF and Fn²⁷. vWbp-activated ProT in the presence of Fbg triggers the non-proteolytic activation of plasma circulating Factor XIII (FXIII) in vitro²⁷. Activated-FXIII catalyses post-translational protein-modification reactions involving the cross-linking of lysine and glutamine residues to produce ϵ -(γ -glutamyl)-lysine isopeptide bonds (transglutaminase activity) and formation of γ -chain dimers and α -chain multimers in fibrin²⁷. This reaction provides stability and resistance to degradation by plasmin of fibrin cables in clots and abscess communities.

In physiological conditions FXIII is directly activated by thrombin and can induce the formation of isopeptide bonds that ligate adjacent fibrin monomers in the clot^{31–33}. Thrombin-activated FXIII also introduces cross-links between FnBPA and Fbg or Fn³⁴.

Apart from FXIII, transglutaminase activity is associated to a number of transglutaminase isoforms, namely TG1, TG2, TG3, TG4, TG5, TG6, TG7³⁵. TG2 (tissue transglutaminase), the most studied isoform, is found in fibroblasts, vascular endothelium, smooth muscle cells, and in the extracellular matrix (ECM) of various tissues and of the arterial walls³⁵. Many intra- and extracellular proteins have been identified as TG2 substrates for TG2-mediated cross-linking reactions. In the ECM, TG2 initiates cross-linking of structural proteins such as collagen, Fn, Fbg and laminin, leading to the formation of an ECM scaffold that displays an increased stability and rigidity^{36–38}.

In this study, we analysed vWbp expression during *S. aureus* growth and the localization of the protein after its secretion. Furthermore, we investigated whether vWbp-activated FXIII and TG2 are able to form cross-links between Fbg and the staphylococcal adhesin FnBPA and demonstrated that the covalent cross-linking reaction is effective when *S. aureus* cells attach to Fbg substrate.

Results

vWbp is predominantly expressed in the exponential growth phase and recognized by IgG in serum from patients with *S. aureus* infective endocarditis. vWbp is a secreted protein^{23,24}. To determine its optimal expression, a mutant LAC strain deficient in the protein A gene (*spa*) of *S. aureus* was grown in BHI during different growth phases. The supernatant was collected from the culture at regular time intervals, immobilized onto ELISA microtiter wells and the level of the protein detected using a polyclonal anti-vWbp antibody. As shown in Fig. 1A, vWbp is predominantly expressed in the exponential phase of growth (2–4 h) with a maximum expression at 3 h post-inoculum. The specificity of the ELISA assay was further confirmed by a Western immunoblotting, where a protein with a molecular mass corresponding to vWbp (60 kDa) was

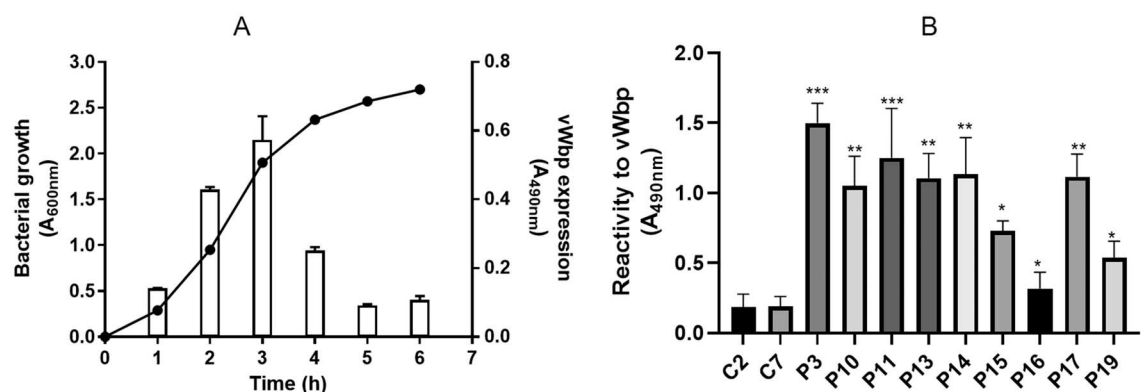


Figure 1. Expression of vWbp during bacterial growth and protein detection in sera from patients with infective endocarditis. **(A)** At the indicated times, the bacterial culture supernatants were collected, filtered and then immobilized onto microtiter wells. The presence of vWbp in the wells was revealed using anti-vWbp IgG followed by secondary HRP-conjugated IgG (right axis). Bacterial growth curve is also reported (left axis). **(B)** To test the immunological reactivity against vWbp, microtiter wells coated with vWbp were probed with IgG isolated from sera of human patients affected by staphylococcal endocarditis (P). IgG from healthy donors were used as controls (C2, C7). Bound antibody was detected by the addition of secondary HRP-conjugated IgG to the wells. Statistically significant differences between patients and controls are indicated (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Data are expressed as means $\pm$ S.D. of tests performed in triplicate.

detected in the supernatants of *S. aureus* LAC *spa* (data not shown). vWbp is involved in the initiation of infective endocarditis in animal models³⁹. Thus, to determine whether vWbp is an immunological active component and expressed in vivo we examined the IgG level against vWbp in previously characterized sera from patients with *S. aureus* infective endocarditis⁴⁰. Although variability from one serum to another was noted, IgG from all patients exhibited a reactivity with vWbp significantly higher than that observed with IgG from healthy donors, indicating the in vivo expression of vWbp and its relevance as an antigen (Fig. 1B).

Secreted vWbp rebinds to the cell surface and maintains its functional activity. To investigate whether vWbp could be captured and retained on the bacteria surface, *S. aureus* LAC cells from both exponential and stationary phases were spotted onto ELISA microtiter wells, incubated with increasing concentrations of biotin-labeled vWbp and association of vWbp to bacteria revealed with avidin-peroxidase. As shown in Fig. 2A, the protein bound to the surface of both exponentially and stationary phase cells, suggesting that the cell surface structures responsible of interaction with the protein are expressed in both stages of growth.

To identify these structures, we compared the rebinding of biotinylated vWbp to the surface of *S. aureus* LAC wt and its sortase A (*srtA*) deletion mutant. In these conditions, no significant difference was observed in binding of vWbp to bacterial surface, suggesting that vWbp binding is not mediated by CWA proteins tethered by sortase A (Fig. 1S). In support of this, none of a collection of sortase A-anchored proteins (ClfA-B, FnBPA-B, CNA, SdrC, SdrD, SdrE, IsdB, IsdH and SpA) displayed interaction with biotin labelled vWbp in an ELISA assays (data not shown).

It should be noted that *S. aureus* cell wall displays on the surface a variety of other molecules such as peptidoglycan (PPG), lipoteichoic acid (LTA), sortase B-anchored IsdC protein and a number of lipoproteins. To test the possible involvement of PPG and lipoteichoic acid (LTA)⁴¹ in vWbp binding, highly purified PPG and LTA from *S. aureus* were spotted onto a PVDF membrane, followed by incubation with biotinylated vWbp. Both LTA and PPG interacted with vWbp, with PPG showing a significantly higher binding than LTA (Fig. 2B). Due to the biochemical complexity of the staphylococcal surface we do not exclude that vWbp can interact with additional components of the cell wall.

Next, we investigated whether soluble and surface attached vWbp retain a comparable ability to activate ProT. As shown in Fig. 2C and D, both the forms of vWbp exhibited similar activating effect on ProT⁴², while a negligible activation of ProT was observed incubating the zymogen with substrate in the absence of vWbp. Likewise, no intrinsic enzymatic activity was observed incubating the substrate with vWbp alone.

vWbp activated-FXIII elicits the formation of cross-links between FnBPA N1N2N3 domain and fibrinogen α chain. Severina et al. demonstrated that thrombin-activated FXIII catalyses covalent cross-

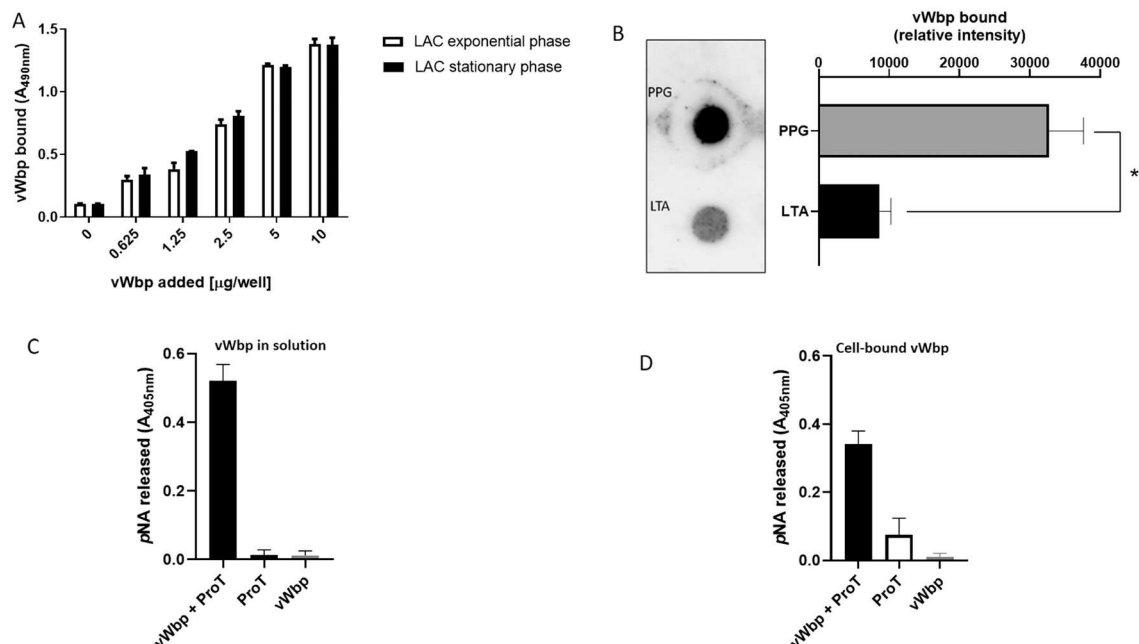


Figure 2. vWbp can associate to the bacterial cell surface via peptidoglycan and lipoteichoic acid and remains functionally active. (A) Binding of increasing concentrations of biotinylated vWbp to *S. aureus* LAC cells from exponential and stationary phases immobilized onto microtiter wells was determined by addition of avidin-peroxidase. (B) Binding of biotinylated vWbp to PPG or LTA dotted onto PVDF membrane was detected incubating the membrane with avidin-peroxidase. Densitometric analysis of vWbp bound to PPG or LTA is shown on the right. Statistically significant difference is reported (* $P < 0.05$). (C) and (D) ProT activation by soluble vWbp (C) or vWbp bound to immobilized *S. aureus* LAC cells (D) was detected by measuring pNA released from S2238 hydrolysis. Data are expressed as means $\pm$ S.D. of triplicate tests.

linking between full-length FnBPA and Fbg⁴³. Along this line, we firstly examined the presence of reactive glutamine or lysine residues in FnBPA N1N2N3 (A domain) assessing the ability of vWbp-activated FXIII to incorporate specific amine-donor (dansylcadaverine) or acceptor (dansyl- ϵ -aminocaproyl-QQIV peptide) synthetic probe into the recombinant bacterial protein. The reaction products were analyzed by SDS-PAGE and revealed under ultraviolet light (Fig. 3A) prior to staining the gel with Coomassie Blue (Fig. 3B). By the image obtained under ultraviolet illumination it appeared that vWbp-activated FXIII catalysed the incorporation of dansylcadaverine (lane 1), but not of dansyl- ϵ -aminocaproyl-QQIV probe (lane 2) (Fig. 3A), suggesting the presence in the A domain of reactive glutamine but not lysine residues.

To further investigate the formation of cross-links between FnBPA and Fbg the substrates were incubated with vWbp-activated FXIII for increased periods of time and the mixtures subjected to SDS-PAGE under reduced conditions and Western immunoblotting using a FnBPA antibody. In these conditions, the formation of high molecular complexes including FnBPA were observed (Fig. 4A). In those mixtures from which Fbg was omitted no high molecular mass complexes were detected. The specificity of the antibody as probe was further assessed by the absence of its reactivity with mixtures containing Fbg only. SDS-PAGE and immunoblotting of mixtures containing FnBPA/Fbg and activated FXIII with a Fbg α chain antibody revealed the production of high molecular weight heteropolymers made of FnBPA and the α -chain (Fig. 4B). No covalent incorporation of the γ chain in the complexes was observed when the mixtures were subjected to SDS-PAGE and nitrocellulose membrane was probed with a Fbg γ chain monoclonal antibody (data not shown).

The specific formation of the FnBPA/Fbg covalent complexes was indicated by the absence of high molecular weight products when A region of FnBPB, ClfA or ClfB proteins were individually incubated for increasing times with Fbg in the presence of vWbp-activated FXIII and then tested by SDS-PAGE and immunoblotting with appropriate antibody (Fig. 4C,D and E).

N1 subdomain of region A of FnBPA is involved in cross-link formation with fibrin(ogen). To localize the site in FnBPA N1N2N3 involved in cross-link formation, recombinant FnBPA N1 subdomain or FnBPA N2N3 protein were incubated for increasing times with Fbg in the presence of vWbp-activated FXIII. Formation of covalent products was assessed subjecting the mixtures to SDS-PAGE and Western immunoblotting using a FnBPA antibody. No covalent complexes were detected incubating N2N3 protein with Fbg (Fig. 5A), while incubation of N1 subdomain with Fbg resulted in the formation of high mol weight complexes (Fig. 5B). Plausibly, the major heterocomplex (110 kDa) that accumulates over time is the result of cross-linking between the N1 subdomain (40 kDa) and the Fbg α chain (70 kDa).

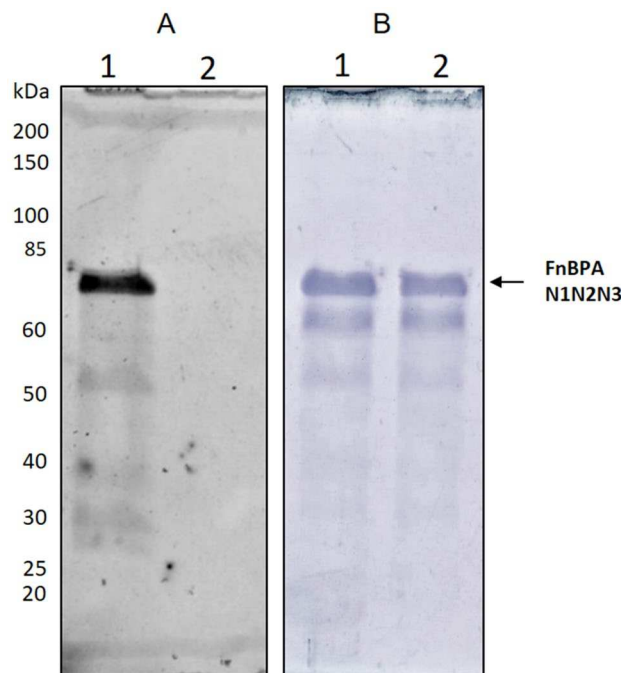


Figure 3. Incorporation of dansyl cadaverine into FnBPA N1N2N3 by action of vWbp-activated FXIII. Mixtures incubated with dansyl cadaverine (lane1) or dansyl- ϵ -aminocaproyl-QQIV peptide (lane 2) were separated by SDS-PAGE and the gel was examined under ultraviolet light (A) and then stained with Bio-Safe Coomassie staining (B). The figures are representative of three independent experiments. Molecular masses of standard proteins are indicated on the left side of the panel. Arrows show the positions of FnBPA N1N2N3 domain.

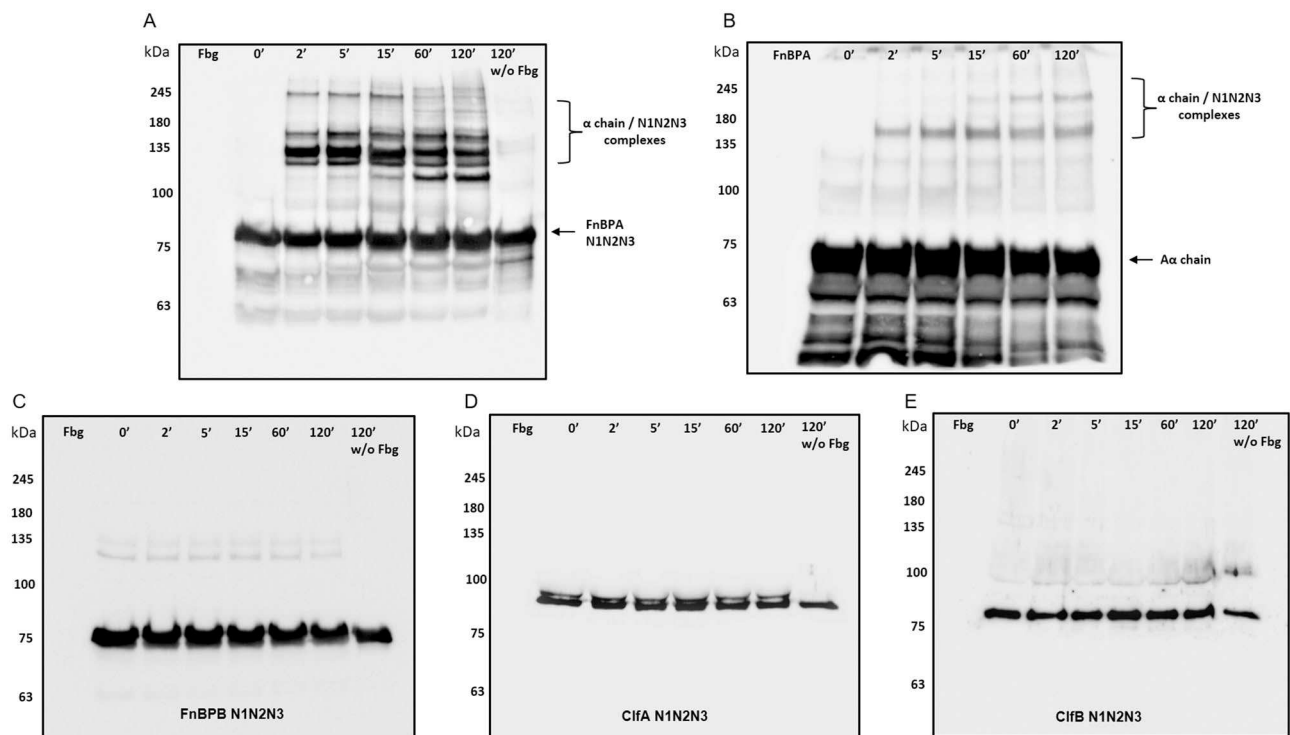


Figure 4. vWbp-activated FXIII elicits cross-links formation between region A of FnBPA and α -chain of Fbg. Panel (A) shows cross-linking of recombinant FnBPA N1N2N3 domain to Fbg by action of vWbp-activated FXIII. After indicated time intervals mixtures were separated by SDS-PAGE under reducing conditions and transferred to a PVDF membrane. The membrane was probed with anti-FnBPA IgG followed by secondary HRP-conjugated IgG. (B) Cross-links formation between FnBPA N1N2N3 domain and Fbg by vWbp-activated FXIII was analyzed by incubating the membrane with anti- α chain Fbg IgG followed by secondary HRP-conjugated IgG. Arrows show the positions of the α chain or α chain/FnBPA N1N2N3 complexes. (C-E) Cross-linking formation of the recombinant A domain of FnBPB (C), ClfA (D) or ClfB (E) with Fbg by vWbp-activated FXIII were analyzed incubating the PVDF membranes with mouse FnBPB, ClfA or ClfB IgG, respectively, followed by secondary HRP-conjugated IgG. The figures are representative of three independent experiments. Molecular masses of standard proteins are indicated on the left side of each panel. Incubation times (0–120 min) on the top of each panel are also specified. Original blots are presented in Supplementary Figure 3.

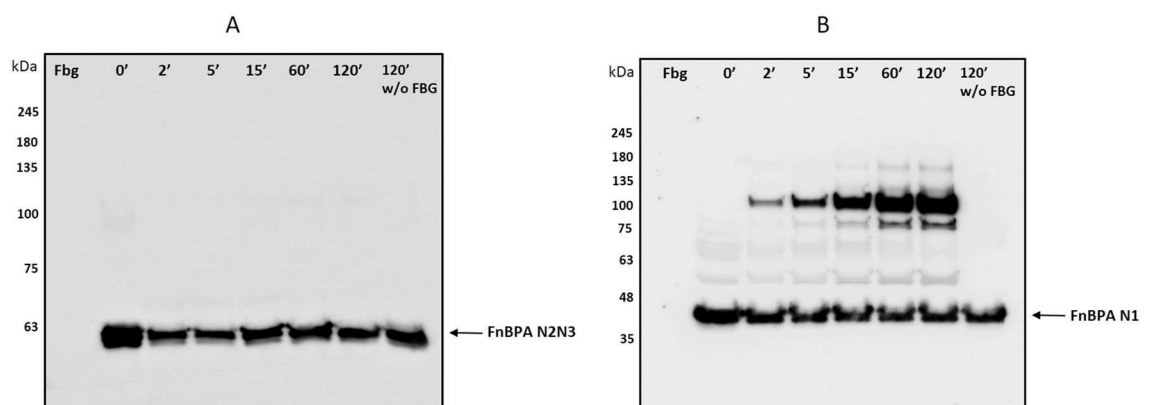


Figure 5. N1 subdomain of region A of FnBPA is involved in cross-link formation with Fbg. Mixtures containing FnBPA N2N3 (A) or FnBPA N1 (B) proteins and Fbg were incubated in the presence of vWbp-activated FXIII for the indicated times (top of each panel) and then separated by SDS-PAGE under reducing conditions and transferred to a PVDF membrane. Membranes were probed with FnBPA IgG followed by secondary HRP-conjugated IgG. Arrows show the positions of FnBPA N2N3 domain (A) or FnBPA N1 subdomain (B). The figures are representative of three independent experiments. Molecular masses of standard proteins are indicated on the left side of each panel. Original blots are presented in Supplementary Figure 4.

TG2 catalyses the formation of cross-links between FnBPA and Fbg. Transglutaminase 2 (TG2) is the most widely distributed and most abundantly expressed member of the transglutaminase family of enzymes³⁵. In view of the fact that TG2 is also highly expressed in several tissues, we decided to investigate the catalytic role of the enzyme in the formation of covalent polymers between FnBPA and Fbg³⁵. To this end, FnBPA N1N2N3, N1 or N2N3 proteins were incubated for increasing time intervals with Fbg in the presence of TG2. The incubation mixtures were then analysed by SDS-PAGE and Western immunoblotting using a FnBPA antibody. As reported in Fig. 6, covalent complexes between N1N2N3 and Fbg with size comparable to those obtained with activated FXIII were observed. Moreover, TG2 induced heteropolymers formation between N1 and Fbg, whereas no cross links were formed when N2N3 was incubated with Fbg.

Interaction of *S. aureus* expressing FnBPA with Fbg is reinforced by the action of vWbp-activated FXIII. Next, we evaluated the potential role of vWbp-activated FXIII on the stabilization of FnBPA-mediated adhesion of *S. aureus* LAC cells to Fbg. Preliminarily, the N1N2N3 and the N2N3 recombinant proteins were allowed to interact with surface-coated Fbg in the presence of vWbp-activated FXIII in an ELISA assay. Then, the wells were washed with increasing concentrations of NaCl (0.15–1.0 M) and then tested for ligand binding to the immobilized substrate. Assessment of Interaction of N1N2N3 protein with Fbg the presence of activated FXIII showed a complete resilience to ionic strength. Conversely, due to its inability to form covalent bonds with Fbg, N2N3 protein dissociates from Fbg as the ionic strength increases (Fig. 7A and B). For incubations where activated FXIII was omitted, a progressive, substantial reduction of ligand binding to Fbg was observed.

To investigate the stabilizing effect of covalent cross links on the attachment of *S. aureus* LAC to Fbg, micro-titer wells coated with Fbg were incubated with bacterial cells in the presence /absence of vWbp-activated FXIII. Wells were then treated with increasing concentrations of NaCl and tested for bacterial adhesion. As expected, only bacteria incubated with activated FXIII showed resilience to the ionic strength. In confirmation of this, cells of the double mutant *S. aureus* LAC *fnbAfnbB* overexpressing FnBPA incubated with vWbp-activated FXIII showed complete resistance to the ionic strength, while cells of the double mutant *fnbAfnbB* expressing high levels of FnBPB demonstrated susceptibility to the ionic strength and inability to remain attached to Fbg. (Fig. 7C).

Discussion

Detailed studies on the cross-linking between FnBPA and Fbg and Fn promoted by thrombin-activated FXIII has been performed by several authors^{43–45}. It has proposed that transglutaminase activity of activated FXIII is likely to serve as a mechanism to incorporate *S. aureus* cells at site of vascular injury and to establish infection^{46,47}. Thrombin-activation of FXIII elicits cross-linking in other bacterial species as well. For example, *Streptococcus pyogenes* cells can be covalently incorporated inside the clot via cross-linking of bacteria to fibrin fibers⁴⁸. In Group B *Streptococcus* (GBS), thrombin-activated FXIII promotes bacterial entrapment within fibrin clots by cross-linking fibronectin to ScpB, a GBS Fn-binding surface protein⁴⁹. Although thrombin-activated FXIII has a primary role in the covalent cross-linking of bacteria to Fbg and fibronectin, incorporation of FnBPA-mediated staphylococcal cells into the clots may also depend on the FXIII activation by secreted staphylococcal coagulases Coa and vWbp. In fact, Coa and vWbp act as nonproteolytic activators of ProT that in turn converts Fbg to fibrin and FXIII precursor into activated FXIII²⁷. Thus, the production of coagulases such as vWbp may implement the pathogenetic potential of this bacterium. In view of these considerations, we investigated the role of vWbp-activated FXIII in the formation of cross-links between Fbg and FnBPA.

Under in vitro conditions we showed that the vWbp is mostly produced in the exponential phase of bacterial growth and that the protein is expressed in vivo and is immunogenic. We also found that secreted vWbp can rebound to the bacterial surface and found that this anchoring is mediated by PPG and LTA. Due to the different structure of PPG and LTA, it is plausible that two distinct binding sites exist on vWbp molecule. Our results also

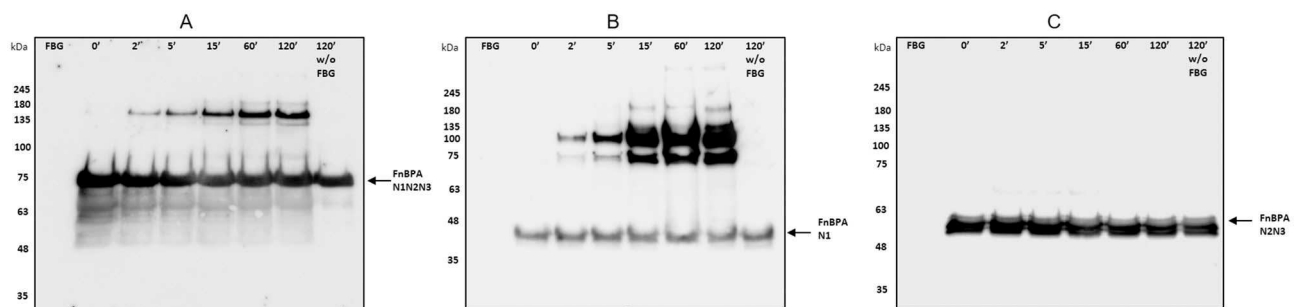


Figure 6. TG2 promotes formation of cross-links between N1 domain of FnBPA and Fbg. Products of cross-linking reactions between FnBPA N1N2N3 (A), N1 (B) and N2N3 (C) proteins and Fbg generated by action of TG2 were separated by SDS-PAGE under reducing conditions and transferred to a PVDF membrane. Membranes were probed with anti-FnBPA IgG followed by secondary HRP-conjugated IgG. Arrows show the positions of unreactive FnBPA proteins. The figures are representative of three independent experiments. Molecular masses of standard proteins are indicated on the left side of each panel. Incubation times (0–120) of the mixtures are indicated on the top of each panel. Original blots are presented in Supplementary Figure 5.

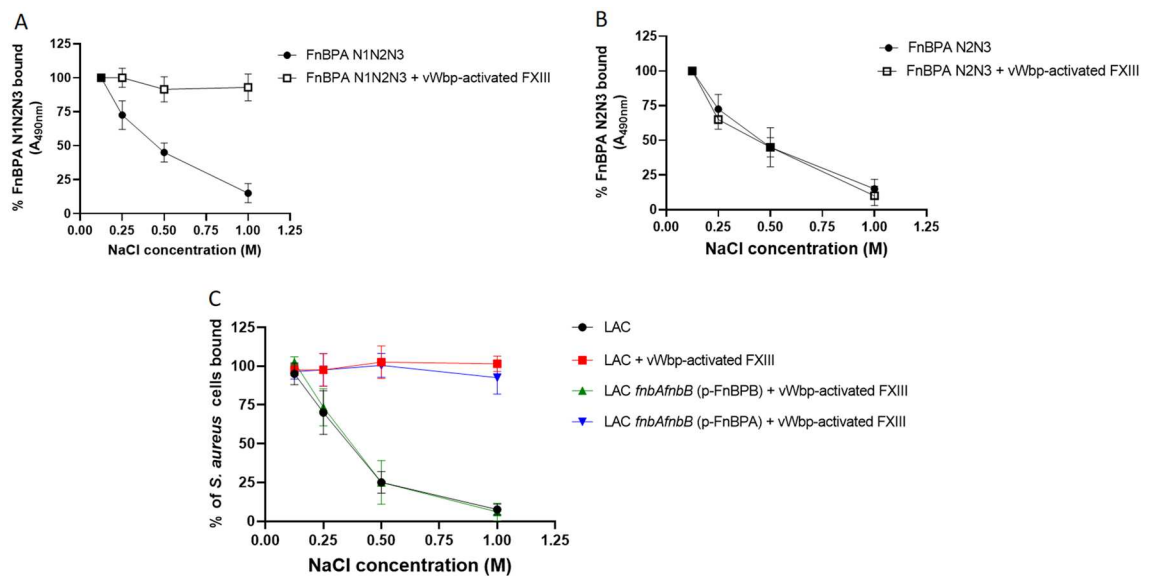


Figure 7. Cross-link formation induced by vWbp-activated FXIII reinforces binding between FnBPA and Fbg-coated surface. Mixtures of vWbp, ProT and FnBPA N1N2N3 (**A**) or FnBPA N2N3 (**B**) were incubated with immobilized Fbg in the presence/absence of FXIII. Wells were extensively washed with increasing concentrations of NaCl. Binding of FnBPA proteins to Fbg was detected by addition of a mouse FnBPA antibody followed by secondary HRP-conjugated IgG to the wells. (**C**) The effect of increasing concentrations of NaCl on the adhesion of *S. aureus* LAC wt cells (red) or *fnbAfnbB* double mutant cells overexpressing FnBPB (green) or FnBPA (blue) to Fbg was evaluated by incubating Fbg-coated microtiter wells with a mixture of vWbp, ProT and bacterial cells in the presence of FXIII. Wells were then washed with the indicated concentrations of NaCl and the attached bacterial cells detected with crystal-violet. The effect of salt concentrations on *S. aureus* LAC adhesion in absence of FXIII is also shown (black). Adhesion levels are expressed as a percentage of controls, where washings of the wells were performed with 150 mM NaCl. Data are expressed as means $\pm$ S.D. of triplicate tests.

indicate that, when bound to the cell surface, vWbp recruits and activates ProT on *S. aureus* surface, possibly generating active FXIII and cross-linking of Fbg to surface-associated FnBPA.

A study performed incubating vWbp-activated FXIII with FnBPA N1N2N3 region in the presence of fluorescent probes dansyl-cadaverine or glutamine-containing peptide revealed the presence of reactive Gln residues in the targeted substrate. Differently than previously reported, no Lys residues potentially involved in cross-link formation were observed in this domain⁴³. vWbp-activated FXIII catalyses the formation of intermolecular $\epsilon(\gamma$ -glutamyl) lysine isopeptide bonds between Fbg and the A region (N1N2N3 domains) of FnBPA. Moreover, the cross-linking site for Fbg in FnBPA was localized in N1 subdomain, which contains the functional reactive Gln103 (Q) residue, previously identified as the main lysine acceptor residue within Fbg⁴³. On the other hand, no cross-linking was observed when incubating the recombinant FnBPA N2N3 domains with Fbg. In line with the work performed by Severina et al.⁴³, we also found that the acceptor glutamine site involved in cross-linking reaction was localized in the α chain of Fbg.

Thus, interaction between Fbg and full length N1N2N3 occurs a) through the non-covalent binding of the Fbg γ chain to the FnBPA N2N3 region via the dock, lock and latch (DLL) mechanism^{19,20} and b) via the covalent interaction of the Fbg α chain with the N1 subdomain.

No isopeptide bond was observed when region A of other Fbg-binding proteins such as ClfA, ClfB or FnBPB was tested for cross-linking reactions in the presence of vWbp-activated FXIII. In accordance with this, multiple alignments of the N-terminal sequence of ClfA, ClfB, FnBPB and FnBPA showed that none of the homologous sequences of ClfA, ClfB and FnBPB adhesins centered on the Gln103 of N1 subdomain of FnBPA possesses a reactive Gln103 residue (Fig. S2), Hence, Gln103 is essential to establish a covalent cross-link with Fbg.

After that, we wondered whether other transglutaminase family members, such as the most ubiquitously expressed tissue TG2³⁵, could have a role in promoting cross-linking of Fbg to FnBPA.

Incubation of N1N2N3 region with Fbg in the presence of TG2 resulted in a rapid and efficient formation of high molecular covalent complexes, with a kinetics similar to that reported when the cross-linking of N1N2N3 to Fbg was examined in the presence of activated FXIII. Of note, as reported for the reaction catalysed by activated FXIII, the complex formation promoted by TG2 involved the N1 subdomain. Thus, although operational in different contexts and exhibiting different biochemical and regulatory properties, the transglutaminase activities of FXIII and TG2 generate almost identical bioproducts.

We also tested whether vWbp-activated FXIII can catalyse covalent attachment and incorporation of *S. aureus* to Fbg. To this end, we primarily demonstrated that the covalent FnBPA/Fbg complex can be formed in the presence of activated FXIII and does not dissociate even in the presence of high levels of ionic strength.

Differently from a mutant strain of *S. aureus* LAC overexpressing FnBPB, the *S. aureus* LAC wt and its mutant overexpressing FnBPA, when incubated with Fbg in the presence of activated FXIII and then treated with increasing amounts of NaCl, showed resilience to ionic strength. Overall, these results indicate that activated FXIII not only elicits a covalent complex formation between recombinant FnBPA and Fbg, but also promotes FnBPA-mediated cross-linking of *S. aureus* cells to immobilized Fbg. Thus, the covalent attachment of bacteria to Fbg induced by activated FXIII may represent a host innate defence mechanism to diminish their dissemination and invasion⁴⁸. Alternatively, covalent entrapment of *S. aureus* cells expressing FnBPA within fibrin fibers may be a prerequisite for the persistence of staphylococci in clots and a potential mechanism to escape the immune defence system²⁶.

The methodological approach described above may be useful to extend the value of covalent cross-links formation between additional bacterial and host components to stabilize tissue colonization and infection. The wealth of *S. aureus* surface proteins and their host ligands could be the natural candidates of future studies.

This study reconfirms the central role of FnBPA at the interface between *S. aureus* and the colonized host. In consideration of previous work demonstrating the formation of covalent bonds between the repetitive region of FnBPA and fibronectin by thrombin-activated FXIII⁴⁴, a further analysis of the molecular aspects of cross-linking of FnBPA to fibronectin may be worth exploring.

The biological significance and the importance in pathogenesis of Fbg cross-linking to FnBPA are still the subject of speculation. We can envisage at least two possible scenarios : i) cross linking reactions could have a role in limiting dissemination of *S. aureus* from the site of infection, such as fibrin clots, to the whole organism minimizing the events that progress to sepsis. In support of this hypothesis, it has been demonstrated that mice infected with GBS exhibit a containment of the bacterial infection, whereas mice deficient in FXIII show a greater susceptibility to systemic infection. Consistent with this observation, administration of exogenous FXIII to mutated mice reduced bacterial spreading and dissemination⁴⁹. Therefore, the catalytic action of transglutaminases could be considered as part of innate defence mechanisms of the host. ii) covalent heteropolymers formed by Fbg and FnBPA through the enzymatic action of vWbp-activated FXIII or TG-2 could contribute to the stabilization of *S. aureus* adherence to the extracellular matrix of tissues constitutively expressing Fbg^{50–52} and to Fbg-rich sites such as vegetations in a host damaged cardiac valve or fibrin(ogen)-coated biomaterials. Thus, through the action of transglutaminases, the bacterium could opportunistically manipulate the host defence mechanisms to its own benefit.

Studies are in progress in our laboratory to determine the value of such important issues.

Methods

Bacterial strains and culture conditions. All strains used in this study are listed in Table 1. *S. aureus* cells were grown overnight in brain heart infusion (BHI) (VWR International Srl, Milan, Italy) at 37 °C with shaking. *Escherichia coli* BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) transformed with vector pQE30 or pET22b (Integrated DNA Technologies, Leuven, Belgium) was grown in Luria agar and Luria broth (VWR International Srl) containing 100 µg/ml ampicillin (Sigma Aldrich) at 37 °C. In those experiments, where a defined number of cells were used, bacteria were harvested from the cultures by centrifugation, washed, suspended in phosphate-buffered saline (PBS), and counted in a Petroff-Hausser chamber.

Plasmid and DNA manipulation. The DNA fragments encoding the N1 (residues 37–193) and N1-3 (residues 37–511) regions of FnBPA were PCR amplified from the genome of *S. aureus* strain 8325–4 using the forward primer 37-193For (5'-GCATCACCATCACCATCACGGATCCGCATCAGAACAAAAGACAAC) for both constructs and the reverse oligonucleotides 37–193 (5'-TAATTAAGCTTGGCTGCAGGTCGACCTACGTTCCACTTTTCGCGTTAC) for N1 region or 37–511 (5'-TAATTAAGCTTGGCTGCAGGTCGACCTAATTTTCTCATTTCGTTTC) for N1-3 region. Amplified fragments were cloned by Gibson assembly (NEBuilder HiFi DNA Assembly Cloning Kit, New England Biolabs) into the expression plasmid pQE30 (Qiagen), between the BamHI and SalI restriction sites, thus generating constructs pFnBPAN1 and pFnBPAN1-3. All constructs were verified by sequencing using primers pQEfor (5'-GTATCACGAGGCCCTTTCGTCT) and pQErev (5'-CATTACTGGATCTATCAACAGGAG). Cloning of N2N3 was performed as previously reported⁹.

Bacterial strain	Relevant properties	Reference
<i>S. aureus</i>		
LAC wt	Community-associated MRSA of USA300 lineage	53
LAC <i>spa</i>	Strain derivative of LAC* deficient in protein A; constructed by transduction of <i>spa</i> ::Kan ^r by phage 85 into strain LAC*	54
LAC <i>srtA</i>	Constructed by the transduction of <i>srtA</i> ::Erm ^r from Newman <i>srtA</i> using bacteriophage 85	11
LAC <i>fnbAfnbB</i> (pFnBA)	<i>fnbA fnbB</i> double mutant transformed with plasmid-expressing FnBPA	55
LAC <i>fnbAfnbB</i> (pFnBB)	<i>fnbA fnbB</i> double mutant transformed with plasmid-expressing FnBPB	55
<i>E. coli</i>		
BL21 (DE3)	<i>E. coli</i> cloning host	Invitrogen

Table 1. List of bacterial strains used in this study. Kan^r, kanamycin resistance; Erm^r, erythromycin resistance.

Expression and purification of recombinant proteins. Recombinant FnBPA N1N2N3, and N1 regions were expressed from pQE30 (Millipore-Sigma, MA, USA) in *E. coli* BL21 (DE3)). Overnight starter culture was diluted at 1:40 in Luria broth containing ampicillin and incubated with shaking until the culture reached the exponential phase ($OD_{600nm} = 0.4-0.6$). Recombinant protein expression was induced by the addition of 1 mM (final concentration) isopropyl 1-thio- β -D-galactopyranoside (IPTG) (Sigma-Aldrich) to the culture and incubated over-night at 28 °C. Bacterial cells were harvested by centrifugation and frozen at -80 °C. Cells were re-suspended in lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, pH 8.0 supplemented with 1 mM $MgCl_2$) containing 1 mM phenyl-methanesulfonyl-fluoride (PMSF) (Sigma-Aldrich) and 20 μ g/mL of protease-free DNase I (Sigma-Aldrich) and lysed by sonication (70% amplitude, 12 $\times$ 30" on/off, 1'30" interval between sonication steps). The cell debris was removed by centrifugation and proteins purified from the supernatants by Ni^{+2} -affinity chromatography on a HiTrap chelating column (GE Healthcare, Buckinghamshire, UK). Protein purity was assessed by SDS-PAGE and Bio-Safe Coomassie staining (BioRad, Hercules, CA, USA). A bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) was used to measure the concentration of purified proteins.

Recombinant proteins FnBPA N2N3⁹, FnBPB N1N2N3⁸, ClfA N1N2N3⁵⁶, ClfB N1N2N3⁵⁷ and vWbp²⁷ were expressed with His₆ N-terminal affinity tags and purified as reported above.

Reagents, proteins and antibodies. BSA (bovine serum albumin), skim milk and dansylcadaverine were purchased from Sigma-Aldrich. Dansyl- ϵ -aminocaproyl-QQIV was synthesized by GenScript (Rijswijk, Netherlands). Human prothrombin, human factor XIII (FXIII) and Fbg were purchased by Prolytix (VT USA). Ultrapure peptidoglycan (PPG) and lipoteichoic acid (LTA) from *S. aureus* were purchased by InvivoGen (CA, USA). The chromogenic substrate S2238 (D-Phe-Pip-Arg-pNA) was obtained from Chromogenix (Milan, Italy). Transglutaminase-2 (TG2) was obtained from Zedira GmbH (Darmstadt, Germany). IgG from patients with infective endocarditis were isolated as previously reported⁴⁰. IgG collection was carried out in accordance with relevant guidelines and regulations; all experimental protocols were approved by the ethical board of the University of Pavia and informed consent was obtained from all human participants. vWbp, FnBPA, FnBPB, ClfA and ClfB polyclonal antibodies were raised in mice by routine immunization procedure using each purified bacterial protein as antigen. The study is reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>). The polyclonal antibodies production method was carried out in accordance with relevant guidelines and regulations; all experimental protocols were approved by the ethical board of the University of Pavia. Monoclonal rabbit anti- α chain and anti- γ chain antibodies were purchased from Bioss Antibodies (MT, USA). Rabbit anti-mouse, rabbit anti-human, goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Dako Cytomation (Glostrup, Denmark). Avidin-peroxidase was purchased from Sigma Aldrich. OPD tablets (*o*-phenylenediamine dihydrochloride) were purchased by ThermoScientific (Rockford, IL, USA).

vWbp expression during bacterial growth phases. The expression of vWbp during different bacterial growth phases was assessed through an ELISA assay. For this purpose, cells of a mutated strain deleted of protein A (*S. aureus* LAC *spa*) were grown for the indicated times. Bacteria were harvested by centrifugation and culture supernatants filtered (pore size 0.22 μ m). 100 μ l of individual supernatants were immobilized onto microtiter wells overnight at 4 °C. The wells were washed three times with 0.5% (v/v) Tween 20 in PBS (PBST) and treated for 1 h at 22 °C with 2% (v/v) BSA in PBS. The binding of vWbp to the surface-coated material was detected incubating the wells for 1 h with a mouse polyclonal anti-vWbp IgG (1:2000) in 1% (v/v) BSA followed by an HRP-conjugated anti-mouse IgG (1:1000) in 1% (v/v) BSA for 45 min. After washing, OPD was added to the wells and the absorbance at 490 nm measured in an ELISA plate reader (Bio-Rad). The bacteria growth curve was obtained evaluating the bacterial density at 600 nm.

Reactivity with vWbp of IgG from patients with infective endocarditis. To test the reactivity of IgGs obtained from sera of patients with endocarditis, recombinant vWbp was immobilized onto microtiter wells (1 μ g/well). After blocking with BSA, the wells were incubated with IgG (1 μ g/well) from patients and healthy donors. The binding of IgG was revealed by the addition to the wells of an HRP-conjugated polyclonal rabbit anti-human IgG (1:1000) and the absorbance was determined as reported above.

Biotin labelling of vWbp. Biotin labelling of vWbp was carried out using a Biotin Conjugation Kit (Fast, Type A) (Abcam, Cambridge, MA, USA). Labelled sample was dialysed and the protein concentration measured using a bicinchoninic acid protein assay (Pierce).

Rebinding of vWbp to the bacterial surface. To measure rebinding of vWbp to the bacterial surface, microtiter wells were coated overnight at 37 °C with 100 μ l of *S. aureus* LAC wt suspensions ($OD_{600nm} = 1.0$) in PBS both stationary and exponential phases. After blocking the wells with BSA, the plates were incubated for 1 h with increasing concentrations of biotinylated vWbp (from 0 to 10 μ g/well). After several washings, the wells were treated with avidin-peroxidase (1:1000) and the absorbance determined as reported above. Cells of *S. aureus* LAC *srtA* from the stationary phase of growth were tested for vWbp rebinding in the conditions reported above.

Effect of increasing concentration of NaCl on the interaction of recombinant FnBPA or of *S. aureus* cells with Fbg. To assess the effect of ionic strength on the binding of recombinant FnBPA N1N2N3

or N2N3 proteins to immobilized Fbg, microtiter wells were coated overnight with 5 µg/well of Fbg. After blocking with BSA, the wells were added with a mixture of vWbp (200 nM), ProT (200 nM), FXIII (20 nM) and FnBPA N1N2N3 or N2N3 (1.5 µM) in presence of 5 mM CaCl₂ and incubated for 2 h at 37 °C. After washing with PBST, the wells were extensively washed with increasing concentration of NaCl (from 0,15 to 1 M) and bound FnBPA detected with a mouse polyclonal anti-FnBPA IgG (1:1000) followed by HRP-conjugated rabbit anti-mouse IgG (1:1000). To measure attachment of *S. aureus* cells to Fbg and the effect of ionic strength on the interaction, Fbg-coated wells were incubated for 2 h at 37 °C with 1×10^8 cells of *S. aureus* strain LAC wt or the double mutant *fnbAfnbB* transformed with plasmid overexpressing FnBPB or FnBPA in the presence of vWbp-activated FXIII. After treatment with increasing concentration of NaCl, bacteria attached to the wells were fixed with formaldehyde (25% v/v), stained with crystal violet (0.5% w/v) and the absorbance at 595 nm determined using an ELISA plate reader.

Activation of ProT by soluble or bacteria-bound vWbp. Activation of human ProT by vWbp was performed incubating human ProT (0.1 mg/ml, 1.38 µM) with equimolar concentration of vWbp in presence of 20 µM S2238 (D-Phe-Pip-Arg-pNa) in TBS-CaCl₂ (25 mM Tris-HCl pH 7.8, 0.15 M NaCl, 5 mM CaCl₂) for 2 h at 37 °C. The absorbance of released pNa (p-Nitroaniline) was determined at 405 nm using an ELISA plate reader. Experiments performed with mixtures where ProT or vWbp was omitted were used as control.

The ability of bacteria-associated vWbp to activate ProT, was determined coating microtiter wells with 100 µl of *S. aureus* LAC suspensions (OD_{600nm} of 1.0) at 37 °C. The plates were incubated with 10 µg/well of vWbp for 1 h and then added with ProT (0.1 mg/ml, 1.38 µM) in presence of 20 µM S2238 in TBS-CaCl₂ for 2 h at 37 °C. The absorbance of released pNA was determined as reported above. Wells coated with bacteria without vWbp and incubated with ProT were used as control. An additional control experiment was performed incubating immobilized bacteria/vWbp complexes with S2238 alone.

Dot blot and western blot assays. *Binding of vWbp to peptidoglycan and lipoteichoic acid.* To evaluate the binding of vWbp to PPG and LTA, 5 µg of each compound was dotted onto a PVDF membrane (BioRad). After overnight incubation at 4 °C with 5% skim milk (w/v) in PBST, the membrane was treated for 1 h at 22 °C with 2 µg/mL of vWbp. The membrane was incubated with a mouse polyclonal vWbp antibody (1:5000) in 2% (w/v) skim milk for 1 h at 22 °C. Following several washings with PBST, the membrane was treated with 45 min at 22 °C with an HRP-conjugated rabbit anti-mouse IgG (1:1000) in 2% (w/v) skim milk and dot blots developed using the Westar Supernova detection kit (Cyanagen srl, Bologna, Italy). An ImageQuant™ LAS 4000 mini-biomolecular imager (GE Healthcare) was used to capture images of the spots. The signal intensities were quantified with ImageJ and plotted on GraphPad Prism.

Cross-linking of recombinant S. aureus proteins to Fbg promoted by vWbp-activated FXIII. To evaluate FnBPA, FnBPB, ClfA and ClfB cross-linking to fibrin(ogen) in the presence of vWbp-activated FXIII, Western immunoblotting assays were performed. Mixtures containing 200 nM ProT, 200 nM vWbp, 15 µg/ml FXIII, 5 µM Fbg and 2.5 µM of each bacterial protein were incubated at 37 °C in 25 mM Tris-HCl buffer, pH 7.5 containing 5 mM CaCl₂ for the indicated times. Reactions were stopped by addition to the mixtures of a buffer containing 62.5 mM Tris-HCl, pH 6.8, 4 M urea, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue. Samples were heated at 95 °C for 10 min, separated by 4.8–10% polyacrylamide gels SDS-PAGE and electroblotted onto a PVDF membrane. After overnight blocking with 5% (p/v) skim milk and washings, the membranes were immunostained with the appropriate mouse antibody against each the bacterial protein. The membranes were treated with an HRP-conjugated rabbit anti-mouse IgG (1:10,000), and the blots developed as reported above.

Detection of the presence of α or γ chain in heteropolymers was determined by using a monoclonal rabbit anti-α chain or anti-γ chain (1:5000) primary antibody and a goat anti-rabbit HRP-conjugated secondary antibody (1:10,000).

Cross-linking of FnBPA proteins to Fbg promoted by TG2. To evaluate isopeptide formation between FnBPA protein and Fbg promoted by the catalytic action of TG2, 2.5 µM of each recombinant FnBPA domain (N1N2N3, N2N3 or N1) was incubated with 5 µM Fbg in the presence of 50 nM TG2 in 25 mM Tris-HCl buffer, pH 7.5 containing 5 mM CaCl₂ for the indicated times. Samples were heated at 95 °C for 10 min, separated by 4.8–10% polyacrylamide gels SDS-PAGE and electroblotted onto a PVDF membrane. After overnight blocking with 5% (p/v) skim milk, membranes were washed and immunostained with a FnBPA mouse polyclonal antibody diluted 1:5000. The bound antibody was detected by treatment of the membrane with an HRP-conjugated rabbit anti-mouse IgG (1:10,000).

Incorporation of dansylcadaverine and dansyl-ε-aminocaproyl-QQIV into FnBPA by vWbp-activated FXIII. To investigate the covalent incorporation of synthetic probes dansylcadaverine and dansyl-ε-aminocaproyl-QQIV into FnBPA, mixtures containing 2 nM FnBPA, 200 nM ProT and 200 nM vWbp were incubated with 500 µg/mL FXIII in the presence of either 2 mM of dansylcadaverine or 2 mM of dansyl-PGGQQIV for 20 min at 37 °C in 20 mM TBS buffer, pH 7.4 containing 150 mM NaCl supplemented with 5 mM dithiothreitol and 5 mM CaCl₂. Reactions were stopped by addition of 2% SDS and 10% β-mercaptoethanol. Controls were performed incubating the above mixtures in the presence of 2 mM EDTA. Samples were analyzed by SDS-PAGE and gels examined under ultraviolet light and then stained with Bio-Safe Coomassie staining.

Statistical methods. Analyses were performed using Prism 4.0 (GraphPad). A minimum of three biological replicates (three independent experiments) were conducted for each experiment. Comparison of more than two groups were performed with the one-way ANOVA followed by Dunnett's test. The two-tailed Student's t-test was employed to compare two groups. P values < 0.05 were considered statistically significant, the following symbols are used, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

Data availability

The datasets generated or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

G.P. and P.S. conceived, designed and coordinated the study. C.M., A.P. and S.C. performed the experiments and analysed the data. G.B. and J.A.G. provided critical resources. C.M., A.P., G.B., J.A.G., P.S. and G.P. wrote the manuscript and prepared figures. All authors reviewed the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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RESEARCH ARTICLE

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Streptococcus pneumoniae binds collagens and C1q via the SSURE repeats of the PfbB adhesin

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Abstract

The binding of *Streptococcus pneumoniae* to collagen is likely an important step in the pathogenesis of pneumococcal infections, but little is known of the underlying molecular mechanisms. Streptococcal surface repeats (SSURE) are highly conserved protein domains present in cell wall adhesins from different *Streptococcus* species. We find here that SSURE repeats of the pneumococcal adhesin plasminogen and fibrinogen binding protein B (PfbB) bind to various types of collagen. Moreover, deletion of the *pfbB* gene resulted in a significant impairment of the ability of encapsulated or unencapsulated pneumococci to bind collagen. Notably, a PfbB SSURE domain is also bound to the complement component C1q that bears a collagen-like domain and promotes adherence of pneumococci to host cells by acting as a bridge between bacteria and epithelial cells. Accordingly, deletion of PfbB or pre-treatment with anti-SSURE antibodies markedly decreased pneumococcal binding to C1q as well as C1q-dependent adherence to epithelial and endothelial cells. Further data indicated that C1q promotes pneumococcal adherence by binding to integrin $\alpha_2\beta_1$. In conclusion, our results indicate that the SSURE domains of the PfbB protein promote interactions of pneumococci with various types of collagen and with C1q. These repeats may be useful targets in strategies to control *S. pneumoniae* infections.

1 | INTRODUCTION

Streptococcus pneumoniae (or the pneumococcus) is an encapsulated Gram-positive organism that is the main cause of community-acquired pneumonia and other frequent infections, such as otitis media and meningitis (Bogaert et al., 2004). Although pneumococci asymptomatically colonize the upper airways, they frequently shift from a commensal to a pathogenic condition within the host. It has been estimated that almost 1 million children below 5 years of age die from, and 15 million become ill with, pneumococcal disease

(O'Brien et al., 2009). The burden of this disease is also high in the elderly and patients with associated immunodeficiencies (Picard et al., 2003). Bacteria make use of various strategies to promote their adhesion to eukaryotic cells during colonization and pathogenesis. Most Gram-positive pathogens express cell-wall surface structures, which directly or indirectly enhance bacterial interactions with host tissues, including cell adherence and invasion (Kline et al., 2009). Extracellular matrix (ECM) components, such as collagens, fibronectin, vitronectin, fibrinogen, and plasminogen, represent fundamental targets for interactions of bacteria with host cells (Bingham

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et al., 2008; Binsker et al., 2017; Hammerschmidt et al., 2019; Singh et al., 2012).

Collagens, the most abundant components of ECM and the main constituents of connective tissues, are also frequently targeted by pathogenic microbes. Collagens consist of the association of three polypeptide α chains that give rise to several molecular isoforms. The 28 types of collagen identified so far include fibril-forming and network-forming collagens. Fibril-forming collagens, such as type I, II, and III, are assembled into triple-helical strands during connective tissue formation and wound healing. Network-forming collagens, such as type IV, VI, and X, determine the structure of basement membranes and are in direct contact with epithelial, endothelial, and muscle cells (Bella & Hulmes, 2017).

C1q, a component of the complement system, is an important part of innate immune defenses against invading pathogens (Ricklin et al., 2010). The C1q molecule has the shape of a flower bouquet and is formed by six identical trimeric proteins that associate to form the "stalk" and then diverge to form six "stems" each ending in a globular "flower head" (Agarwal & Blom, 2015). Each of the six proteins consists of three polypeptides whose collagen-like domains form a characteristic triple helix structure. The globular flower head has the function to bind antibodies as well as some acute-phase proteins, while the stalk binds to the C1r and C1s components of the complement system. The binding of the globular region to pathogen-bound antibodies or acute phase proteins induces a conformational change of C1r and activates the classical complement activation pathway (Agarwal & Blom, 2015). At least two surface proteins with C1q-binding properties have been identified in *S. pneumoniae*, namely the peptidoglycan-associated glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Terrasse et al., 2012; Terrasse et al., 2015) and pneumococcal endopeptidase O. Binding of the latter to C1q can promote bacterial adherence to and invasion of epithelial cells (Agarwal et al., 2013) and modulate activation (Agarwal et al., 2014).

Collagen-binding proteins of several species of staphylococci and streptococci have been studied in detail (Arora et al., 2021; Philominathan et al., 2012). Less is known about the ability of *S. pneumoniae* to bind collagen and of the functional significance of this interaction. This pathogen was found in an early study to bind collagen type I and IV in a strain-dependent manner (Kostrzynska & Wadstrom, 1992). Moreover, collagen VI has been proposed as a putative adhesive target for pneumococcal binding to respiratory epithelium, even though the capacity of surface pneumococcal adhesins to bind this type of collagen has been incompletely characterized (Bober et al., 2010). A few proteins with type I collagen-binding activity have been described in *S. pneumoniae*. The tip protein of the pneumococcal pilus-1, RrgA, has been demonstrated to bind to collagen I as well as laminin (Hilleringmann et al., 2008). Using atomic force microscopy, the pilus backbone protein RrgB was also found to bind to human collagen I in a manner influenced by the orientation of collagen fibrils (Becke et al., 2019). Moreover, DiiA (Dimorphic invasion-involved A), a novel pneumococcal dimorphic cell wall protein involved in invasive disease, is capable of binding collagen I

(Escolano-Martinez et al., 2016). However, the precise contribution of these proteins to the overall ability of pneumococci to bind collagen has not been assessed.

Streptococcal surface repeats (SSURE) have been described as 148–152 amino acid long tandem repeats in several species of streptococci (Bumbaca et al., 2004). *Streptococcus agalactiae* (group B streptococcus or GBS) expresses on its surface PbsP (standing for plasminogen-binding surface protein), a cell-wall protein bearing two SSURE domains (Buscetta et al., 2016). *S. pneumoniae* also expresses a protein named PfbB (plasminogen and fibronectin-binding protein B) (Papasergeri et al., 2010) or PavB (pneumococcal adherence and virulence factor B) (Jensch et al., 2010) containing 2 to 6 tandem SSURE domains (Figure 1a). This protein was previously identified as a significant target of antibody responses in patients with pneumococcal pneumonia and invasive infections (Beghetto et al., 2006). The C-terminal and core SSURE repeats of PfbB are >95% identical but differ from the N-terminal repeat (Figure 1a). SSURE-containing proteins endow streptococci with the ability to bind multiple ligands in the extracellular matrix. Group B streptococcal PbsP can bind to plasminogen and vitronectin (Buscetta et al., 2016; De Gaetano et al., 2018; Lentini et al., 2018) while PfbB can interact with plasminogen, fibronectin and thrombospondin via SSURE repeats (Binsker et al., 2015; Jensch et al., 2010; Kanwal et al., 2017; Papasergeri et al., 2010).

The present study was undertaken to better understand the molecular mechanisms underlying the interactions between pneumococci and collagen by focusing on the role of the SSURE-containing protein PfbB. We found here that the SSURE domains of PfbB can interact with various types of collagen and with C1q. Moreover, the binding of PfbB to C1q allows *S. pneumoniae* to adhere to and invade epithelial and endothelial cells by a mechanism involving the collagen-binding integrin $\alpha_2\beta_1$.

2 | RESULTS

2.1 | Optimal binding of *S. pneumoniae* to collagen requires PfbB

In initial experiments, we assessed the ability of the reference D39 pneumococcal strain to bind collagen I in comparison with fibronectin (Fn) and fibrinogen (Fbg), two ECM components that were previously shown to interact with these bacteria (Holmes et al., 2001; Speziale et al., 2019; Vassal-Stermann et al., 2014). In this assay, plates were sensitized with host proteins, incubated with pneumococcal cells, and bacterial adhesion was detected using mouse anti-pneumococcal serum. *S. pneumoniae*, strain D39, interacted with type I collagen with an adhesion efficiency that was intermediate between Fn and Fbg binding (Figure 1b). In addition, we found that the DP1004 strain, an unencapsulated D39 mutant, adheres to collagen I, Fn, and Fbg with moderately greater efficiency than the parental strain (Figure 1b), indicating that the presence of a capsule partially masks the interaction of bacteria with such ligands. We also tested

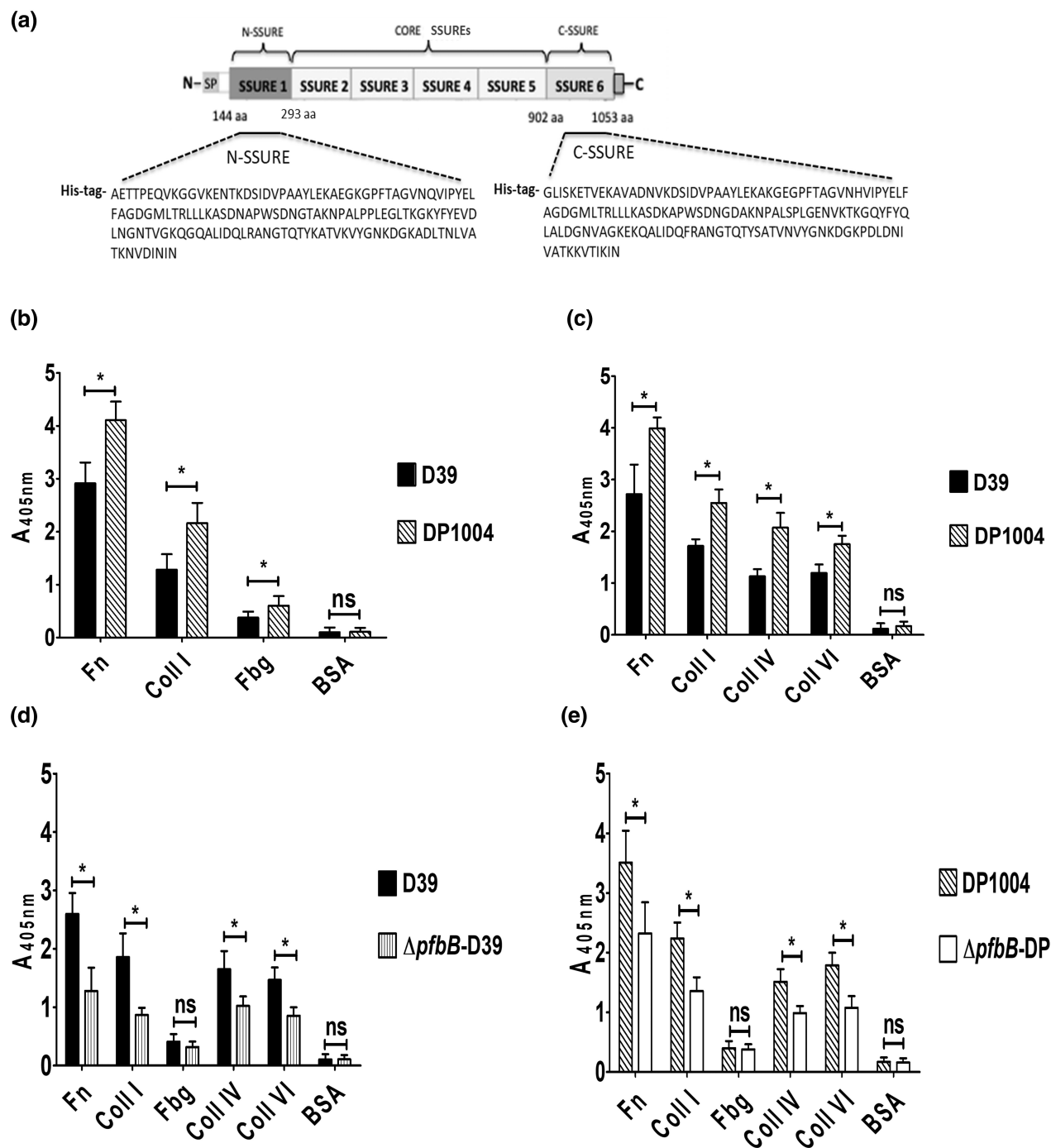


FIGURE 1 *S. pneumoniae* adhesion to collagens. (a) Schematic representation of the primary structure of PfbB, displaying a region (amino acid residues 144–1053) containing six SSURE repeats and including N-SSURE (N-terminal repeat or SSURE 1), CORE SSUREs (SSURE 2–5), and C-SSURE (C-terminal SSURE repeat or SSURE 6). N, N-terminus; SP, signal peptide; C, C-terminus; aa, amino acid. Shown are the amino acid sequences fused to a polyhistidine tail (his-tag) of the recombinant C-terminal SSURE (C-SSURE) and N-terminal SSURE (N-SSURE) domains used in this study. (b) Adhesion of encapsulated D39 and unencapsulated DP1004 strains to wells sensitized with fibronectin (Fn), fibrinogen (Fbg), type I collagen (Coll I), or bovine serum albumin (BSA), used as control. Bacterial adhesion was detected by ELISA using an anti-pneumococcus serum. (c) Adhesion of the same strains shown in (b) to collagen type I (Coll I), collagen type IV (Coll IV), collagen type VI (Coll VI), fibronectin (Fn), and bovine serum albumin (BSA), used as control. Bacterial adhesion was detected by ELISA using an anti-pneumococcus serum. Encapsulated D39 (d) and unencapsulated DP1004 (e) strains were compared to their respective isogenic *pfbB* deletion mutants ($\Delta pfbB$ -D39 and $\Delta pfbB$ -DP) for their ability to adhere to immobilized collagen type I (Coll I), collagen type IV (Coll IV), collagen type VI (Coll VI), fibronectin (Fn) and bovine serum albumin (BSA), used as control. Bacterial adhesion was detected by ELISA using an anti-pneumococcus serum. Shown are means $\pm$ SDs of three independent experiments conducted in triplicate. * $p < 0.05$ by Mann–Whitney test.

encapsulated and unencapsulated bacteria for adhesion to other collagen types, such as type VI (Bober et al., 2010), and type IV (Bella & Hulmes, 2017). Pneumococci bound efficiently to the different collagen types and the encapsulated strain showed a significantly reduced adhesion also to these ligands (Figure 1c). We next investigated the role of PfbB, a Fn-binding adhesin (Papaserghi et al., 2010), by analyzing the effects of *pfbB* deletion on pneumococcal binding to collagen. A $\Delta pfbB$ mutant (Papaserghi et al., 2010) was significantly impaired compared to the wild-type D39 parental strain in its ability to bind to various collagen types and Fn, used as a positive control, but not to Fbg (Figure 1d). Since surface expression of PfbB is partially masked in the encapsulated strain (Papaserghi et al., 2010), we also determined the impact of *pfbB* deletion in the unencapsulated strain. As expected, *pfbB* deletion in the background of the unencapsulated DP1004 strain also resulted in a significant reduction of collagen adhering ability (Figure 1e). Overall, these data indicate that the PfbB protein significantly contributes to the ability of pneumococci to bind to collagens.

2.2 | Role of PfbB in pneumococcal growth on collagen

Pneumococci can form biofilms in the upper respiratory tract of patients with recurrent infections, a process that is thought to be important in the pathogenesis of the pneumococcal disease (Domenech et al., 2012). Therefore, we investigated whether pneumococci could grow as biofilm-like aggregates on collagen-coated surfaces and whether the PfbB adhesin can play a role in the process. To this end, we measured surface-adherent growth in an assay in which bacteria were resuspended in a fresh culture medium and added to microtiter plates pre-coated with collagen type I. Pneumococci were then allowed to grow overnight at 37°C. Under these conditions, coating with collagen significantly promoted the growth of wild type D39 bacteria as surface-adherent aggregates, compared to BSA-coated control wells (Figures 2a,b). Moreover, bacteria lacking *pfbB* ($\Delta pfbB$ -D39) showed a 40% decrease in their

ability to form such aggregates on collagen-coated surfaces relative to the parental D39 strain (Figure 2a). Similarly, *pfbB* deletion significantly decreased the ability of the unencapsulated DP1004 strain to grow as sessile aggregates on collagen-coated wells (Figure S1). These data indicate that collagen promotes the growth of pneumococci as surface-adherent masses and that PfbB is at least partially involved in this process.

2.3 | SSURE antibodies inhibit pneumococcal binding to collagens

The PfbB protein of the D39 strain contains six SSURE domains that contribute to 78% of the protein structure. Therefore, it was of interest to explore the effects of SSURE-specific antibodies on bacterial adherence. To this end, we immunized mice with C-SSURE, a recombinant PfbB fragment encompassing the C-terminal SSURE domain (Figure 1a), which is >95% homologous to the four core repeats and tested the effects of immune sera on bacterial adherence. Pretreatment of encapsulated pneumococci with SSURE antibodies resulted in significant inhibition of their ability to bind to Fn as well as type I and IV collagen, but not to Fbg (Figure 3a). Collectively, these data suggest that the PfbB SSURE repeats are involved in the binding of pneumococci to collagen.

2.4 | *S. pneumoniae* SSURE domains bind to collagen and C1q

Keeping in mind the ability of SSURE antibodies to inhibit *S. pneumoniae* adherence to collagen, we investigated whether SSURE domains directly bind to collagen. In a previous study, we found that two isolated PfbB fragments, each containing one SSURE domain, bound weakly to type I collagen (Papaserghi et al., 2010). However, it could not be excluded from those data that the presence of a relatively bulky glutathione-S-transferase tag in the PfbB fragments interfered with such binding (Papaserghi et al., 2010). Therefore, we

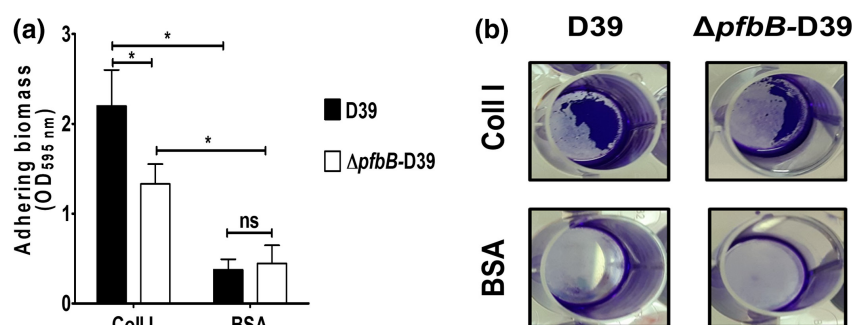


FIGURE 2 *S. pneumoniae* grows in the sessile form on surfaces coated with type I collagen by a mechanism involving PfbB. (a) Effect of *pfbB* deletion on sessile growth of the encapsulated D39 strain (D39) in wells coated with type I collagen (Coll I) or bovine serum albumin (BSA). $\Delta pfbB$, D39 *pfbB* deletion mutant. Columns indicate optical density at 595 nm (OD_{595nm}) of solubilized crystal violet. Shown are means $\pm$ SDs of three independent experiments conducted in triplicate. * $p < 0.05$ by the Mann-Whitney test. (b) Adhering aggregates (purple) of the encapsulated D39 strain and its isogenic *pfbB* deletion mutant ($\Delta pfbB$ -D39) before crystal violet solubilization.

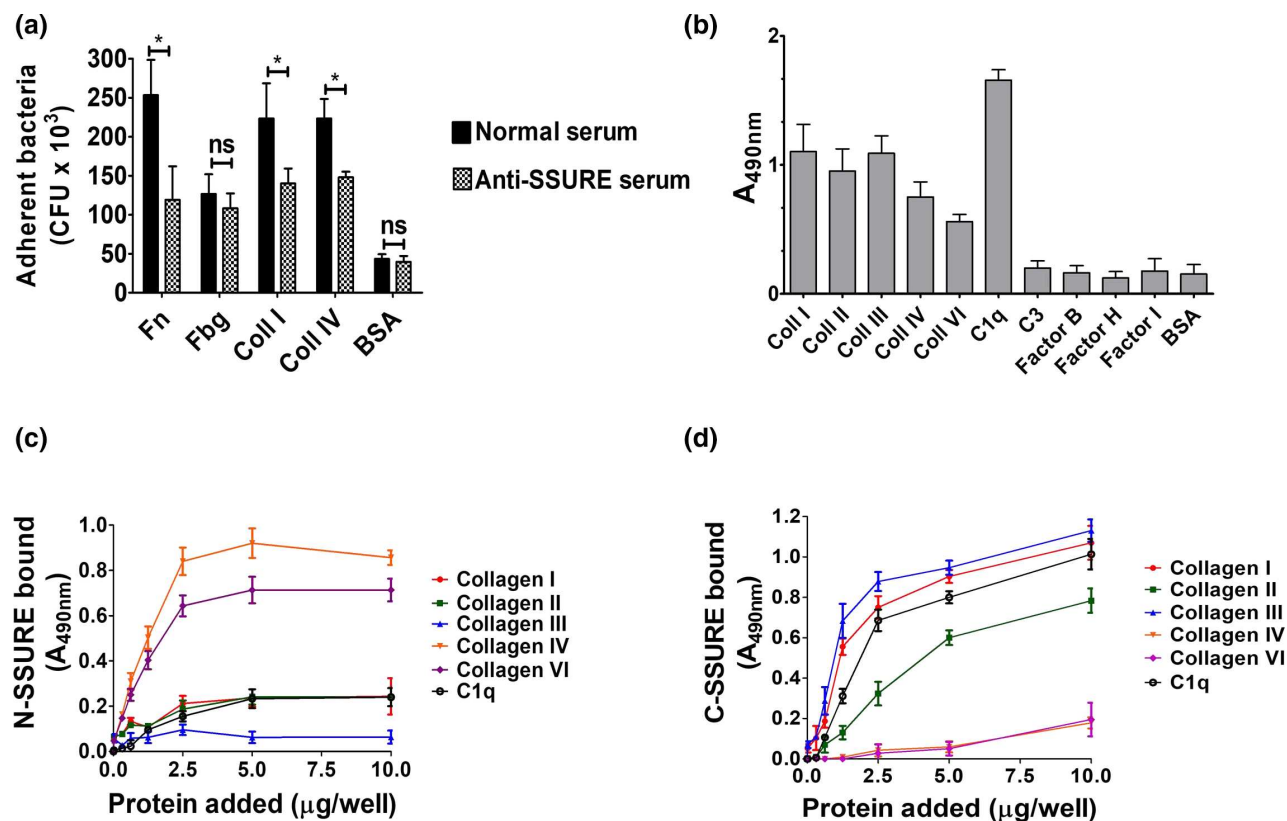


FIGURE 3 The C-terminal PfbB SSURE domain binds to collagens and C1q. (a) Inhibition of binding of D39 pneumococci to collagens after bacterial pretreatment with serum raised in mice against recombinant PfbB C-SSURE (anti-SSURE serum). Serum from unimmunized mice (Normal serum) was used as a control. Bacterial binding was detected by counting colony-forming units (CFU) after detachment of bacteria with trypsin. (b) Binding of histidine (his)-tagged recombinant PfbB C-SSURE to collagens and complement components. Binding was detected by ELISA using an anti-his antibody. Plates were sensitized with 5 μg/ml of collagen types I, II, III, IV, VI, complement protein 1q (C1q), complement protein 3 (C3), factor B, factor H, factor I or bovine serum albumin (BSA), used as control. (c and d) Dose-dependent binding of N-SSURE (c) and C-SSURE (d) domains to wells sensitized with 1 μg/ml of collagen type I, II, III, IV, VI and complement protein 1q (C1q). The binding of increasing concentrations of recombinant proteins fused to histidine (his) was revealed by ELISA using an anti-his antibody. Shown are means ± SDs of three independent experiments conducted in triplicate.

produced two His-tagged recombinant polypeptides encompassing the C-terminal and the N-terminal SSURE domains (C-SSURE and N-SSURE, respectively), of the PfbB protein (Figure 1a) and tested them for binding to collagens (types I, II, III, IV, and VI).

Since the complement system plays an important role in pneumococcal pathogenesis, we also tested the C-SSURE fragment for binding to various complement factors (C3 and factors B, H, and I). In these experiments, plates were coated with human proteins and binding of C-SSURE was detected by addition to the wells of a His tag antibody. Figure 3b shows that C-SSURE is efficiently bound to collagen I, II, and III. Notably, C-SSURE also efficiently bound C1q, but not the other complement components. To better define the binding activities of different SSURE domains, we tested graded doses of C-SSURE and N-SSURE, which differ significantly in amino acid sequence (Figure 1a), for their interactions with different types of collagens and C1q. Surprisingly, N-SSURE is efficiently bound to type IV and VI, but not to type I, II, or III collagens or to C1q, while the opposite was true for C-SSURE (Figure 3c,d). The binding of both recombinant fragments to their respective ligands was dose-dependent and saturable. Data from these titration ELISA experiments were then

used to calculate dissociation constants. C-SSURE bound collagen I, III, and C1q with dissociation constants of, respectively, 1.72 ± 0.2 , 1.16 ± 0.1 , and 5.21 ± 0.4 μM, while N-SSURE bound collagen IV and VI with dissociation constants of, respectively, 1.58 ± 0.1 and 1.20 ± 0.3 μM. Collectively, these data indicate that the two types of SSURE domains found in pneumococci bind to human collagens and C1q with an unexpected degree of specificity. C-SSURE more efficiently recognizes fibrillar collagens, such as type I and III, as well as C1q compared with other collagen types. Conversely, N-SSURE, which is only 73–75% identical to C-SSURE, binds network-forming collagens such as type IV and VI, but not fibrillar collagens or C1q.

2.5 | Pneumococci bind to C1q via PfbB

In view of the ability of the C-SSURE repeat to bind C1q, we assessed the contribution of the SSURE-containing PfbB protein to the overall ability of pneumococci to bind C1q. As previously reported (Agarwal et al., 2013), we first observed that wild type D39 bacteria recognize and bind to C1q in a dose-dependent manner (Figure 4a),

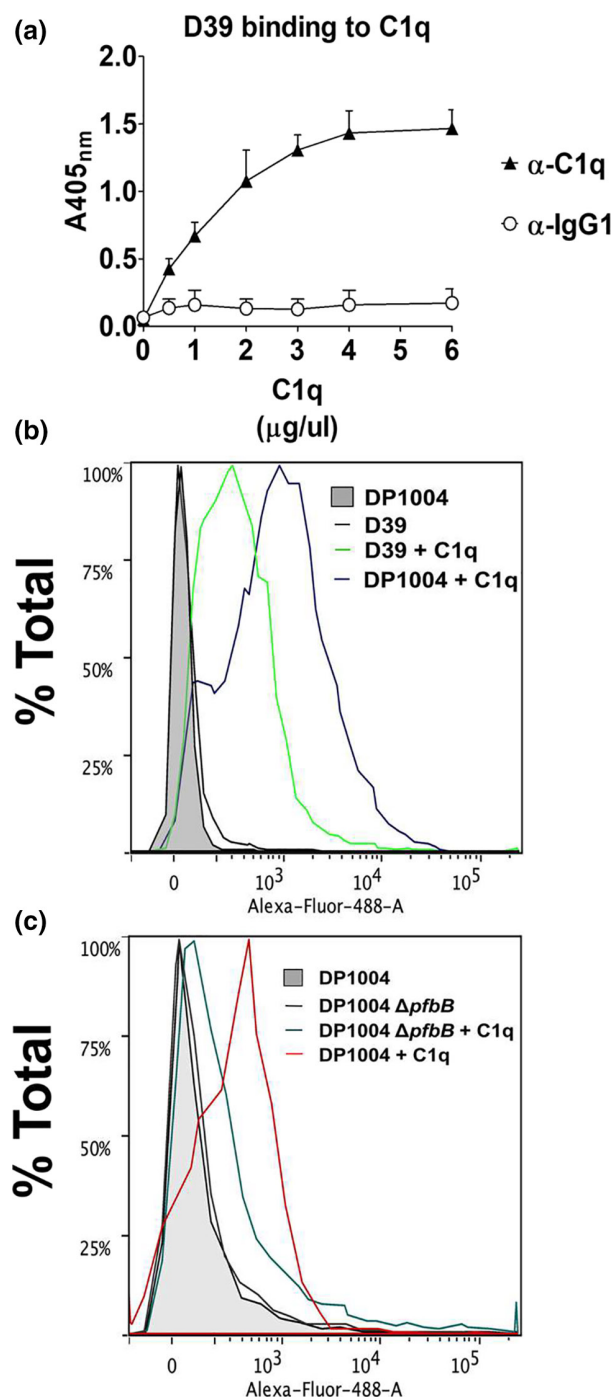


FIGURE 4 Role of PfbB in pneumococcal binding to C1q. The binding of C1q to pneumococci was measured by ELISA (a) or immunofluorescence flow cytometry (b and c). (a) Multi-well plates sensitized with pneumococci (5×10^7 CFU/ml) were incubated with increasing concentrations of C1q. Bound C1q was detected using anti-C1q antibodies (α -C1q). IgG1, isotype control. Shown are means $\pm$ SDs of three experiments performed in triplicate. (b) Binding of C1q (5 μ g/ml) to encapsulated D39 (green line) and unencapsulated DP1004 (purple) strains as detected by immunofluorescence flow cytometry using an Alexa-Fluor-488-labeled antibody. (c) Binding of C1q (5 μ g/ml) to unencapsulated DP1004 (red) and its isogenic *pfbB* deletion mutant (Δ *pfbB*-DP, dark green), as detected by immunofluorescence flow cytometry using an Alexa-Fluor-488-labeled antibody.

and that unencapsulated bacteria were more efficient in C1q binding relative to the unencapsulated DP1004 strain (Figure 4b). Notably, the Δ *pfbB*-DP deletion mutant was considerably impaired in C1q binding, with mean fluorescence intensity values that were considerably lower than those observed with the DP1004 unencapsulated parental strain (Figure 4c). All together, these data indicate that the PfbB protein plays a significant role in pneumococcal binding to C1q.

2.6 | Role of PfbB in C1q-mediated pneumococcal invasion of host cells

Pneumococcal adhesins may interact directly with host cell receptors or bind to host proteins that function as a bridge between the pathogen and host tissue. Previous studies have demonstrated that C1q bound to the pneumococcal surface promotes adherence to and invasion of host cells (Agarwal et al., 2013). Therefore, we investigated the role of the PfbB protein in promoting C1q-mediated interactions between pneumococci and host cells. In these experiments, we used two human epithelial cell lines, namely A549 (lung) and Hep-2 (larynx), as well as the brain microvascular endothelial cell line hCMEC/D3. In agreement with previous data (Agarwal et al., 2013), pretreatment of pneumococci with C1q considerably increased adherence of encapsulated wild type pneumococci to the cell lines tested (Figure 5a-c). Conversely, this effect was observed to a significantly lower extent or not at all using the Δ *pfbB*-D39 mutant (Figure 5a-c) or when pneumococci were pretreated with SSURE antibodies (Figure 5d,e). When the role of the PfbB protein in promoting C1q-mediated invasion was examined, the ability of encapsulated bacteria to invade microvascular endothelial cells as well as laryngeal or alveolar epithelial cells significantly increased in the presence of C1q. Interestingly, C1q was able to increase to similar degrees pneumococcal adhesion and invasion, indicating that C1q-mediated adherence is followed by bacterial internalization (Figure 6). The invasion-promoting activity of C1q was reduced in comparison with the parental strain when the *pfbB* mutant was tested (Figure 6a-c). A similar reduction of invasion was observed when pretreating wild type bacteria with C1q in the presence of SSURE antibodies (Figure 6d,e). Collectively, these data confirm previous studies indicating that, in the presence of C1q, pneumococci bind more efficiently to, and invade, epithelial and endothelial cells (Agarwal et al., 2014). Moreover, our data indicate that the ability of C1q to promote these interactions depends at least partially on the expression of PfbB on the bacterial surface.

2.7 | C1q promotes pneumococcal adherence by binding to the $\alpha_2\beta_1$ integrin

Previous studies have determined that the $\alpha_2\beta_1$ integrin interacts with C1q and collagen via its I domain (Merle et al., 2021). By this mechanism, C1q acts as a bridge between *Bacillus anthracis* spores and $\alpha_2\beta_1$ integrin expressed on the surface of respiratory cells to

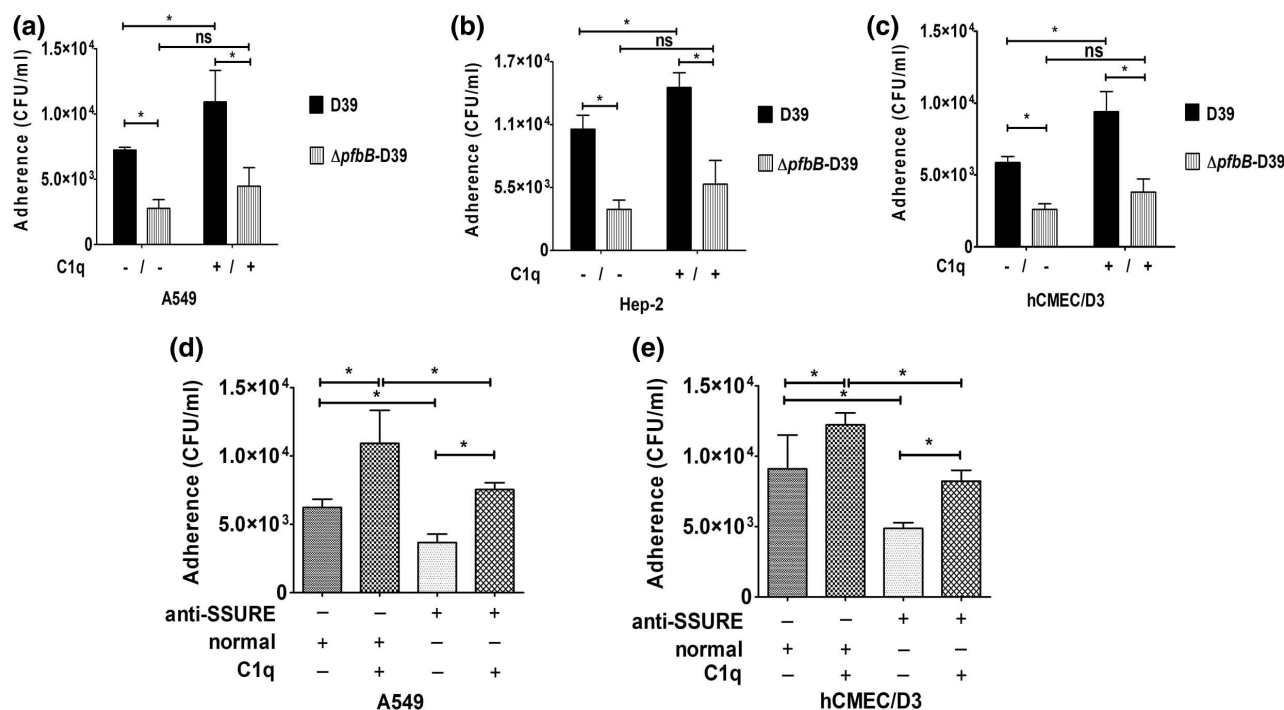


FIGURE 5 PfbB promotes C1q-dependent adhesion to epithelial and endothelial cells. (a–c) monolayers of respiratory epithelial (A549, hep-2) and brain microvascular endothelial (hCMEC/D3) cell lines were infected with pneumococcal strain D39 or its isogenic *pfbB* mutant in the presence (10 μ g) or in the absence of C1q. Adhering bacteria were measured by colony-forming units (CFU) counts. In some experiments (panels d and e) bacteria were pretreated with a pool of sera from animals immunized with C-SSURE recombinant repeat (anti-SSURE) or with a normal serum pool (normal). Shown are means $\pm$ SDs of three independent experiments conducted in triplicate. * $p < 0.05$ by Mann-Whitney test.

increase spore invasion of these cells (Xue et al., 2011). Therefore, it was of interest to ascertain whether C1q can also act as a bridge between the PfbB SSURE domains and the $\alpha_2\beta_1$ integrin thereby promoting pneumococcal adherence. We first analyzed the interactions between C1q, isolated SSURE domains, and recombinant $\alpha_2\beta_1$ integrin. C1q is readily bound to $\alpha_2\beta_1$ integrin, as expected (Figure 7a). The further addition of C-SSURE, but not N-SSURE, resulted in the formation of a ternary complex with C1q and $\alpha_2\beta_1$ integrin (Figure 7a). Next, we investigated the involvement of $\alpha_2\beta_1$ in C1q-mediated pneumococcal adherence using anti- $\alpha_2\beta_1$ antibodies. Figure 7b shows that pretreatment of epithelial cells with these antibodies, but not with control anti- $\alpha_v\beta_3$, markedly reduced C1q-mediated adherence to A549 pneumocytes. Notably, anti- $\alpha_2\beta_1$ or IgG pretreatment did not affect bacterial adherence in the absence of C1q, indicating that these integrins do not function as direct receptors for pneumococci. Collectively, these data suggest that the ability of C1q to increase pneumococcal interactions with epithelial cells is at least partially related to the formation of a ternary complex involving the SSURE domains of PfbB, C1q, and $\alpha_2\beta_1$ integrin.

3 | DISCUSSION

Collagens are the body's most abundant proteins across the animal world and are likely to have played an important role in vertebrate

evolution (Boot-Handford & Tuckwell, 2003). Not surprisingly, symbiotic bacteria have co-evolved various means to bind collagen, including the expression of specific cell-wall adhesins that endow them with the ability to colonize a variety of tissues and cause persistent infections (Nobbs et al., 2009). Relatively little is known of the ability of pneumococci and their surface proteins to bind collagen. Investigations have focused on the classical striated fibrillar collagen type I with the exception of one study showing strong interactions of pneumococci with collagen type VI, which is abundantly expressed in the subepithelial surfaces of the upper and lower respiratory airways (Bober et al., 2010). However, pneumococcal proteins capable of binding collagen type VI have not been identified. In pneumococci, the presence of type-1 pili has been associated with virulence and enhanced the ability to interact with epithelial and endothelial cells (Iovino et al., 2020). Proteins RrgA and RrgB were shown to be expressed along the entire pilus-1 surface (Hilleringmann et al., 2008) and to consecutively interlock with collagen type I fibrils, as shown using atomic force microscopy (Becke et al., 2019). However, pilus-1 is present in only 20–30% of pneumococcal clinical isolates.

We find here that SSURE domains from the PfbB protein, which is highly conserved among pneumococcal strains, can bind various types of collagen. Notably, we detected a considerable degree of selectivity in SSURE interactions with different collagen types. A recombinant fragment encompassing the C-terminal repeat, which is

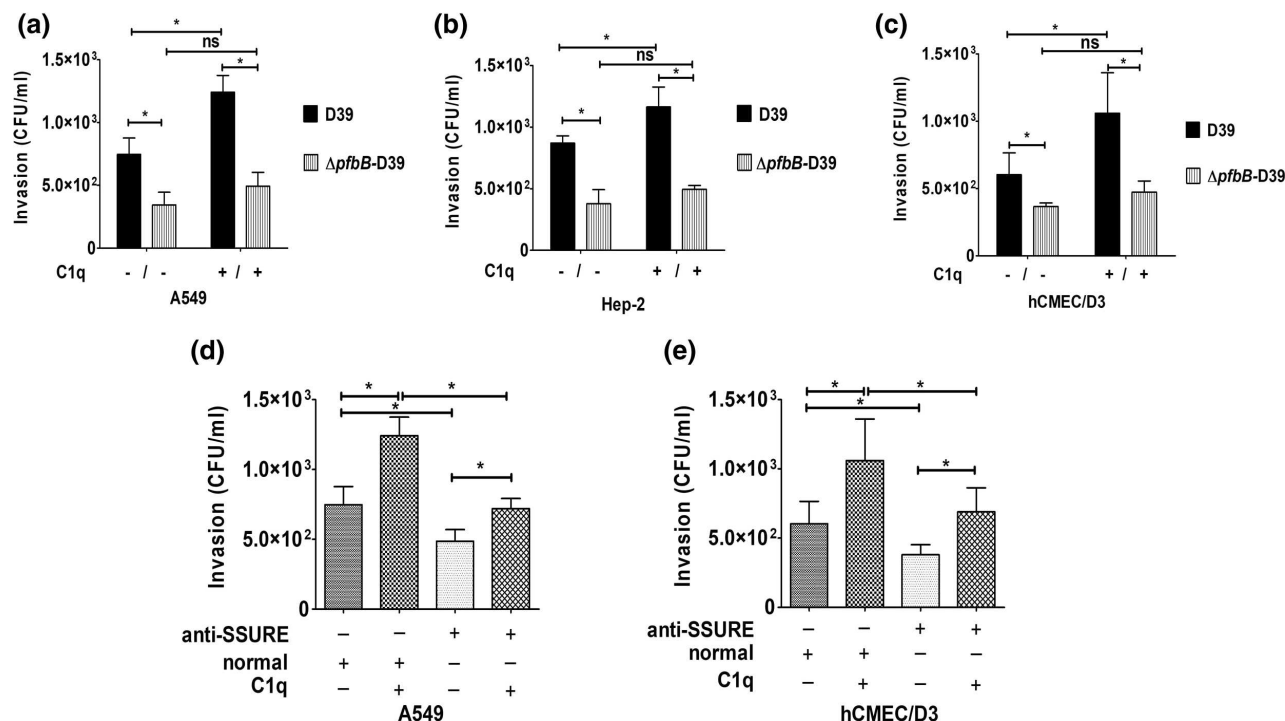


FIGURE 6 PfbB promotes C1q-dependent invasion of epithelial and endothelial cells. (a–c) monolayers of respiratory epithelial (A549, hep-2) and brain microvascular endothelial (hCMEC/D3) cell lines were infected with pneumococcal strain D39 or its isogenic *pfbB* mutant in the presence (10 μ g) or the absence of C1q. Intracellular bacteria were measured by colony-forming units (CFU) counts in an antibiotic protection assay. In some experiments (panels d and e) bacteria were pretreated with a pool of sera from animals immunized with the C-SSURE recombinant domain (anti-SSURE) or with a normal serum pool (normal). Shown are means $\pm$ SDs of three independent experiments conducted in triplicate. * p < 0.05 by Mann–Whitney test.

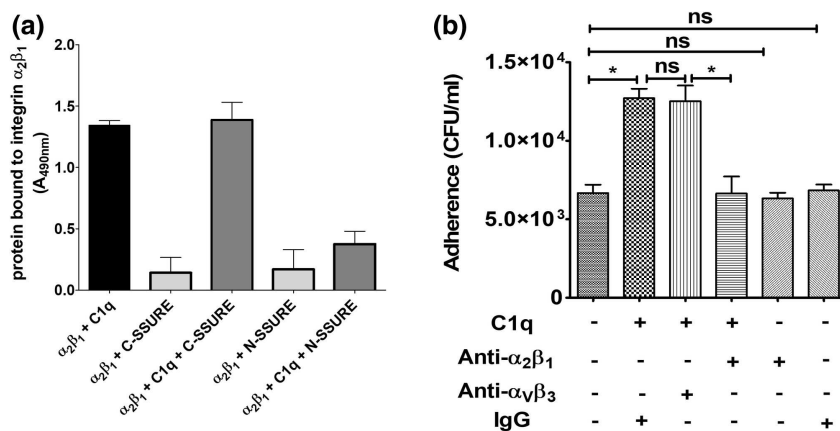


FIGURE 7 C1q acts as a bridge between the C-SSURE domain and $\alpha_2\beta_1$ integrin in mediating pneumococcal adhesion. (a) Interactions between C1q, recombinant SSURE domains (C-SSURE or N-SSURE), and $\alpha_2\beta_1$ -integrin in ELISA assays. Binding was detected with anti-C1q (black column) or anti SSURE antibodies. Shown are means $\pm$ SDs of three independent experiments conducted in triplicate. (b) Inhibitory effect of anti- $\alpha_2\beta_1$ antibodies on C1q-mediated adhesion. Monolayers of respiratory epithelial cells (A549) were pretreated with antibodies against $\alpha_v\beta_3$ or $\alpha_2\beta_1$ integrins before being infected with bacteria in the presence (10 μ g) or absence of C1q. Adhering bacteria were measured by colony-forming units (CFU) counts. Shown are means $\pm$ SDs of three independent experiments conducted in triplicate. * p < 0.05 by Mann–Whitney test.

>95% homologous to the core repeats, is bound more efficiently to fibril-forming collagens (types I, II, and III) than to network-forming collagens (type IV and VI). The latter was, in contrast, preferentially recognized by the N-terminal repeat, which differs at least by 25% or more in amino acid sequence from the other repeats. Recognition

of different collagen types by different SSURE domains, as shown here, seems to provide a good example of how bacteria acquire the ability to interact with an increasing number of ECM components by continuous duplication and variation of existing repeats rather than de novo protein creation. Moreover, repeat oligomerization can

increase the capacity of bacteria to interact with host molecules. Indeed, although the collagen-binding affinities of single SSURE domains are low (with dissociation constants in the micromolar range, as estimated here), the presence of multiple repeats is likely to increase PfbBB binding efficiency, as previously demonstrated for Fn and plasminogen binding (Jensch et al., 2010; Kanwal et al., 2017). Accordingly, PfbB deletion impaired the ability of encapsulated and unencapsulated pneumococci to interact with different collagen types, suggesting that this protein significantly contributes to the overall collagen-binding activity of these bacteria.

SSURE domains are present not only in pneumococci but also in other pathogenic and commensal streptococcal species, such as *S. agalactiae* and *S. gordonii* (Buscetta et al., 2016). Since the ECM binding ability varies among SSURE domains from different streptococcal species, it will be of interest to investigate in future studies whether SSURE domains from *S. agalactiae* and *S. gordonii* also bind collagen and whether they display selectivity for different collagen types, as shown here for PfbB SSUREs. Although the PfbB protein plays a significant role in the ability of pneumococci to adhere to collagen, other proteins also seem to contribute to this activity as suggested by the residual binding activities of PfbB-deletion mutants. DiiA is a highly conserved protein that efficiently binds to collagen type I (Escolano-Martinez et al., 2016). Moreover, while in the present study, we used a non-piliated reference strain, the RrgA and RrgB adhesins might make an important contribution to the ability of pilated strains to bind collagen, as mentioned above. Therefore, future studies should assess the relative contribution of PfbB, DiiA, and pilus-1 to the ability of pilated and non-piliated pneumococci to bind collagen. Currently available data suggest that adhesion of pneumococci to collagen involves the coordinated activity of different adhesins, in an analogous manner to adherence to fibrinogen of group B streptococci (Buscetta et al., 2014; Gutekunst et al., 2004; Papasergi et al., 2013). However, the structural basis of interaction with collagen of the above-mentioned pneumococcal adhesins, which differ in sequence and structural organization, remains to be determined.

Well-organized pneumococcal biofilms are frequently found in the nasopharynx of individuals with persistent pneumococcal carriage. Moreover, the propensity of single strains to form biofilms is inversely related to the amount of capsular material (Chao et al., 2015). Accordingly, we found here that the encapsulated D39 strain has little ability to grow as surface-adherent aggregates on plastic surfaces. This strain, however, did grow in the sessile form on collagen-coated wells, in a manner that was partially dependent on the expression of the PfbB protein. It will be of interest to determine whether sessile growth on collagen, as described here, has common features with in vivo biofilm formation (Chao et al., 2015) and with in vitro biofilm models that depend on the presence of competence stimulating peptide and its receptor (Oggioni et al., 2006). It will also be of interest to assess whether PfbB expression is increased in biofilms and promotes in vivo biofilm formation.

Our study shows that SSURE repeats from *S. pneumoniae* also bind C1q, the first component of the classical complement pathway.

This is reminiscent of collagen-binding proteins from other bacterial pathogens. Collagen adhesin (Cna) from *Staphylococcus aureus* (Valotteau et al., 2017) and Cna-like proteins make up a large family of structurally related virulence factors from Gram-positive (Kreikemeyer et al., 2005; Krishnan & Narayana, 2011; Patti & Hook, 1994; Sillanpaa et al., 2009; Xu et al., 2004) and Gram-negative (Fink et al., 2002; MacKichan et al., 2008; Patti et al., 1994; Wagner et al., 2007) bacteria. These proteins bind both collagen and C1q and inhibit the classical complement activation pathway (Kang et al., 2013). Interestingly, Cna-like proteins are found in streptococcal species, such as *Streptococcus mutans*, *Streptococcus equi*, *Enterococcus faecalis*, and *Enterococcus faecium*, that do not possess SSURE-containing proteins (Kang et al., 2013; Sillanpaa et al., 2009). Studies are underway to ascertain whether SSURE repeats from *S. gordonii*, *S. mitis*, and *S. agalactiae* bind C1q and inhibit, by these means, the classical complement activation pathway.

In the present study, we focused on another recently described functional feature of C1q, namely its ability to promote pneumococcal adherence to and invasion of host cells. Notably, functionally active complement components have been detected in airway secretions (Sarma et al., 2006). In this scenario, pneumococci can recruit C1q on their surface and use it as a bridge to interact with epithelial and endothelial cells (Agarwal et al., 2013; Agarwal et al., 2014). In a study performed by Xu et al. (2004), it was found that *Bacillus anthracis* spores interact via the LPXTG surface protein BclA with C1q, which in turn binds to the $\alpha_2\beta_1$ integrin on host cells and promotes by this mechanism spore entry into epithelial cells (Xue et al., 2011). Thus, it was of interest to ascertain whether a similar mechanism is involved in the pneumococcal invasion of respiratory epithelial cells. This seems to be the case, as shown here by the formation of a ternary complex comprising C-SSURE, C1q, and $\alpha_2\beta_1$. SSURE domains were, however, unable to directly interact with this integrin. Moreover, pretreatment of respiratory epithelial cells with anti- $\alpha_2\beta_1$ largely prevented C1q-dependent, but not C1q-independent, pneumococcal adherence. These data indicate that, albeit not a primary or direct receptor for pneumococci, $\alpha_2\beta_1$ integrin is involved in adherence and subsequent entry of these bacteria in the presence of C1q. Therefore, our data add pneumococci to the growing list of pathogens that target $\alpha_2\beta_1$ integrin for entry into epithelial cells, including group A streptococci, *B. anthracis*, echovirus 1, and rotavirus (Bergelson et al., 1992; Caswell et al., 2007; Ciarlet et al., 2002; Xue et al., 2011). Our data do not exclude, of course, the possibility that C1q bound to the surface of pneumococci interacts with receptors other than $\alpha_2\beta_1$, such as CD91 and gC1q-R (receptor for the globular head of C1q; Ogden et al., 2001), a well-characterized bacterial internalization receptor (Braun et al., 2000). Finally, it should be noted that other proteins, such as the pneumococcal PepO protein that also binds C1q, can participate in C1q-mediated interactions with host cells (Agarwal et al., 2014). This notion is supported by our data showing that anti-SSURE antibodies were unable to completely block C1q-mediated adherence and internalization.

In conclusion, our data indicate an important role of the PfbB SSURE domains in promoting interactions of pneumococci with

collagens, C1q and host cells. Since the colonization of epithelial cells and invasion of collagen-rich subepithelial tissues are essential steps in pneumococcal pathogenesis, these data may be useful to devise alternative strategies to control infections caused by antibiotic-resistant strains.

4 | MATERIALS AND METHODS

4.1 | Bacterial strains and reagents

The encapsulated strain D39, serotype 2, (Iannelli et al., 1999; Shoemaker & Guild, 1974), and its unencapsulated DP1004 derivative (Iannelli et al., 2004) were used in this study. Deletion of the *pfbB* gene was accomplished by transformation of D39 or DP1004 strains, as described previously (Papaseri et al., 2010), yielding strains $\Delta pfbB$ -D39, and $\Delta pfbB$ -DP. All bacteria were grown in Todd-Hewitt broth (Difco Laboratories) supplemented with 0.5% yeast extract (THY, Thermo Fisher Scientific) at 37°C in 5% CO₂. We used in this study human fibronectin (Sigma-Aldrich; F0895), human fibrinogen (Fluka Analytical; 46,313), and C1q (Calbiochem; 204,876). Type I and III collagens from calfskin were generously donated by Dr R. Tenni, Department of Molecular Medicine, University of Pavia, Italy. Collagen type II was prepared from bovine nasal cartilage according to Stawich and Nimni (Stawich & Nimni, 1971). Human type IV and VI collagens were from Sigma-Aldrich. Complement Factors B, H, and I were purchased from R&D (Minneapolis MN, USA).

4.2 | Recombinant PfbB SSURE fragment and antisera

In the present study, we used two recombinant fragments designated C-SSURE and N-SSURE encompassing the C-terminal and N-terminal SSURE domains of the PfbB protein, respectively. C-SSURE is a recombinant polyhistidine-tagged polypeptide encompassing amino acids 902–1053 of the PfbB protein, while N-SSURE is an identically tagged polypeptide encompassing amino acids 144–293 (Figure 1a). The encoding DNA fragments were cloned from genomic *Streptococcus pneumoniae* D39 DNA into the pET21b vector (Thermo Fisher Scientific, Milan, Italy). The C-SSURE encoding genomic fragment was amplified using the primers c-ter_up: 5'-AAACATATGGGTTAATTTCTAAAGAACTGTCGAAAAAG-3' and c-ter_low: 5'-AGTGCTCGAGTTCTTAACATTATCTTAATAG-3'. The N-SSURE encoding genomic fragment was amplified using the primers N-ter_up: 5'-AAACATATGGCAGAACTACTCTGAAC-3' and N-ter_low: 5'-AGTGCTCGAGATTGATGTTGATGTCTACATT-3'. Both fragments were cloned into the *Xho*I and *Nhe*I restriction sites of pET21b. The recombinant plasmids were used to transform *E. coli* BL21(DE3) bacteria that were grown in the Luria Bertani medium (LB; Life Science) at 37°C with ampicillin (100 µg/ml) until their mid-log phase (OD_{560nm} = 0.6). Next, the bacteria were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich;

16758) under shaking for 18 h at 30°C. The bacteria were then washed with phosphate-buffered saline (PBS: 137 mM NaCl; 2.7 mM KCl; 10 mM Na₂HPO₄; 1.8 mM KH₂PO₄), resuspended in lysis buffer (PBS; 100 µg/ml lysozyme, Fluka Analytical, 62,970; 25 mM imidazole, Sigma-Aldrich, I5513; 1 tablet per 50 ml of complete, EDTA-free protease inhibitor cocktail, Roche Diagnostics, 11,873,580,001) and lysed using a sonicator (MSE Soniprep 150, 4 1-min bursts). Both recombinant SSURE fragments were purified from lysate supernatants using a nickel affinity column (HisTrap HP; GE Healthcare, Italy). The purity of these preparations was assessed by polyacrylamide gel electrophoresis (Figure S2a). Anti-SSURE serum was produced by immunizing 6-week-old specific pathogen-free CD1 mice (Charles River Laboratories, Italia) by the intraperitoneal injection of 50 µg of the C-SSURE fragment in alum on days 0, 14, and 28. Sera were collected 15 days after the last immunization. The PfbB-specificity of SSURE sera was verified in ELISA assays by observing their lack of reactivity against bacteria deleted for *pfbB* (Figure S2b). To obtain anti-pneumococcus serum, mice were immunized using 50 µg of a crude pneumococcal extract obtained as previously described (Papaseri et al., 2010). Immunizations were conducted according to the European Union guidelines for the handling of laboratory animals and to the Italian law, as detailed below under "Ethics Approval."

4.3 | Bacterial binding to immobilized host proteins

Bacterial adhesion to extracellular matrix components was determined by ELISA assays. For this purpose, 96 well of Nunc MaxiSorp flat-bottom plates (Thermo Fisher Scientific; 44-2404-21) were sensitized at 4°C overnight with different substrates (5 µg/well) dissolved in 0.05 M carbonate buffer (pH 9.5). After blocking with 5% BSA in Tris-Buffered Saline pH 7.5 (TBS; 50 mM Tris-Cl; 150 mM NaCl), pneumococci (10⁷/well) were added to the plates and incubated for 2 h at room temperature (RT). After washing, pneumococcal binding to the wells was detected with anti-pneumococcus serum diluted 1:500 in TBS-1% milk and incubated for an additional 1 h at 37°C. To detect antibody binding, anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich; A7434) diluted 1:2000 in TBS-1% milk was added and left for 1 h at 37°C. After the addition of para-phenyl phosphate, absorbance at 405 nm was determined in an ELISA plate reader. In some experiments, bacterial attachment to host extracellular matrix components was assessed by colony-forming units (CFU) counting adherent pneumococcal cells. To this end, pneumococci were preincubated for 15 min at 37°C with sera and then added to substrate-coated-wells and incubated for 1 h at 37°C in 5% CO₂. After extensive washing, the wells were treated with trypsin (2.5 mg/ml; Sigma-Aldrich) for 10 min at 37°C in 5% CO₂ to release the attached bacteria, which were enumerated on agar plates.

4.4 | Growth on collagen-coated surfaces

Pneumococci were grown in THY broth to the early exponential phase (OD_{600nm} ~ 0.3), diluted 1:5 in fresh THY medium, and added

to 6-well polystyrene flat-bottom plates that had been coated with human collagen type I (5 µg/ml) or BSA (5 µg/ml), used as control. Plates were incubated at 37°C in 5% CO₂ for the following 16 h. Unattached bacteria were then gently decanted from the wells that were carefully washed with PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) and air-dried. Adherent bacteria were stained for 15 min with a 0.1% (w/v) solution of crystal violet (Sigma-Aldrich). After rinsing with PBS, the bound dye was released from stained cells using ethanol/acetone 80:20 (vol/vol) and quantified by measuring A_{595 nm} values.

4.5 | C-SSURE binding to human collagens and complement factors

To test the binding of the recombinant C-SSURE and N-SSURE fragments to immobilized substrates, wells were coated overnight at 4 °C in 0.1 M sodium carbonate, pH 9.5, with the indicated concentrations of human collagens or complement factors. The plates were washed three times with 0.5% (v/v) Tween 20 in PBS. To block additional protein binding sites, the wells were treated for 1 h at 22 °C with 2% (v/v) BSA in PBS. Increasing concentrations of C-SSURE or N-SSURE (0.016–10 µg/ml) were then added to the wells. Binding was detected by the addition of a mouse monoclonal antibody directed against the His-tag (Sigma-Aldrich) in 1% BSA-PBS for 1 h at RT, followed by a 45 min incubation with anti-mouse IgG horseradish peroxidase-conjugated antibody. Absorbance at 490 nm was determined after the addition of 3, 3', 5, 5'-tetramethylbenzidine or o-phenylenediamine dihydrochloride (Sigma-Aldrich) as a substrate.

4.6 | Complex formation with α₂β₁ integrin

The binding of 1 µg of C1q to surface coated α₂β₁ integrin (R&D systems; 250 ng/well) was detected by the addition of rabbit anti-human C1q IgG (1:1000) followed by HRP-conjugated IgG secondary antibodies as described above. Binding of SSURE fragments to the integrin was detected using mouse monoclonal anti-His IgG (1:1000) followed by rabbit HRP-conjugated anti-mouse IgG, as described above. To assess ternary complex formation between α₂β₁ integrin, C1q, and SSURE domains, microtiter wells coated with 250 ng of α₂β₁ integrin were incubated with 1 µg of C1q for 1 h followed by incubation with 1 µg of either C-SSURE or N-SSURE. After 1 h, the wells were incubated with a mouse monoclonal anti-His IgG followed by rabbit HRP-conjugated anti-mouse IgG as described above.

4.7 | Analysis of C1q binding to bacteria

To investigate the binding of C1q to pneumococci, bacteria were grown in THY broth to the early exponential phase (OD_{600 nm} ~0.3),

washed in TBS, and distributed in 96-well plates (5 × 10⁷ CFU/well). Afterward, wells were blocked with TBS-2% milk and incubated with the indicated doses of human recombinant C1q (Calbiochem; 204,876) for 1 h at 37°C. Cell-surface bound C1q was revealed with a mouse anti-C1q monoclonal antibody (Abcam; ab71940) diluted 1:100 in TBS-1% milk followed by incubation with AP-conjugated anti-mouse IgG (Sigma-Aldrich; A7434).

For flow cytometry immunofluorescence analysis, bacteria were incubated with recombinant human C1q (5 µg/ml) for 1 h at room temperature in constant agitation. Washed bacteria were incubated with anti-C1q antibody (Abcam; ab71940) diluted 1:100 in 1% milk-PBS for 1 h at room temperature in constant agitation. Afterward, to detect the binding of C1q to the bacteria, an Alexa-Fluor-488-labeled anti-mouse antibody was used. Bacteria were analyzed with a Flow Cytometer (BD FACS canto II) using the Flowlogic software.

4.8 | Bacterial adhesion and invasion assays

The human cell lines A549 (type II alveolar epithelial cells, ATCC CCL-185), HEp-2 (laryngeal epithelial cells, ATCC CCL-23), and hCMEC/D3 (brain microvascular endothelial cells, kindly provided by P.O. Couraud, INSERM, Paris, France) were grown in, respectively, F-12 medium (ATCC 30-2004) and Eagle's Minimum Essential Medium (ATCC 30-2003) with 10% (vol/vol) fetal bovine serum (1203C, Sigma-Aldrich) and Endothelial Cell Medium 2 (C-22011, Promo Cell), supplemented with SupplementMix (C-39216, Promo Cell), at 37 °C in a humidified 5% CO₂ incubator.

For adherence and invasion assays, cells were dispensed into 12-well plates at a density of 5 × 10⁴/well and grown in 5% CO₂ for 48 h before the assay. The monolayers were then washed three times with Dulbecco's PBS (DPBS; Euroclone) to remove any residual medium. Bacteria were grown to the early log phase (OD_{600 nm} ~0.3), washed, resuspended in RPMI without FBS, and applied to the monolayers at a multiplicity of infection of 30. For adherence assays, infected monolayers were incubated for 1 h at 37°C in 5% CO₂ and washed three times to remove non-adherent bacteria. In preliminary experiments, we determined that, under these conditions, there is no significant bacterial internalization. For studying C1q-mediated pneumococcal adhesion, bacteria were incubated for 20 min with 10 µg human recombinant C1q in a total volume of 100 µl at 37°C in 5% CO₂ prior to addition to the monolayers. Post-infection, cells were washed three times with DPBS to remove unbound bacteria. After the addition of cold H₂O and scraping, cell lysates were serially diluted and plated onto agar plates for colony counting. For invasion assays, after 1 h of adhesion, monolayers were washed and further incubated for 1 h with a medium containing bactericidal amounts of penicillin and streptomycin (200 U/ml and 200 µg/ml, respectively), as previously described. Adherence and invasion values were expressed as the number of cell-associated or invading bacteria. Blocking antibodies against integrin α₂β₁ and α_vβ₃ and control non-immune

antibodies were obtained in rabbits as previously described (De Gaetano et al., 2018). To assess the effects of blocking antibodies on pneumococcal adherence, cells were pre-incubated for 1 h with the appropriate antibodies diluted 1:1000 in a cell culture medium and then incubated with bacteria.

4.9 | SSURE immune sera

To obtain sera, mice were immunized at the animal facilities of the Department of Pathology of the University of Messina, according to the European Union guidelines for the handling of laboratory animals. The procedure was approved by the local animal experimentation committee (OPBA) and by national authorities (Ministero della Salute, permit no.-785/2018-PR).

4.10 | Statistical analysis

All experiments were repeated at least three times and the data were expressed as means $\pm$ standard deviations (SD). Data were analyzed for statistical significance by the Mann-Whitney test. A p-value of 0.05 was used as the threshold for significance.

AUTHOR CONTRIBUTIONS

Concetta Beninati, Pietro Speciale, Giuseppe Teti and Giampiero Pietrocola conceived the study. Giuseppe Valerio De Gaetano, Giampiero Pietrocola, Germana Lentini, Agata Famà, Concetta Beninati and Giuseppe Teti designed the experiments. Giuseppe Valerio De Gaetano, Germana Lentini, Agata Famà, Chiara Cullotta, Ivana Raffaele, Francesco Coppolino and Chiara Motta carried out the experiments and analyzed the data. Concetta Beninati, Giuseppe Teti and Giuseppe Valerio De Gaetano wrote the paper. All authors gave approval to the final version of the paper.

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CONFLICT OF INTEREST

Concetta Beninati and Giuseppe Teti act as scientific advisors for, respectively, Scylla Biotech Srl. and Charybdis Vaccines Srl. without receiving any compensation for these activities. Charybdis Vaccines S.r.l. and Scylla Biotech S.r.l. did not provide funding for this study and had no role in its conduction. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICS APPROVAL

The procedure for obtaining serum samples from immunized animals was approved by the local animal experimentation committee

(OPBA) and by the relevant national authority (Ministero della Salute, permit no.-785/2018-PR). Human samples, except for cell lines, were not used in this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Review

Colonization and Infection of Indwelling Medical Devices by *Staphylococcus aureus* with an Emphasis on Orthopedic Implants

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Abstract: The use of indwelling medical devices has constantly increased in recent years and has revolutionized the quality of life of patients affected by different diseases. However, despite the improvement of hygiene conditions in hospitals, implant-associated infections remain a common and serious complication in prosthetic surgery, mainly in the orthopedic field, where infection often leads to implant failure. *Staphylococcus aureus* is the most common cause of biomaterial-centered infection. Upon binding to the medical devices, these bacteria proliferate and develop dense communities encased in a protective matrix called biofilm. Biofilm formation has been proposed as occurring in several stages—(1) attachment; (2) proliferation; (3) dispersal—and involves a variety of host and staphylococcal proteinaceous and non-proteinaceous factors. Moreover, biofilm formation is strictly regulated by several control systems. Biofilms enable staphylococci to avoid antimicrobial activity and host immune response and are a source of persistent bacteremia as well as of localized tissue destruction. While considerable information is available on staphylococcal biofilm formation on medical implants and important results have been achieved on the treatment of biofilms, preclinical and clinical applications need to be further investigated. Thus, the purpose of this review is to gather current studies about the mechanism of infection of indwelling medical devices by *S. aureus* with a special focus on the biochemical factors involved in biofilm formation and regulation. We also provide a summary of the current therapeutic strategies to combat biomaterial-associated infections and highlight the need to further explore biofilm physiology and conduct research for innovative anti-biofilm approaches.

Keywords: indwelling medical devices; *Staphylococcus aureus*; biofilm; infection



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1. Introduction

Advances in surgery and biomaterials science have provided us with a wide range of artificial body parts that can be used to replace/repair their natural counterparts [1–5]. The absolute number of implant-related infections has been documented as steadily growing for the aging population. This is particularly true in orthopedics as the prevalence of osteoarthritis, the principal cause of joint diseases and implant surgery, increases indefinitely with age. As recalcitrant to antibiotic therapy and surgical curettage, infection is a serious complication of implant orthopedic surgery, often leading to implant failure [6]. Moreover, it must be mentioned that each substitution of a failed prosthesis involves a much higher risk of relapse (up to 30%) than that for primary surgery. A number of medical procedures involve the insertion of a variety of catheters through the skin or orifices, and other clinical

areas are fertile grounds for the application of biomaterials. Biomaterials may be natural or synthesized in the laboratory using a variety of chemical approaches depending on what they will be used for. Naturally derived biomaterials can be classified in at least two groups including protein-based (collagen, gelatin, silk) and polysaccharide-based biomaterials (cellulose, chitin/chitosan). Synthetic biomaterials are generally grouped into metals, ceramics, plastic, glass and polymers. Furthermore, significant research has investigated creating composites of these materials to combine their benefits [7]. Biomaterials can be functionally passive, such as those used for a heart valve, or may be bioactive with a more interactive functionality such as hydroxyapatite-coated hip implants [8].

On the one hand, biomedical implants have revolutionized medicine and opened new avenues in human health progress, but they have also increased the risk of infection of body tissues. In fact, while in the absence of a foreign implant, body contamination by an opportunistic pathogen is usually cleared by host immune defenses, the implantation of a medical device exposes the body to the risk of a permanent colonization and potential replacement [9,10].

Bacterial adhesion is the first step of biomaterial-associated infections. Staphylococci are the most frequent causes of device-associated infections, with *Staphylococcus aureus* and *Staphylococcus epidermidis* representing the leading species of contamination [11]. This successful action is due to the large variety of virulence factors expressed by these pathogens [12,13]. In many circumstances, indwelling medical devices are contaminated by bacteria during surgery. The source can be the skin or other colonized body sites of the patients or the health care workers. In an alternative route, the already implanted devices can be infected via hematogenous seeding. Moreover, immunocompromised patients, such as AIDS patients or premature newborns, are at increased risk of developing infections when they receive a medical implant.

The ability of *S. aureus* to promote the formation of a three-dimensional structure composed of the bacteria and extracellular biopolymers (polysaccharides and/or proteins) referred to as biofilm is central to the biomedical infection process [14]. Biofilm can be defined as a microbial community of bacteria that is attached to a substratum and embedded in a matrix that they have produced. A prerequisite for biofilm formation is the coating of medical devices with plasma proteins [6,14]. Bacterial cells in a biofilm exhibit a phenotype that is different in terms of growth rate and gene transcription with respect to that exhibited by planktonic bacteria. The physiological features and structure of bacteria in biofilm may explain the increased resistance to antimicrobial agents and less susceptibility to biological attacks by the immunological defenses of the host [14,15]. In continuity with our previous report [6], in this review we focus on the types of biomedical implants, with a special emphasis on the orthopedic materials, and update the role of adhesive plasma proteins in coating biomaterials and the function of *S. aureus* virulence factors in the colonization and infection of indwelling medical devices. We also discuss in more detail the structure and function of biofilm as an important means against immune evasion of the host. Finally, we illustrate the potential strategies to prevent and therapeutically treat biomaterial-associated infections by *S. aureus*. We believe that the holistic approach implemented in this study where microbiological, biochemical, and material science data were integrated may offer a useful cultural framework for microbiologists, experts in biochemistry and clinicians.

2. Most Used Medical Devices: Chemical Composition, Applications and Infection

Medical devices particularly prone to *S. aureus* infection include, in addition to prosthetic joints, central venous catheters, urinary catheters, endotracheal tubes, mechanical heart valves, pacemakers and contact lenses.

Prosthetic joints. A variety of materials have been used for orthopedic implants. High-molecular-weight polyethylene is the main polymer used for artificial joints, and it is widely used as an acetabular material component due to its superior biocompatibility, non-toxicity and high impact strength. Due to their good chemical stability, excellent resistance to corrosion and reliable long-term behavior, alumina and zirconia ceramics

have been extensively used in total joint bearings [16]. Silicon carbide, classified as a non-oxide ceramic, has higher strength and hardness characteristics than those of alumina. Even better, stainless steel, Co-based alloys and titanium and its alloys, due to their good elasticity, superior biocompatibility and wear resistance, are the most widely used metallic materials in orthopedic replacement [17,18]. Polymethyl methacrylate (PMMA) is the well-known orthopedic fixation cement. It has been used extensively over the last 50 years to make stable joint implants (mainly hip or knee) or as a bone filler. The main function of an orthopedic biomaterial is to bear load, also providing structural integrity and stability to the human body. The nanocoating of metals and alloys are promising orthopedic materials for controlling protein adsorption and tissue integration at the bone-implant interface, improving implant functionalities, and strengthening mechanical properties of orthopedic implants. *S. aureus* and the coagulase-negative staphylococci are the major cause of prosthetic joint infection [19]. Hematogenous seeding or surgery interventions of arthroplasty, where the articular surface of a musculoskeletal joint is remodeled or replaced, can cause the infection of the orthopedic device by *S. aureus*, the development of bacterial macroscopical biofilm-like clumps in the synovial fluid present in the joints and consequent recalcitrance of infection to antibiotics [20].

Mechanical heart valves. Mechanical heart valves are made of materials such as titanium and carbon and are commonly used to replace aortic and mitral valves damaged with age, by certain diseases or congenital abnormalities. They usually consists of two leaflets and a metal ring surrounded by a ring of knitted fabric, which is sewn onto the heart in place of the original valve. Both *S. epidermidis* and *S. aureus* form biofilms on mechanical heart valves and the surrounding cardiovascular tissues, and in some cases this can result in serious diseases such as prosthetic valve endocarditis [21–23].

Central venous catheters (CVCs). CVCs are used to consign blood products, nutrients and medications, as well as facilitating dialysis treatment. Virtually all current central venous catheters (particularly long-term catheters) are made of silicone rubber or polyurethane. The internal jugular vein, common femoral vein and subclavian veins are the preferred sites for temporary central venous catheter placement, while for mid-term and long-term central venous access, the basilic and brachial veins are utilized for peripherally inserted central catheters. CVCs are predominantly infected by *S. epidermidis* and *S. aureus* [24]. In the case of *S. aureus* infections, typical clinical signs include purulence, erythema and tenderness [25].

Urinary catheters. Urinary catheters are tubular silicone or latex devices used to empty the bladder and collect urine in a drainage bag. Biofilms can readily develop on the inner or outer surfaces of urinary catheters upon insertion, making it difficult the prevention of bacterial colonization by using mere hygienic procedures [26]. The dominant organisms isolated from urinary catheters are *S. epidermidis*, *S. aureus*, *Enterococcus faecalis*, and *Escherichia coli* [27,28].

Endotracheal tube. An endotracheal tube is a flexible polyvinyl chloride tube that is placed between the vocal cords through the trachea. The endotracheal tube is then connected to a ventilator, which delivers oxygen to the lungs. Endotracheal tubes directly link the outside environment and the lungs, making them susceptible to bacterial infection. For this reason, biofilms can rapidly develop on endotracheal tubes [29]. *S. aureus* is the most frequent pathogen infecting ventilator-associated pneumonia, next to *Pseudomonas aeruginosa* [30].

Cerebrospinal shunts. A shunt is a hollow tube surgically placed in the brain (or occasionally in the spine) to allow the drainage of cerebrospinal fluid and redirect it to another location in the body. Cerebrospinal shunt infections remain the most significant complication in the treatment of hydrocephalus and significantly contribute to excess morbidity and mortality. Skin flora organisms such as *S. epidermidis*, *S. aureus*, and *Propionibacterium acnes* are the most common infecting organisms, and the clustering of approximately 70% of infections within the two-month postoperative period strongly suggests that colonization during shunt placement is an important initiating event [31].

Contact lenses. Contact lenses (CLs) can be classified as hard and soft. Conventional hard CLs are made of fairly stiff plastic (polymethyl methacrylate or PMMA) and are rigid and impermeable to oxygen [32,33]. Hard permeable CLs are made of silicone-containing compounds that allow oxygen to pass through the lens material to the cornea [34]. The first type of soft CLs are hydrogels made of 2-hydroxyethylmethacrylate (HEMA) cross-linked to ethylene glycol dimethacrylate and contain up to 38% water [35]. Silicone hydrogel contact lenses are more advanced soft lenses that allow more oxygen to pass through the lens to the cornea than regular hydrogel contacts and may be up to 70% water [36]. During contact lens wear, proteins in the tear film deposit on the CLs and promote microbial adherence. Tears contain more than a thousand proteins that exert various functions, such as antimicrobial defense, lubrication and wound healing. Recently, Osei et al. reported that the adsorption of the glycoprotein Gp340, a normal component of tears, on two common contact lens materials promotes the adhesion of *S. aureus* and *Pseudomonas aeruginosa*. This study also identified staphylococcal surface protein SraP as a receptor for Gp340 [37]. Since *S. aureus* is among the most common causes of ocular infections this information could have potential therapeutic implications. Table 1 summarizes the major biomaterials used in medicine and their properties.

Table 1. Most used devices in human medicine.

Device	Chemical Composition	Application	Type of Infective Agent	Refs
Prosthetic joints	Polyethylene, alumina and zirconia ceramics, silicon carbide, stainless steel, polymethyl methacrylate, titanium	An artificial joint is implanted to replace a damaged or diseased natural joint due to arthritis or other causes such as injuries or obesity	<i>S. aureus</i> , coagulase-negative staphylococci,	[16–20]
Mechanical heart valves	Titanium and carbon	Artificial heart valves are used to replace heart valves that have become damaged with age or by specific diseases (endocarditis) or congenital abnormalities	<i>S. epidermidis</i> , <i>S. aureus</i> , streptococci spp.	[21–23]
Central venous catheters	Silicone rubber, polyurethane	Central venous catheter is used to give intravenous fluids, blood transfusions, chemotherapy, and other drugs	<i>S. epidermidis</i> , <i>S. aureus</i> , Enterococci, Aerobic Gram-negative bacilli	[24,25]
Urinary catheters	Silicone, latex	A urinary catheter is used to empty the bladder and collect urine in a drainage bag	<i>S. epidermidis</i> , <i>S. aureus</i> , <i>Enterococcus faecalis</i> , <i>Escherichia coli</i>	[26–28]
Endotracheal tube	Polyvinyl chloride	Endotracheal tube keeps the airway open in order to give oxygen, medicine, or anesthesia. It supports breathing in certain illnesses, such as pneumonia, emphysema.	<i>S. aureus</i> , <i>Pseudomonas aeruginosa</i>	[29,30]
Cerebrospinal shunts	Silicone	Cerebrospinal shunts are used to help drain cerebrospinal fluid and redirect it to another location in the body where it can be reabsorbed	<i>S. epidermidis</i> , <i>S. aureus</i> , <i>Propionibacterium acnes</i>	[31]
Contact lenses	Polymethyl methacrylate/silicone, 2-hydroxyethylmethacrylate/ethylene glycol dimethacrylate	Contact lenses are used to correct nearsightedness, farsightedness, astigmatism and age-related loss of close-up vision, as well as an irregular corneal curvature (keratoconus).	<i>S. aureus</i> , <i>P. aeruginosa</i>	[32–36]

3. Staphylococcal Biofilm Development on the Surface of Biomaterials

As reported above, biofilms are communities of sessile bacteria tightly adhering to biomaterial surfaces, encased in a matrix of extracellular polymeric substances [6,38]. In many bacteria including *S. aureus* biofilm formation involves several stages: initial attachment; accumulation and maturation; dispersal (Figure 1). In all these stages proteinaceous and non-proteinaceous compounds are expressed and their expression is tightly controlled [39,40].

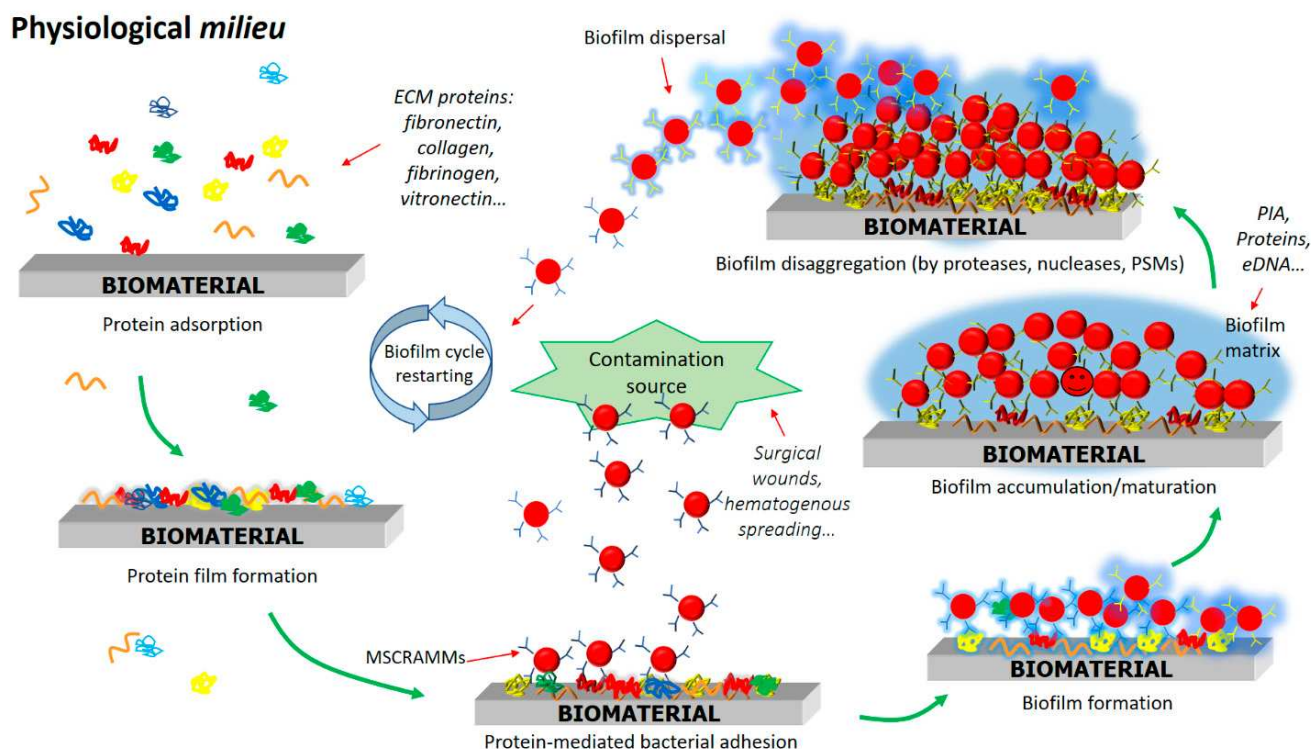


Figure 1. Schematic representation of *S. aureus* biofilm lifecycle. In the first stage *S. aureus* cells directly attach to the abiotic surface of biomaterials, followed by their robust, specific interaction via adhesins (MSCRAMMs) which specifically recognize plasma proteins covering the device. In the second step bacteria proliferate, accumulate and produce a biofilm matrix composed of polysaccharides, DNA and proteins. In the third, dispersal stage several staphylococcal factors disperse the bacteria and disseminate infection. For more details see the text and Refs. [14,15].

3.1. Attachment of *S. aureus* Cells to Plasma Protein-Coated Biomaterials

Staphylococcal biofilm formation on medical devices begins with the attachment of staphylococcal cells to the abiotic surface followed by adhesion to the plasma protein-coated surface of the biomaterials. Interactions between staphylococci and the biomaterial surface are not specific and are driven by hydrophobic, electrostatic and Van der Waals forces (Figure 1). The hydrophobic character of a few staphylococcal surface components, such as wall teichoic acids and the surface proteins Atl from *S. aureus* and *S. epidermidis*, also contribute to the formation of hydrophobic interactions between bacteria and the material surface [41–43]. However, in vivo direct interaction of *S. aureus* cells with the abiotic implant surface is not considered significant, because implants are rapidly covered by host plasma proteins (Figure 1). The type and amount of plasma proteins adsorbed and retention on the biomaterial's surface depend on the physical and chemical properties of the surface such as wettability and charge of the material and the characteristics of the plasma proteins such as the relative abundance of plasma, size, stability and charge [44]. The adsorption of plasma proteins, in particular those with adhesive properties such as fibronectin, fibrinogen, vitronectin and von Willebrand factor, on the surface of implanted material provides a physiological microenvironment for the integration of diverse materials into the host tissues.

Coating can also concur to modulate the biocompatibility of the biomaterials and influences interfacial events such as blood clotting and thrombogenesis. Beyond such actions, coating with plasma proteins produces a perfect scaffold for the colonization and infection of biomaterials by several species of both Gram-positive (staphylococci) and Gram-negative (for example, *Pseudomonas aeruginosa*) bacteria. For this purpose, bacteria have evolved a series of surface-anchored proteins called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), each binding to a specific set of host proteins.

Fibronectin (Fn), a multidomain glycoprotein (440 kDa) found in almost all tissues and organs and in biological fluids (plasma concentration, 200 µg/ml), is an important player in processes such as adhesion, differentiation, growth, and migration of cells and is also involved in blood clotting and wound healing [45]. Fn is present in soluble form in various body fluids and in insoluble form in extracellular matrices and basement membranes. Hepatocytes produce soluble Fn and secrete it into the bloodstream, whereas the insoluble cellular Fn is synthesized by fibroblasts and endothelial cells. Fn is a dimer made of two nearly identical chains linked by a pair of disulfide bonds. The protein has a modular organization and is composed of a combination of three types of homologous domains, i.e., type I (FnI), II (FnII), and III (FnIII) (Figure 2). Each monomer consists of 12 type I repeats, 2 type II repeats, and 15–17 type III repeats or modules. Cellular Fn can include two variable proportions of alternatively spliced FnIII modules (EIIIB and EIIIA, also termed EDB and EDA, respectively) and one FnIII connecting segment (IIICS). On the other hand, these alternative spliced type III repeats lack in the soluble form. Fn is organized in contiguous domains, each consisting of several modules, and each domain contains binding sites for specific ligands. Five independently folded type I modules (modules FI1–5) constitute the N-terminal domain (NTD) which binds, among others, bacterial receptors and fibrin. The region downstream of the NTD consists of the combination of FnI6FnIII1–2FnI7–9 repeats and binds gelatin/collagen. The wide following region including 15 constitutive type III repeats comprises a number of modules involved in interactions with other extracellular molecules. Among these, the RGD motif is critical for integrin binding and consequent cell adhesion to the extracellular matrix. The interaction of the RGD motif with integrin $\alpha 5 \beta 1$ facilitates the internalization of *S. aureus* by non-professional phagocytic cells. The extreme C-terminal of Fn (FnI10–12) contains a second fibrin binding site and a cysteine residue involved in the interchain disulfide bond (Figure 2) [45–47].

Fibrinogen (Fbg) is a large glycoprotein (340 kDa) synthesized in the liver and circulating at a concentration of 2.6 mg/mL. The molecule appears as a trinodular rod and contains three pairs of non-identical chains, A α (67 kDa), B β (56 kDa) and γ (47 kDa). The assembly of Fbg occurs through complex interchain disulfide bonding involving the N-terminal regions of the three polypeptides; together, these form the central nodular E domain. The globular end of the molecule represents the D domain, containing the C-termini of the B β and γ chains (Figure 2). Thrombin can cleave an Arg-Gly bond in the N-terminus of the α chain, releasing the 16 residue fibrinopeptide A (FPA). The new exposed N-terminal end of the α chain interacts with sites in the γ chain C-termini of neighboring molecules, initiating the non-covalent end-to-end and lateral assembly of the two molecule thick fibrin protofibrils. Subsequently, a second thrombin cleavage releases a 14-residue peptide (FPB) from the N-terminus of the B β chain and the exposed new β N-terminus reinforces lateral aggregation resulting in the formation of thick fibrin fibers. Fbg binds specifically to integrin receptors on platelets, endothelial cells and many other cells. Binding to activated platelets is specific and mediated by the platelet integrin $\alpha II \beta 3$. Residues in the γ chain of Fbg from 400 to 411 are necessary for binding to platelets. The adhesion of endothelial cells to surface-coated-Fbg appears to be mediated by $\alpha_v \beta_3$ integrin and the RGD sequence in the C-terminus A α chain [48].

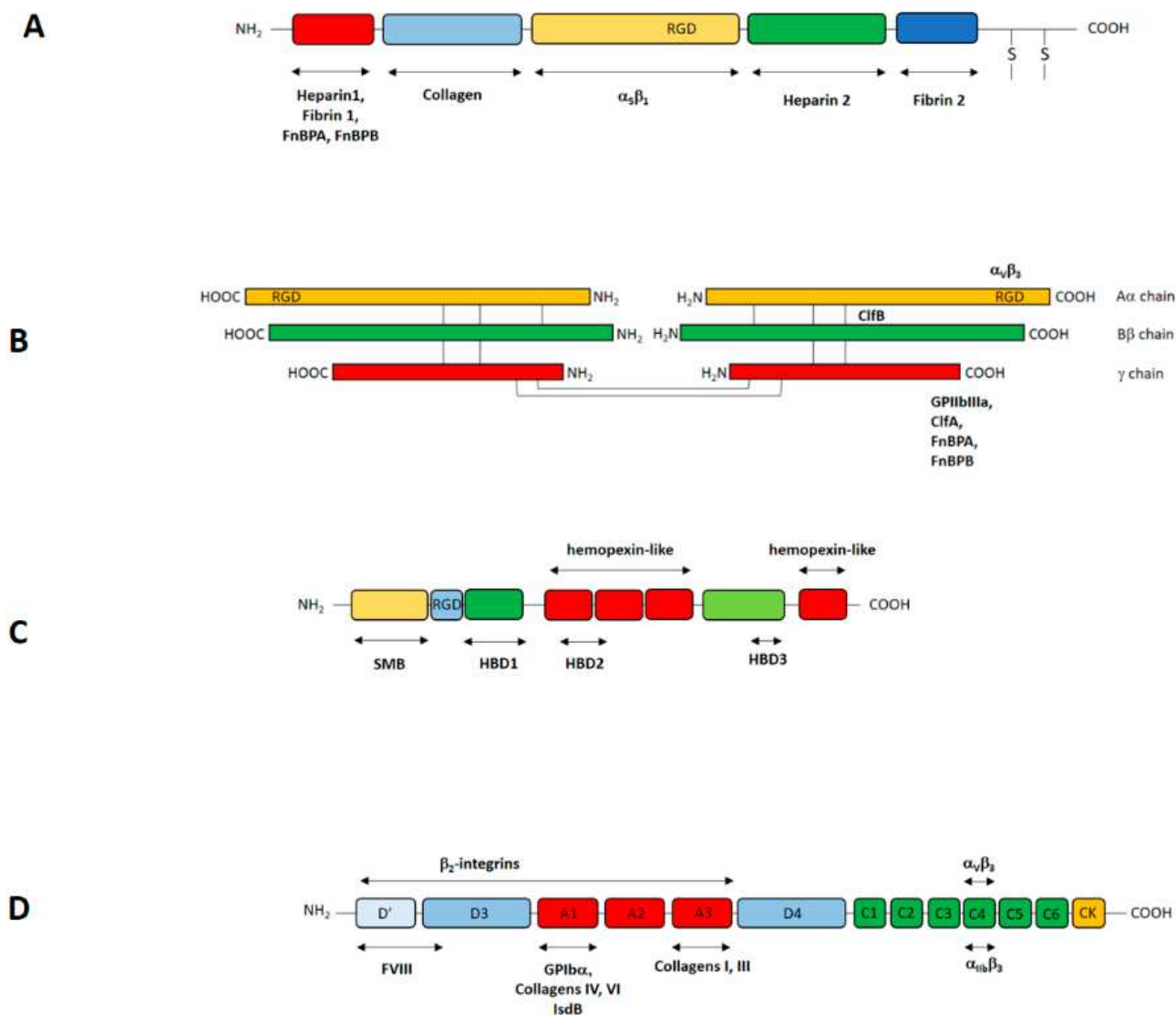


Figure 2. Schematic depiction of the extracellular matrix / plasma human proteins. (A) fibronectin; (B) fibrinogen; (C) vitronectin; (D) von Willebrand factor; For each protein, domain organization is indicated. The binding sites for specific ligands on each protein are also reported.

Vitronectin (Vn), a glycoprotein synthesized in the liver, is found at a high concentration in plasma (200–700 $\mu\text{g}/\text{mL}$) [49] and is also present in the extracellular matrix of different tissues [50]. The N-terminal portion of mature Vn (43 aa residues) consists of a somatomedin B (SMB) domain followed by the integrin-binding RGD motif. Several members of the integrin family are engaged in Vn binding, among these, integrin $\alpha_3\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ [51]. The next domain comprises four hemopexin-like domains with putative heme-binding motifs. In addition, Vn has three heparin-binding domains corresponding to residues 82–137, 175–219, and 348–361 [52–54]. Vn is present in animal organisms as a native, folded monomer in plasma/serum and as a multimeric unfolded form in the extracellular matrix [49,55,56] (Figure 2). Conformational change from the monomeric to the activated, multimeric state is promoted by binding to physiological ligands such as the thrombin-antithrombin complex. Vn conformational activation reveals a number of cryptic sites, including the full exposure of the heparin-binding site at the C-terminal domain of the protein [57,58] and the RGD cell-binding motif [59,60].

Von Willebrand factor (vWF) is a large glycoprotein produced by activated endothelial cells and megakaryocytes, the precursor cells of platelets. vWF circulates in the bloodstream

at concentrations between 8–14 µg/mL. The mature 2050 amino acid long monomer consists of three homologous units arranged in the following order: D'D3–A1–A2–A3–D4–C1–C2–C3–C4–C5–C6–CK [61,62] (Figure 2). Each domain can specifically bind to several ligands. The D'D3 domain interacts with factor VIII, the A1 domain binds to the platelet GPIb receptor, heparin, and collagens type IV and VI. The A2 domain contains a cryptic cleavage site for ADAM13 protease, the A3 domain binds fibril-forming collagens I and III and C4 is the binding domain for integrins α II β 3 and α v β 3 through an RGD motif.

As reported above, plasma protein-coated biomaterials are the specific substrates for a variety of MSCRAMMs expressed by *S. aureus* cells. The MSCRAMM protein family is the most prevalent group of surface proteins that bind to the extracellular matrix and plasma proteins. MSCRAMMs have a conserved overall structure, which includes, from N terminus to C terminus, a signal peptide followed by one or more ligand-binding domains, a cell wall LPXTG anchoring motif, a membrane-spanning region and a short positively charged intracellular tail [63]. The N terminus of several MSCRAMMs contains an A domain made of three separated IgG-like folded subdomains N1, N2 and N3. The combination of N2 and N3 forms a structure that binds the ligand by the “dock, lock and latch” (DLL) mechanism [64,65].

The archetypal examples of staphylococcal MSCRAMMs are clumping factor A (ClfA) [66,67] and B (ClfB) [68,69]. Both the proteins bind Fbg using a variant of the DLL mechanism [66,69]. Linking the A domain to the wall-spanning region W is the region R composed of Ser-Asp repeat (SD repeat) (Figure 3). Other members of the Clf subfamily are SdrC, SdrD and SdrE which contain additional B repeats inserted between the A domain and the SD repeat region [70,71]. Fibronectin-binding protein A (FnBPA) and FnBPB have A domains that are structurally and functionally similar to the A domain of the Clf group and bind several ligands including Fbg [72] and plasminogen [73]. Located in place of the SD repeat region are tandemly repeated motifs (11 in FnBPA and 10 in FnBPB), each binding the NTD of each Fn subunit [74,75] (Figure 3). Importantly, the bridging activity of Fn between FnBPA or FnBPB of *S. aureus* and $\alpha_5\beta_1$ integrin on the surface of non-phagocytic cells promotes the internalization of *S. aureus* into the host cells [76–78].

An important highly expressed MSCRAMM in *S. aureus* is protein A (SpA). The N terminus of SpA comprises a tandem array of five separated folded three-helical bundles, each of which can bind to different ligands (Figure 3). The A1 domain of vWF, tumor necrosis factor receptor 1 and the Fc region of IgG bind to the interface between helices E and D, while the Fab region of IgM binds to the interface of helices D and A [79–82]. IgG- and vWF-coating of medical device surface can also induce the SpA-mediated attachment of *S. aureus* to implants and qualifies SpA as a genuine adhesin.

A significant number of *S. aureus* strains express CNA, an MSCRAMM that contains a collagen-binding A region at the N-terminus followed by repeated sequences, the B repeats, and a C-terminal cell wall anchoring elements [83] (Figure 3). CNA recognizes collagen by a variation of the DLL mechanism called a collagen hug, a multistep process where the triple helical structure of monomeric collagen is critical for binding [84]. CNA also binds to laminin and C1q and interactions of CNA-laminin and CNA-C1q are weaker than the binding of CNA to collagen [85,86]. CNA binds to the collagenous domain of C1q and interferes with the interaction of C1r with C1q. As a result, C1r2C1s2 is displaced from C1q, and the C1 complex is deactivated [87]. Thus, the dual function of CNA as adhesin and subverter of the classical pathway provides a benefit to pathogen survival and helps establish infection both on the tissues and implants. Interestingly, an epidemic cluster of *S. aureus* from orthopedic periprosthetic infections was identified and characterized from the *bbp-cna* gene tandem for the adhesins binding bone matrix proteins [87]. The cluster corresponded to an epidemic clonal complex, the historical CC30 responsible for worldwide epidemics. The ability to bind bone matrix proteins explains the invasive success of the clone in periprosthetic bone tissue [88].

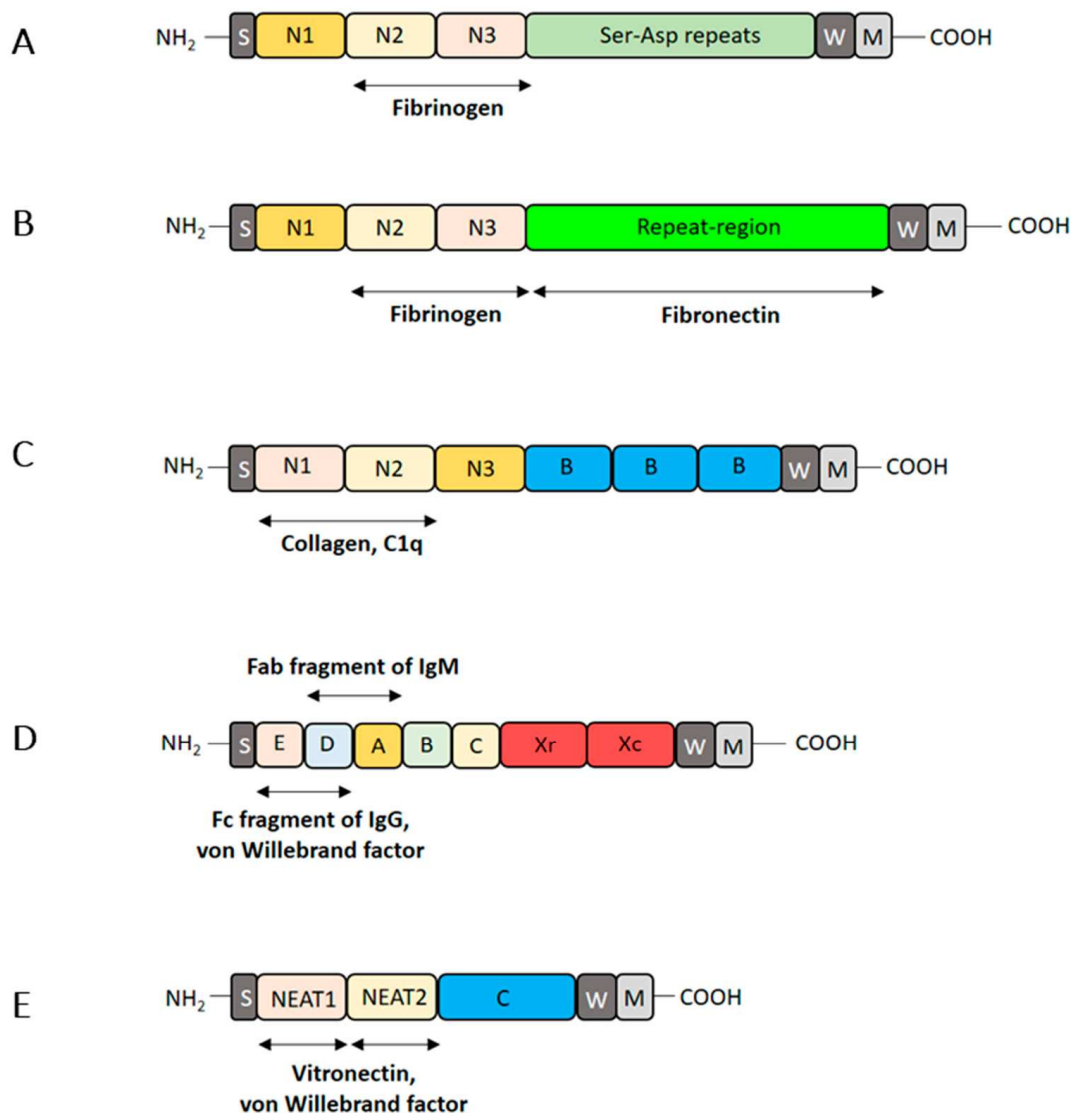


Figure 3. Depiction of *S. aureus* adhesins. **(A)** ClfA (or ClfB); **(B)** FnBPA (FnBPB); **(C)** CNA; **(D)** SpA; **(E)** IsdB. The N-terminal A region of ClfA, ClfB, FnBPA, FnbpB and CNA contains three separate folded subdomains, named N1, N2 and N3. The serine-aspartate region of ClfA and ClfB is composed of a variable number of serine-aspartate dipeptide repeats. The repeat region of FnBPA and FnBPB comprises 11/10 39-residue fibronectin-binding units. The B region of CNA includes a variable number of B repeats. The Xr and Xc of SpA correspond to repetitive and non-repetitive domains, respectively. All the proteins contain a wall binding region (W) and a membrane spanning region (M) at the C-terminus. For each adhesin the binding sites for specific host proteins are indicated.

In iron restricted conditions, *S. aureus* cells express a set of MCSRAMMs named iron-regulated surface determinant (Isd) proteins that have one (IsdA), two (IsdB) or three (IsdH) NEAT (Near iron transporter) domains that bind hemoglobin and heme. Each NEAT motif of IsdB also binds to Vn and vWF, suggesting a role as an iron transporter and adhesin for this protein [89,90] (Figure 3).

The MSCRAMM-mediated adhesion to protein-coated biomaterial is propedeutic to the bacterial colonization and development of biofilm on the surface of medical devices.

3.2. Proliferation and Production of Biofilm Matrix

After attachment to the plasma protein-covered device is accomplished, bacteria grow by proliferation and produce polymeric molecules to form a scaffolding extracellular matrix

(Figure 1). Biofilm polymers include polysaccharides, proteins, extracellular DNA (eDNA), teichoic acids and other molecules on the whole constituting an aqueous gel-like material with proper mechanical properties and resistance to external shocks [91].

S. aureus and *S. epidermidis* produce one main biofilm exopolysaccharide, named polysaccharide intercellular adhesin (PIA). PIA is a β 1-6-linked homopolymer of N-acetylglucosamine, which has a net positive charge due to the enzyme-catalyzed removal of 15 to 20% of N-acetyl groups after the polymer is secreted. The cationic character of PIA allows electrostatic interaction with other negative charged biofilm components such as teichoic acids. PIA biosynthesis is accomplished by the products of the *ica* locus, which comprises the *icaA*, *icaD*, *icaB* and *icaC* genes [92].

The enzyme IcaA, an N-acetylglucosaminyltransferase, synthesizes PIA from UDP-N-acetylglucosamine precursor. IcaD is required to enhance the efficiency of IcaA while IcaC allows the translocation of PIA across the cell membrane [93]. The bacterial surface-associated enzyme IcaB is responsible for the above-mentioned limited deacetylation of PIA [94]. In addition to the *ica* biosynthetic genes *icaADBC*, the PIA biosynthesis locus contains a negative regulatory gene, *icaR* [95].

Several strains of *S. aureus* and *S. epidermidis* have been reported to not produce PIA but still form biofilm both in vivo and in vitro. In those strains, several staphylococcal surface proteins can promote the accumulation/maturation phase of biofilm development [96–98]. The *S. aureus* surface protein G (SasG) [99], clumping factor B [100], serine aspartate repeat protein (SdrC) [101], the biofilm-associated protein (Bap) [102,103] and the fibronectin-binding proteins (FnBPA and FnBPB) [104,105] are individually involved in biofilm matrix formation.

SasG, a surface anchored biofilm-forming protein, contains an N-terminal A domain followed by a region composed of a variable number of B repeats. Each B repeat comprises a nearly identical 78 residue G5 sequence and an E region of 50 residues. The mechanism for SasG-mediated cell association in biofilm is based on homophilic protein–protein interactions. Specifically, SasG is enzymatically cleaved within B region to remove the N-terminal A domain and the exposed B repeats on neighboring cells interact with each other in a Zn^{2+} -dependent manner, leading to cell–cell adhesion [106].

The A domains of SdrC [101] and FnBPs [105,107] engage in homophilic interactions and promote cell–cell accumulation of staphylococcal cells during biofilm formation. The A domain of SdrC can also promote attachment to abiotic hydrophobic surfaces and could thus be involved in both the early attachment to an indwelling medical device and in accumulation phase of biofilm [108]. Notably, the properties of the PIA- and protein-dependent biofilms show marked morphological differences in structures. The PIA-dependent biofilm is characterized by the presence of fibrous, string-like structures that are absent in proteinaceous biofilm [109,110] and have a higher strength than protein-based biofilm [111].

A number of secreted proteins in the extracellular matrix have been reported to be involved in staphylococcal biofilm formation. Among these we can mention the giant extracellular matrix binding protein (Embp), the extracellular adherence protein (Eap) and the extracellular matrix binding protein (Emp) [14]. The specific molecular mechanism with which these proteins intervene in the process is unknown.

Besides the proteinaceous components, extracellular DNA (eDNA) works in biofilm as an electrostatic net, thus stabilizing and strengthening the matrix [112–116]. Studies on the mechanism of eDNA production and release have revealed that bacteria produce eDNA either through altruistic suicide or fratricide killing and that bacterial autolysis is mediated by murein hydrolase [117]. In the presence of eDNA β -toxin, a sphingomyelinase of *S. aureus*, forms covalent cross-links to itself, producing an insoluble nucleoprotein and stabilizing the biofilm structure [118].

3.3. Dispersal Phase of Biofilms

In the dispersal phase the biofilm structure is disrupted by enzymatic degradation of the matrix components, most notably by proteases, nucleases and soluble modulins (PSMs)

(Figure 1). Among the biofilm-degrading enzymes, proteases are the most important ones [119]. *S. aureus* produces at least ten proteases, each having a proper substrate specificity and expression profile. The major proteases that modulate biofilm integrity include V8, aureolysin and staphopains and their identified targets can be FnBPs and ClfB [100,105,120–122]. Cathepsin G released during neutrophil degranulation contributes to the degradation of biofilm into fragments [123] and possibly promotes biofilm dispersal.

The biofilm matrix component eDNA is susceptible to degradation by *S. aureus* nucleases. *S. aureus* is known to produce two secreted nucleases Nuc1 and Nuc2 [124]. The expression of Nuc1 reduces biofilms and a *nuc1* mutation in strain USA300 shows increased in vitro biofilm development [125]. On the other hand, purified Nuc2 prevents biofilm formation in vitro and modestly decreased biomass in dispersal experiments [126].

A group of small α -helical peptides known as phenol soluble modulins (PSMs) functioning as surfactants are also involved in biofilm dispersal. PSMs can be classified in α -type peptides (20–25 amino acids in length) and β -type peptides (45 amino acids in length) [127]. PSMs work on growing biofilms, while they have a limited effect on the dispersal of mature biofilms [128]. They are also involved in the formation of internal channels in biofilms and affect the structure of both PIA-dependent and -independent biofilms [111]. In contrast to these actions, PSMs form amyloids that stabilize *S. aureus* biofilms and block the enzymatic degradation of the biofilm structure [129].

4. Regulation of Biofilm Formation

A central regulator controlling biofilm development in staphylococci is a peptide quorum-sensing system, also called the accessory gene regulator or *agr* system. The *agr* locus consists of two divergent transcriptional units, RNAII and RNAPIII, whose transcription is driven by the P2 and P3 promoters, respectively. The RNAII locus contains four genes, *agrB*, *agrD*, *agrC* and *agrA*. The *agrD* transcript encodes a peptide precursor of autoinducing peptide (AIP). In its mature form, AIP is 7–9 amino acids long and contains a thiolactone ring between the centrally located cysteine and the C terminus. The *agrB* gene product is a transmembrane endopeptidase that processes AgrD into the final structure with assistance of the type I signal peptidase SspB. Outside the cell, the AIP signal accumulates at particular concentrations level (usually in the low nM range) and activates AgrC. AgrC, the product of the *agrC* gene, is a transmembrane histidine kinase sensor that self-phosphorylates upon binding AIP. Activated AgrC in turn phosphorylates the cognate response regulator AgrA, which then binds to the P2 promoter region for RNAII leading to the autoinduction loop and the P3 promoter region for RNAPIII, as well as the promoters controlling the expression of the PSMs. RNAPIII is the intracellular effector molecule of the *agr* system responsible for the control of the Agr targets. RNAPIII directly inhibits the production of predominantly surface proteins such as protein A and others. Moreover, through the inhibition of the repressor Rot, RNA III leads to the prompt density-dependent upregulation of enterotoxins, α -toxin, leukocidins, degradative exoenzymes such as staphopain proteases, and down-regulation of surface proteins such FnBPs and coagulase. In *Staphylococcus*, the *agr* system appears to influence biofilm formation at structuring and dispersal stages through its effect on PSM and protease expression. Moreover, the *agr* system controls the expression of surface proteins (adhesins) involved in the initial stage of biofilm development. Notably, the *agr* system does not affect PIA-dependent biofilm formation [130–134].

Besides the *agr* system, biofilm formation is regulated by SarA, a protein which controls *ica* [135,136] and serine protease loci [137]. SarA also indirectly impacts biofilm development through the enhancement of *agr* system activity [138] and expression of nuclease [139]. Sigma B (Sig. B), an alternative sigma factor of RNA polymerase, increases *sar* gene expression and down regulates the activity of the *agr* system [140]. Specifically, the downregulation of RNAPIII expression and production of secreted proteases affect the formation of FnBPs-dependent biofilm [141].

5. Evasion of the Immune System by Biofilm and Resistance to Antibiotics

Many studies have investigated the effects of staphylococcal biofilms on immune cell function. In the innate immune system, neutrophils and macrophages are the first line of defense against staphylococcal infections. One study reported that neutrophils have a limited influx in a murine model of catheter-associated *S. aureus* biofilm growth [142], while other investigations have demonstrated that activated neutrophils are prevalent at the site of infection in human patients with orthopedic device-associated staphylococcal biofilm infections [143,144]. Moreover, several investigations have reported that human neutrophils in vitro cocultured with *S. aureus* localize to the biofilm and can phagocytose bacteria [145,146]. On the other hand, it has been demonstrated that the *agr* system may facilitate resistance of staphylococcal biofilm to neutrophil killing. Consistent with this, an *agr* mutant resulted in being less cytotoxic to neutrophils in coculture than its wildtype strain, suggesting that bacteria expressing *agr* could kill neutrophils and consequently evade phagocytosis [145].

Along this line, *S. aureus* extracellular nuclease has been reported to degrade NETs (Neutrophil Extracellular Traps), which are secreted thread-like structures composed of DNA and granular antibacterial components [147–149].

The anti-biofilm action of macrophages has also been the subject of investigation. Biofilm mode of growth promotes activation of the macrophage phenotype M2 which is characterized by high arginase activity and the production of IL-10, IL-4 and IL-13 [150]. This will create a microenvironment that favors little phagocytosis and even the death of those macrophages that were close to the biofilm [151].

To investigate how *S. aureus* biofilms respond to macrophages and neutrophils gene expression, patterns were profiled using microarrays. The incubation of macrophages with *S. aureus* biofilms led to a global downregulation of genes involved in metabolism, cell wall synthesis/structure and transcription/translation/replication. Unlike coculture with macrophages, coculture of *S. aureus* biofilms with neutrophils did not affect the biofilm transcriptome [152].

The biofilm also increased resistance to antibiotics. This is mainly due to the presence of a matrix in the biofilm that functions as a physical barrier for some antibiotics and to the reduced metabolic processes targeted by antibiotics. Additionally, during prosthetic infection, the *agr* system is downregulated. The consequent absence of PSMs would lead to the pronounced aggregation of bacteria and increased resistance of the infected joint prosthesis and adjacent tissues to the treatment with antibiotics. Hence, novel effective anti-biofilm antibiotics should penetrate the biofilm structure and show high antimicrobial resistance [14,15].

6. Therapeutic Strategies to Fight Biomaterial-Associated Staphylococci

Several experimental efforts have been put forth to identify antimicrobials that can treat biomaterial-associated *S. aureus* biofilms. Treatment with DNAase I, which degrades DNA in biofilm, combined with trypsin and proteinase K efficiently disrupts biofilm matrix [122,152–154]. Likewise, dispersin B, a glycoside hydrolase, can break down the polysaccharide components of staphylococcal biofilms and can promote penetration of antibiotics [155–157]. Hydrolases such as α amylase and cellulase and lysostaphin have also been shown to significantly reduce the matrix biomass of *S. aureus* biofilm [158,159]. However, the in vivo use of exoenzymes as therapeutics may have limited application due to the immunological and inflammatory implications of these treatments.

cis-2 decenoic acid (C2DA), a fatty acid produced by *Pseudomonas aeruginosa*, causes a release of planktonic bacteria from *S. aureus* biofilm, suggesting that it can be utilized as a dispersal agent. C2DA combined with traditional antibiotics increases the eradication of *S. aureus* biofilm [160].

Antimicrobial peptides (AMPs), such as a derivative of the cathelicidin peptide LL-37 named OP-145, when incorporated into a medical device, were shown to prevent implant-associated *S. aureus* infections in rabbits [161]. Another LL-37 derivative, SAAP-148, shows

to be effective in the eradication of methicillin-resistant *S. aureus* biofilm both in vivo and in vitro [162]. An even better therapeutic efficacy was demonstrated by the combination of AMPs IB-367 and BMAP-28 in the treatment of catheter-associated biofilms [163,164]. As reported for antibiotics, bacteria, including *S. aureus*, have developed specific resistance mechanisms to neutralize the action of AMPs. Thus, caution should be taken when AMP-based treatments are considered.

Other compounds such as aryl rhodamine, D-amino acids, metal chelators and benzimidazole have been successfully tested as anti-biofilm agents in vitro, suggesting the promising use of a variety of small inhibitors of biofilm associated with implanted medical devices [154,165–167].

Iron is critical for a variety of cellular processes including energy production, DNA synthesis and biofilm. Analogs of iron have been used to effectively disrupt staphylococcal biofilms: among these, gallium nitrate has been shown to be effective at reducing bacterial biofilm in vitro [168,169]. The metalloporphyrin gallium-protoporphyrin IX (GaPP) is a compound capable of mimicking heme and facilitating metal ion uptake by bacteria. Once inside the bacterial cell, GaPP can block iron-dependent activities of cytochromes, catalases and peroxidases [170]. GaPP administered together with the iron chelator deferiprone demonstrates therapeutical efficacy against biofilm produced by MRSA and MSSA bacteria [171]. Moreover, this combination of compounds strengthened the anti-biofilm activity of antibiotics [172].

Coating with antiadhesive or antibacterial compounds, such as metal ions (copper, silver and zinc) and nanoparticles technology, have also been used as therapeutic strategies to neutralize biomaterial infections [173,174]. However, these approaches do not result in a full clinical success, possibly due to the fact that the medical devices are rapidly covered by plasma proteins so that the anti-biofilm compounds are masked and their efficacy reduced.

Synthetic and natural compound targeting products of the *agr* system have been evaluated for their efficacy against *S. aureus* infections. Among these compounds we can mention ambuic acid, a fungal metabolite that interferes with the biosynthesis of AIP peptide and has been successfully tested in a murine model of skin and soft tissue infection [175]. The plant-derived hamamelitannin and analogues have been shown to potentiate the antibiotic susceptibility of *S. aureus* biofilms by affecting peptidoglycan biosynthesis and eDNA release [176–178]. Byaril hydroxyketones, which inhibit the binding of AgrA to P3 promoter, when tested in a *Galleria mellonella* insect larvae infection model, showed increased larval survival. A combination therapy of these quorum-quenching agents with the antibiotics cephalothin or nafcillin revealed additional survival benefits [179]. The antibiofilm value of these blockers in animal bearing implant-associated *S. aureus* infections remains to be determined.

Bacteriophages have also evolved as potential bactericidal agents. The efficacy of phage therapy has been explored for a wide range of *S. aureus* diseases, including biofilm formation on indwelling medical devices. For example, bacteriophage K treatment significantly reduces the *S. aureus* infection of a central venous catheter compared to controls in a rabbit model [180]. Moreover, precoating orthopedic implant surface with phages has been found to prevent *S. aureus* adhesion to medical devices [181] and to reduce bacterial load in adjacent tissues [182]. Finally, orthopedic wires, which are smooth stainless pins used to hold bone fragments together, when coated with phages, were colonized less by *S. aureus* in a murine model of experimental joint infection, and the inhibitory effect could be increased when phage coating was combined with linezolid treatment [182]. In conclusion, although conducted at an experimental level and with animal models, these studies suggest that phage prophylaxis could be an additional tool to prevent *S. aureus* infection of human medical implants.

Vaccination strategies have also been used to contrast the biofilm formation into the medical devices. DNABII proteins are essential components of the biofilm produced by Gram-positive and Gram-negative bacterial species. Estellès et al. produced a monoclonal antibody (mAb) named TLR1068 that cross-reacts with a DNABII epitope and showed

that the antibody effectively disrupts the *S. aureus* biofilm in vitro as well as the catheter-associated biofilm in a rat model of infection. Due to this effect, TLR1068 has been proposed as a candidate for the treatment of implant-associated infections in combination with antibiotics [183]. Moreover, immunization with PIA promotes clearance of *S. aureus* from the blood [184] and anti-PIA antibodies are under investigation to treat a variety of biofilm infections in several pathogens [185]. Table 2 refers to the major non-conventional anti-microbial agents used to combat infections by *S. aureus*.

Table 2. List of non-conventional anti-microbial agents used for treating biomaterial-associated *S. aureus* infections.

Antimicrobial Agent	Chemical Composition	Mechanism of Action	Refs
DNAase I/Proteinase K	Enzyme	Disrupt DNA/protein content of biofilm	[122,152–154]
Dispersin B	Enzyme	A glycoside hydrolase that can breakdown the polysaccharide component of biofilm	[155–157]
Cis-2-decanoic acid	Unsaturated fatty acid	Causes release and dispersal of planktonic bacteria from biofilm	[160]
Cathelicidin LL-37 derivatives: OP-145, SAAP-148, IB-367/BMAP-28	Peptide	Disrupt bacterial membrane	[161–164]
Gallium protoporphyrin IX	A protoporphyrin derivative	Can block iron-dependent activities- of bacterial enzymes	[168–172]
Ambuic acid	A highly functionalized cyclohexenone	Interferes with the biosynthesis of AIP peptide	[175]
Hamamelitannin	2',5-Digalloylhamamelofuranose	Affects peptidoglycan biosynthesis and DNA release	[176–178]
Byaril hydroxyketonecompounds	-	Inhibit the binding of AgrA to P3 promoter	[179]
Phages	Phages are composed of a nucleic acid molecule that is surrounded by a proteinaceous coat	Phage penetrates the cell wall and its DNA is drawn into the bacterium and effectively blocks the bacterium's ability to function or replicate.	[180–182]
TLR1068	Monoclonal antibody	Disrupts the <i>S. aureus</i> biofilm in vitro as well as the catheter-associated biofilm in a rat model of infection	[183]

7. Concluding Remarks

Progress in current medicine increasingly relies on the use of foreign bodies, but their implantation in many cases creates conditions that favor opportunistic infections by several bacteria, mainly represented by staphylococci. The biofilm mode of growth adopted when *S. aureus* initiates device-associated infections, the persistence of this bacterium in the implanted material due to increased recalcitrance to antibiotics, and lastly, the plethora of immune evasion mechanisms implemented by the bacterium seriously interfere with the success of medical device implantation. Therefore, besides new antibiotics, novel therapeutic approaches need to be pursued, including drugs which disrupt biofilms and the development of anti-biofilm vaccines. The generation of monoclonal antibodies targeting the individual steps of biofilm formation and the single components of biofilm regulatory machinery and their use in passive immunization can broaden the range of therapeutic options.

However, every single intervention to treat patients with infection-associated medical devices is destined to have limited success and for this reason it is desirable to combine and integrate different therapeutic strategies to combat staphylococcal infections. Importantly, a

focus on the in vivo evaluation of anti-biofilm therapeutic efficacy deserves better attention. Thus, the use of animal models that reflect conditions closer to the implant-associated infections in humans is highly advisable. Finally, further investigation of the molecular aspects of biofilm development and regulation and a more intense basic and preclinical and clinical research of new compounds and methods will be required.

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Article

Structural Insights into the Ligand–LsrK Kinase Binding Mode: A Step Forward in the Discovery of Novel Antimicrobial Agents

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Abstract: LsrK is a bacterial kinase that triggers the quorum sensing, and it represents a druggable target for the identification of new agents for fighting antimicrobial resistance. Herein, we exploited tryptophan fluorescence spectroscopy (TFS) as a suitable technique for the identification of potential LsrK ligands from an in-house library of chemicals comprising synthetic compounds as well as secondary metabolites. Three secondary metabolites (**Hib-ester**, **Hib-carbaldehyde** and **(R)-ASME**) showed effective binding to LsrK, with KD values in the sub-micromolar range. The conformational changes were confirmed via circular dichroism and molecular docking results further validated the findings and displayed the specific mode of interaction. The activity of the identified compounds on the biofilm formation by some *Staphylococcus* spp. was investigated. **Hib-carbaldehyde** and **(R)-ASME** were able to reduce the production of biofilm, with **(R)-ASME** resulting in the most effective compound with an EC₅₀ of 14 mg/well. The successful application of TFS highlights its usefulness in searching for promising LsrK inhibitor candidates with inhibitor efficacy against biofilm formation.

Keywords: LsrK; antimicrobial resistance; tryptophan fluorescence spectroscopy; circular dichroism; docking; screening; LsrK binders; biofilm inhibitors



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1. Introduction

Antimicrobial resistance (AMR) and the worldwide increase in superbug infections are recognized by the World Health Organization (WHO) as global concerns for public health and the sustainability of healthcare systems [1]. Superbug bacteria are responsible for about 25% of infections and almost 30% of AMR-related deaths, and they are expected to become the primary cause of death by the year 2050 [1]. To challenge this inauspicious perspective, one of the strategic objectives of the WHO is the research of new pharmaceutical tools and medicines [2]. Overuse, inappropriate prescription, and extensive agricultural use of antibiotics have clearly been exposing bacteria to strong, selective evolutive pressure, leading to the development of protective mechanisms to inactivate, remove, and in general circumvent the toxicity of antibiotics [3]. Conventional antibacterial drugs usually interfere with bacterial cell wall biosynthesis, protein synthesis, or DNA replication, and the search for new antimicrobials is still mainly focused on these strategies, thus exposing these new drugs to the same resistance mechanism [4–6]. The search for new targets and innovative antibacterial strategies and weapons has become mandatory. An innovative strategy to deal with AMR is to interfere with biofilm formation and with bacterial quorum sensing (QS). About 80% of all human bacterial infections are complicated by biofilms, within which bacteria have a 1000-fold higher tolerance to antibiotics [6,7]. The QS signaling is the most

effective mechanism that bacteria use to communicate and organize in biofilms. QS is mediated by diverse autoinducers. [5] Particularly, AI-2 is produced and sensed by both Gram- and Gram+ bacteria and plays a crucial role in maintaining QS. LsrK is a pivotal key kinase in triggering the QS cascade [8–10]. LsrK is responsible for the phosphorylation of Autoinducer-2 (AI-2, the QS messenger most exploited by both Gram-positive and Gram-negative bacteria) to phospho-4,5-Dihydroxy-2,3-pentanedione (P-DPD) [8,11]. This represents the key triggering step in the AI-2-mediated QS cascade (Figure 1). P-DPD binds to the transcriptional repressor LsrR. LsrR dissociates from the promoter region of the *lsrRK* and *lsrACDBFG* operons, inducing the transcription of genes involved in bacterial virulence and in the auto-sustainment of QS [12]. Several studies proved the role of LsrK in bacterial QS [13,14]. LsrK mutants do not activate *lsr* transcription because of the lack of P-DPD with reduced expression of the Lsr transporter and extracellular AI-2 accumulation [13]. Furthermore, when LsrK and ATP were added in the extracellular medium, the phosphorylation of AI-2 outside the cell avoids the internalization of P-DPD due to its negative charge. As a result, a reduction in *lsr* gene expression and attenuation of QS were observed [13].

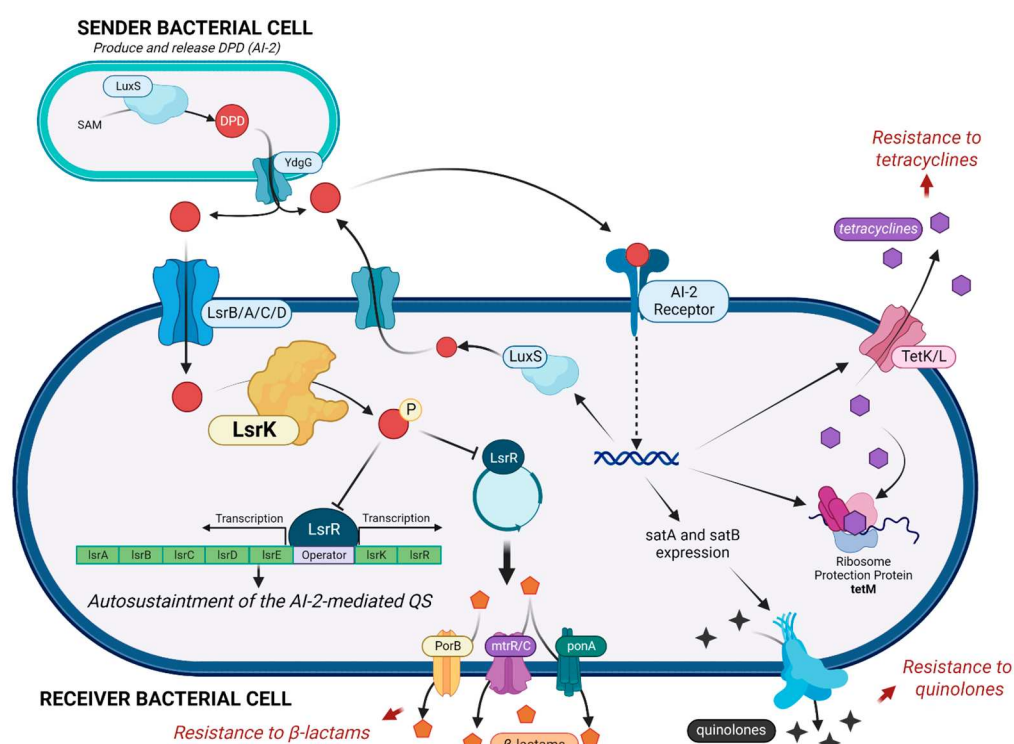


Figure 1. AI-2 mediated cascade, with a focus on the key role of LsrK kinase in controlling the auto-sustainment of QS and the diverse mechanisms involved in antimicrobial resistance. (Created with BioRender).

The multifunctional capabilities of LsrK in the bacterial virulence and AMR make this kinase an attractive candidate for drug development. To date, this target is almost unexplored, and few compounds have been proposed as LsrK inhibitors. However, these pilot studies pave the way for a more complete understanding of the role of the bacterial LsrK kinase inhibition in the autoinducer-2-mediated QS cascade and will allow for the discovery of novel innovative non-antibiotic therapies [8,11]. To date, LsrK inhibitors have been identified by exploiting different indirect strategies (Figure S1) [12]. Briefly, meclofenamic acid (**1**) and its halogenated derivatives **3–8** (Figure S1A) were identified via a structure-based virtual screening approach performed on a LsrK homology model developed before the X-ray structures of the kinase were resolved [15]. Compounds **9–12** (Figure S1B) were designed by a member of our group by exploiting the structure–activity

relationship deciphered around the main backbone of DPD; a spirocyclohexyl-dioxolane moiety replaced the diol portion essential for LsrK-mediated phosphorylation, whereas the diketo group was embedded in diverse heteroaromatic rings [16]. Lastly, several structural unrelated LsrK inhibitors (Figure S1C) were identified through an automatized target-based HTS, with hapargoside and rosolic acid being active in a cell-based AI-2 QS interference assay [17]. Although the LsrK inhibitory activity for these compounds is reported, no experimental evidence of their binding to the protein was still investigated. Moreover, to complicate the canvas, we still lack experimental evidence of the binding mode of DPD or LsrK inhibitors with the protein, and the putative catalytic binding site has just been postulated thanks to the three LsrK crystallographic structures (PDB ID: 5YA0, 5YA1, and 5YA2) [18] together with the homology comparison with other bacterial kinases, such as glycerol kinase (GK) or xylulose kinase (XK) [18]. As part of our ongoing research on the bacterial LsrK, in this paper we present the first application of the tryptophan fluorescence spectroscopy (TFS) for studying the ligand–LsrK binding, and we applied this biophysical assay to cherry-pick compounds that potentially target the bacterial kinase from an in-house library of small molecules and secondary metabolites. In addition, the conformational changes of LsrK upon binding with the identified ligands were discussed on the basis of circular dichroism (CD) and the binding of the ligands to the protein was lastly rationalized via molecular docking. These facile methods can provide binding mechanisms between LsrK kinase and potential inhibitors and may facilitate the discovery of novel drugs for fighting AMR through an innovative mechanism of action.

2. Results and Discussion

Among the diverse techniques useful for studying the protein–ligand binding, we selected the TFS. Indeed, tryptophan is known to emit intrinsic fluorescence that can be measured via fluorescence spectroscopy [19]. Interactions with binding ligands can change the tryptophan microenvironment, resulting in shifts of the maximum fluorescence peak and variations in intensity [20]. Bacterial LsrK is rich in tryptophan (Figure 2A), with thirteen highly conserved tryptophan residues among diverse bacteria species (e.g., *Enterobacter* sp., *Klebsiella pneumonia*, *Yersinia pestis*, *Escherichia coli*, *Salmonella choleraesuis*, and *Salmonella typhimurium*). Only *E. coli* and both *Salmonella* spp. LsrKs possess an additional tryptophan residue in positions 356 and 471, respectively. The location of tryptophan residues on the protein was highlighted on the crystallographic structures of *Escherichia coli* LsrK (*ecLsrK*). As illustrated in Figure 2B,C, four of the thirteen conserved tryptophan residues (Trp43, Trp277, Trp320, and Trp435) are situated close to the catalytic binding pocket, while the remaining are distributed across the protein's surface. In particular, Trp435 and Trp277 are in close contact with ATP, whereas Trp43 and Trp320 are part of the catalytic cleft, where DPD is supposed to bind.

The ubiquitous presence of tryptophan residues in the LsrK led us to hypothesize that changes in the intrinsic tryptophan fluorescence, upon ligand binding, might effectively reflect the strength of the LsrK–ligand interactions and the protein conformational change [20]. Based on these premises, we firstly investigated via TFS the secondary metabolite produced by some species of lichen, namely fumarprotocetraric acid (FP), which has been isolated by our group within a nature-aided drug discovery (NADD) program. FP was used as pilot molecule to investigate its binding with LsrK, since it has been demonstrated as a promising LsrK inhibitor (IC_{50} of 7 μ M) [17] and strong antimicrobial agent [21]. Recombinant LsrK was expressed as previously reported [9]. LsrK was titrated by the successive addition of FP (from 0.01 nM up to 4 μ M). An amount of 1 μ L of the appropriate concentration of ligands was progressively added so as to contain the overall dilution of the protein solution below 1%. The fluorescence quenching at 330 nm (the maximum in fluorescence emission) was analyzed. Figure 3A shows the quenching of the fluorescence emission of the Trp residue of *ecLsrK* by the addition of aliquots of FP solution. The LsrK fluorescence decreased with the increasing compound concentrations, which suggests that this decrease reflected the interaction of the bacterial kinase with FP. The absence of considerable changes in the

The analysis of the data using the modified Stern–Volmer equation is the classical model used in the interpretation of some results of fluorescence quenching in protein solution when the Stern–Volmer plot negatively deviates from linearity, [26–33] since it allows for investigating in detail the quenching mechanism that is occurring between ligands and proteins. Indeed, the linearity of the modified Stern–Volmer plots indicates static quenching (i.e., binding) in the presence of an inaccessible population of fluorophores and the plot allows for the calculation of the dissociation constant (K_D) and the percentage of fluorophores accessible to the quenchers. f_a is calculated as the reciprocal of the intercept, whereas K_D is given by the product of f_a and the slope of the line interpolating the points in the graph [34]. Accordingly, FP binds LsrK with static quenching to 26% of accessible Trp residues, and results in a K_D of 217 ± 8 nM (Figure 3B).

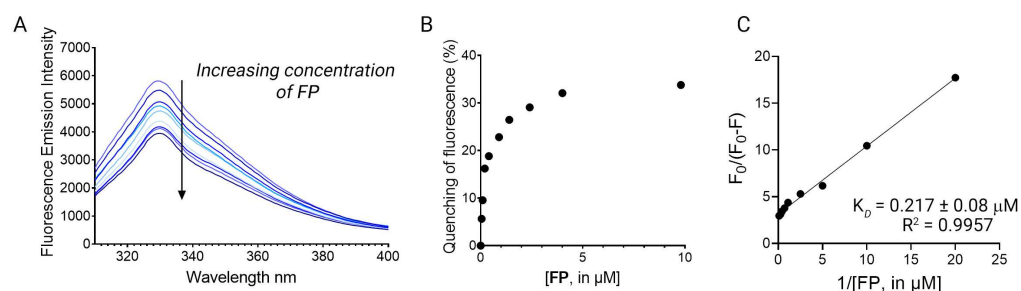


Figure 3. (A) The fluorescence quenching spectra of LsrK in the presence of FP, [LsrK] = 0.1 μM, [ligand] ranging from 0 to 10 μM, T = 298 K, pH = 7.4, λ_{ex} = 280 nm; the experiments were performed in triplicate; (B) % of fluorescence quenching vs. ligand concentration. (C) Modified Stern–Volmer plot for the binding of FP to LsrK. The points are interpolated using linear regression for the quantification of the K_D ; the values ($\pm$ SD) are the results of three independent measurements.

Once the suitability of TFS in studying the ligand–LsrK interaction was proven, following the encouraging results obtained with FP we screened our in-house compound library for potential ligands that could bind to LsrK. Such chemical libraries are usually assembled from compounds and intermediates produced during other medicinal chemistry investigations performed by research groups in academia and industry. The test’s small molecules were primarily obtained from an in-house collection of compounds (MedChemLab, MCL, Department of Drug Sciences, University of Pavia, Pavia, Italy). The MCL compound library consists of non-commercial small molecules synthesized and assessed during several drug discovery projects performed in the past years by our research group, including several secondary metabolites extracted from the proper plant drugs during nature-aided drug discovery programs [35–44]. Representative compounds of the MCL library were initially assessed in a low-throughput primary screening against recombinant LsrK via TFS. All the compounds were screened in duplicate at a 100 μM concentration against a 0.1 μM solution of LsrK. The intrinsic fluorescence spectra were then collected in the range of 300–400 nm by keeping the excitation wavelength at 295 nm and the fluorescence emission reduction at 330 nm (the maximum of the emission spectra) was measured. To establish a cutoff point for quenching, we chose FP as a reference compound (e.g., reduction in the fluorescence emission > 30% at the tested concentration). Only four compounds (Figure 4) significantly decreased the LsrK fluorescence by more than 30%, namely dimethyl (2R,3S)-3-hydroxy-5-oxotetrahydrofuran-2,3-dicarboxylate (**Hib-ester**), 5-hydroxy-2H-pyran-6-carbaldehyde (**Hib-carbaldehyde**), (R)-(-)-aloesaponol III 8-methyl ether [(**R**)-ASME], and (2S,3R)-3-(6-fluoronaphthalen-2-yl)-1,2-dimethylpyrrolidin-3-ol (**MCL-022**), and they were selected for further characterization. Interestingly, **Hib-ester** and **Hib-carbaldehyde** are two secondary metabolites isolated from the calyces of *Hibiscus sabdariffa*, [35–37] whereas (**R**)-ASME was isolated from *Eremurus persicus* [38]. Conversely, **MCL-022** is a synthetic compound initially designed as an antinociceptive agent [39,40].

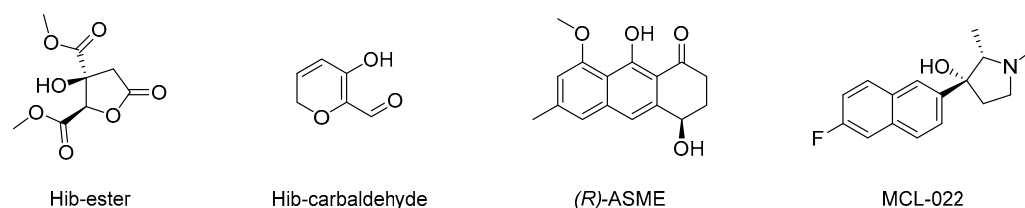


Figure 4. Chemical structure of dimethyl (2*R*,3*S*)-3-hydroxy-5-oxotetrahydrofuran-2,3-dicarboxylate (**Hib-ester**), 5-hydroxy-2H-pyran-6-carbaldehyde (**Hib-carbaldehyde**), (*R*)-(-)-aloesaponol III 8-methyl ether [(*R*)-**ASME**], and (2*S*,3*R*)-3-(6-fluoronaphthalen-2-yl)-1,2-dimethylpyrrolidin-3-ol (**MCL-022**).

The four primary hits were validated using dose-response studies to avoid false-positive results, and the K_D was determined. The intrinsic fluorescence of the four primary hits alone was recorded first, to confirm the absence of interference between the fluorescence of the compounds with that of the protein. **Hib-ester**, **Hib-carbaldehyde**, and (*R*)-**ASME** did not emit fluorescence at 330 nm when excited at 295 nm. Conversely, **MCL-022** possessed intrinsic fluorescence in the experimental conditions and it was therefore marked as false-positive and discarded. LsrK was titrated by the successive addition of selected compounds as described for **FP**. Figure 5A shows the fluorescence emission spectra in the range 300–400 nm of LsrK in the presence of increasing concentrations of the ligand. **Hib-ester**, **Hib-carbaldehyde**, and (*R*)-**ASME** quenched the intrinsic fluorescence of LsrK in a dose-dependent manner, whereas no quenching of fluorescence emission was detected when only PBS was added to the mixtures of the recombinant protein. Analysis of the tryptophan quenching data using the modified Stern–Volmer equation showed the dependence of $F_0/\Delta F$ on the reciprocal values of the quencher concentration $[Q]^{-1}$ exhibited a good linear relationship, thus confirming that the quenching mechanism between the ligands and LsrK occurred through a static quenching, resulting in K_D values of 78, 140, and 190 nM for **Hib-ester**, **Hib-carbaldehyde**, and (*R*)-**ASME**, respectively (Figure 5B,C). To assess whether these compounds might compete with ATP for the binding to LsrK, the binding of ATP for LsrK was studied via TFS, resulting in a K_D of 5.07 ± 0.23 μ M (Figure S2). The identified secondary metabolites possess a binding affinity 26–63-fold higher than ATP, and therefore they might considerably compete with and displace ATP from its binding site. This suggests a possible ATP-competitive inhibition of the LsrK kinase.

To characterize in more detail the interaction between the selected natural products and LsrK, we referred to circular dichroism (CD) [45,46]. More specifically, we measured the Far-UV CD spectrum of the bacterial protein in the presence or absence of a saturating concentration of each compound. The results were expressed as the mean residue ellipticity (MRE in $\text{deg cm}^2 \text{ dmol}^{-1} \text{ res}^{-1}$, Equation (S1)). Far UV-CD spectra of apo-LsrK at 2 μ M revealed a positive band at 195 nm and a negative band with two minimum at 208 nm ($n \rightarrow \pi^*$) and 223 nm ($\pi \rightarrow \pi^*$), characteristic of an α -helix structure. Deconvolution of the CD spectrum by using BestSel [47] confirmed that the protein is a helix-strand protein and allowed us to estimate the relative content of the secondary structure elements of the protein (Table S1). This result agrees with the crystallographic structure of the protein and the secondary structure content calculated from the available crystallographic structure of the apo-LsrK (PDB ID: 5YA0) [18]. Figure 6 shows the CD spectra of LsrK in the complex with the ligands, in comparison with the spectra of LsrK alone. The presence of each compound modifies the spectrum of the protein, in terms of both an increase in the maximum and a decrease in the minimum, suggesting a change in its conformation as a result of an interaction with the ligands. Briefly, the CD spectra of LsrK upon binding with the four ligands at a molar ratio ligand:LsrK of 1:1 showed an increased ellipticity value at 195 nm and decreased ellipticity at 208 and 223 nm, thereby illustrating that an increase in the α -helical content occurred. Conversely, a slight reduction in the β -sheets and, more importantly, in the turns and the random portions of the protein, was observed. No further variations were observed at a ligand concentration ten times higher than the protein, thus revealing the reaching of saturation binding at 2 μ M, as already observed

during the TFS studies. This variation was much more marked for **FP**, **Hib-carbaldehyde**, and **(R)-ASME** than for **Hib-ester**. This suggests a shift of LsrK towards a more ordered conformation, upon complex formation with the ligands. The variation in the secondary structural components of LsrK upon binding with the four ligands was calculated. When **FP** was present, the contents of the α -helix, β -sheet, β -turn, and random coil structures were changed to 49.5%, 17.6%, 2.6%, and 30.3%, respectively. In the presence of **(R)-ASME**, the contents of the α -helix, β -sheet, β -turn, and random coil structures were changed to 47.0%, 16.8%, 0%, and 36.2%, respectively. Similarly, in the presence of **Hib-ester**, the contents of the α -helix, β -sheet, β -turn, and random coil structures were changed to 47.2%, 15.6%, 6.4%, and 30.8%, respectively. Only **Hib-carbaldehyde** induced little variation in the LsrK conformation. Therefore, the CD experimental analysis showed that the interaction of the identified hit compounds with LsrK clearly lead to conformational changes in the protein, thus confirming their binding.

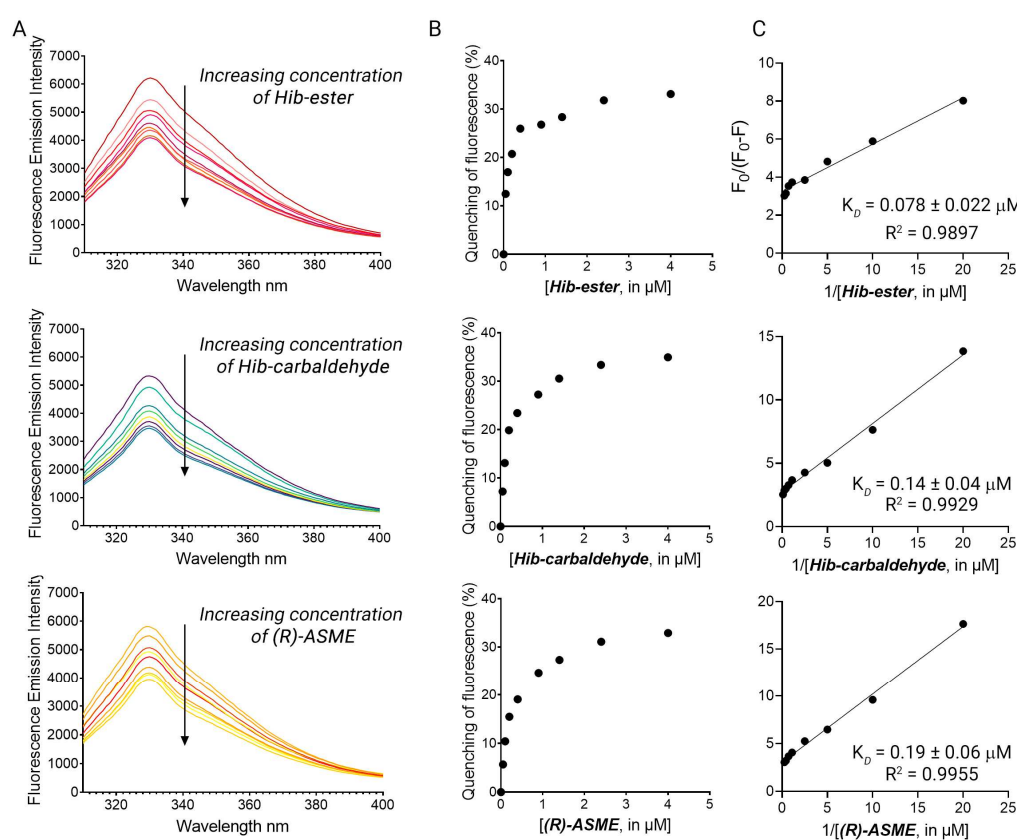


Figure 5. (A) The fluorescence quenching spectra of LsrK in the presence of **Hib-ester**, **Hib-carbaldehyde**, and **(R)-ASME**, [LsrK] = 0.1 μM, [ligand] ranging from 0 to 4 μM, T = 298 K, pH = 7.4, λ_{ex} = 280 nm; the experiments were performed in triplicate; (B) % of fluorescence quenching vs. ligand concentration. (C) Modified Stern–Volmer plot for the binding of **Hib-ester**, **Hib-carbaldehyde**, and **(R)-ASME** to LsrK. The points are interpolated using linear regression for the quantification of the K_D; the values (±SD) are the results of three independent measurements.

To rationalize the spectroscopic results at the molecular level and to identify the likely binding mode of **FP**, **Hib-ester**, **Hib-carbaldehyde**, and **(R)-ASME** to LsrK, molecular docking was performed with Glide, which is part of the Schrödinger Platform: Maestro [48–50]. More specifically, an X-ray structure of LsrK (in a complex with ATP; PDB ID: 5YA1) was used as receptor for docking [18]. The receptor grid was centered onto the co-crystallized ligand, and ATP and the docking of the four identified secondary metabolites was performed in standard precision mode (“Glide SP”). The docking results indicate that the four investigated small molecules are likely to bind to LsrK in an ATP-competitive manner (Table 1 and Figure 7). With the exception of **Hib-ester**, all of the generated dock-

ing poses extend to the deepest part of the ATP binding pocket and are characterized by good protein-ligand surface complementarity. The most convincing and, at the same time, top-ranked docking pose obtained for any of the investigated ligands was obtained for **(R)-ASME** (GlideScore—8.184 kcal mol^{−1}). It is noted that the docking scores (Table 1) do not correlate with the measured K_D values. Considering the facts that (i) the highest and lowest measured K_D values measured for these compounds differ only by a factor of 2, (ii) the capacity of scoring functions to predict binding free energies is inherently limited, and (iii) the number of data points to test for any correlation is extremely low, this result is well within expectations. Focusing on the binding mode, ATP and all investigated ligands are predicted to form aromatic (or hydrophobic) interactions with Y341. Ligand binding is expected to be sustained by a network of hydrogen bonds (Table 1). Indeed, all investigated ligands are predicted to form at least two hydrogen bonds, primarily with R319, K431, T342, Y342, and/or D339, of which at least one hydrogen bond is known to be formed between ATP and the protein (namely, R319). Overall, the results obtained from docking indicate an ATP-competitive binding of the four compounds of interest.

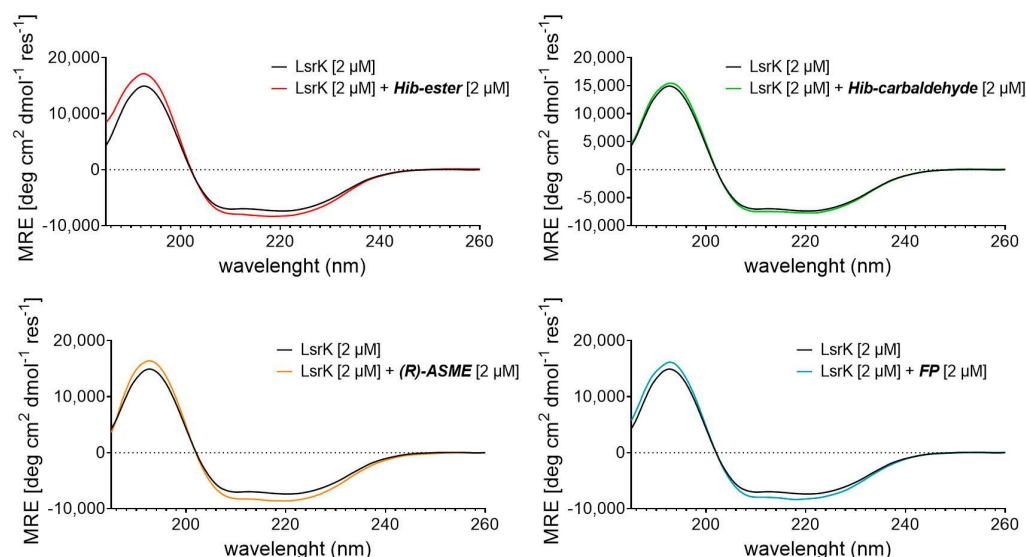


Figure 6. Far-UV CD spectra of LsrK in presence or absence of the indicated compounds. The reported spectra are the average of six scans and corrected for buffer blank.

Table 1. Docking scores and (predicted) interactions of ATP, FP, Hib-ester, Hib-carbaldehyde, and **(R)-ASME** with LsrK.

Compound	Residues Involved in the Binding	GlideScore [kcal mol ^{−1}]
ATP	G315 ^{b,d} , R319 ^{a,c} , R322 ^{a,c} , Y341 ^{a,c} , K431 ^{a,c} , Y341 ^{b,c}	n/a
FP	G315 ^{b,d} , R319 ^{a,c} , R322 ^{a,c} , Y341 ^{a,c} , Y341 ^c	−6.490
Hib-ester	G315 ^{b,d} , R319 ^{a,c} , Y341 ^{b,c}	−4.984
Hib-carbaldehyde	T342 ^{a,c} , K431 ^{a,c} , Y341 ^{b,c}	−6.169
(R)-ASME	D339 ^{a,c} , K431 ^{a,c} , E345 ^{a,c} , Y341 ^{b,c}	−8.184

^a H-bond; ^b aromatic/hydrophobic interaction; ^c interactions formed with the side chain; ^d interactions formed with the backbone. n/a not available.

Once the capability in binding LsrK was demonstrated, the efficacy of the three identified compounds in inhibiting the biofilm formation was evaluated on the culture of *Staphylococcus* spp. [7]. **Hib-ester**, **Hib-carbaldehyde**, and **(R)-ASME** were initially assessed at 40 µg/well and the biofilm inhibitory activity was reported as percentages of the biofilm produced by the positive control (Figure 8A). Despite the sub-micromolar affinity toward LsrK, **Hib-ester** did not exert any effect on biofilm formation at the tested concentration. Conversely, **Hib-carbaldehyde** and **(R)-ASME** caused more than 40% biofilm

inhibition at the tested concentration. Clearly, the most active compound was **(R)-ASME**, with a biofilm inhibition rate of $60\% \pm 15$ at $40 \mu\text{g}/\text{well}$, followed by **Hib-carbaldehyde** that exerted a biofilm inhibition rate of $47\% \pm 22$ at the same concentration. Statistical analysis confirmed the significance of the results. **(R)-ASME**, which resulted as the most effective compound, was further assessed in a dose-response study resulting in an EC_{50} of $14 \pm 4 \mu\text{g}/\text{well}$, thus indicating its high potential as an effective inhibitor of biofilm formation (Figure 8B). Interestingly, both **Hib-carbaldehyde** and **(R)-ASME** did not negatively affected the bacterial viability (measured as planktonic bacterial growth), thus demonstrating that the antibiofilm activity is not correlated with any intrinsic antibacterial activity of the compounds.

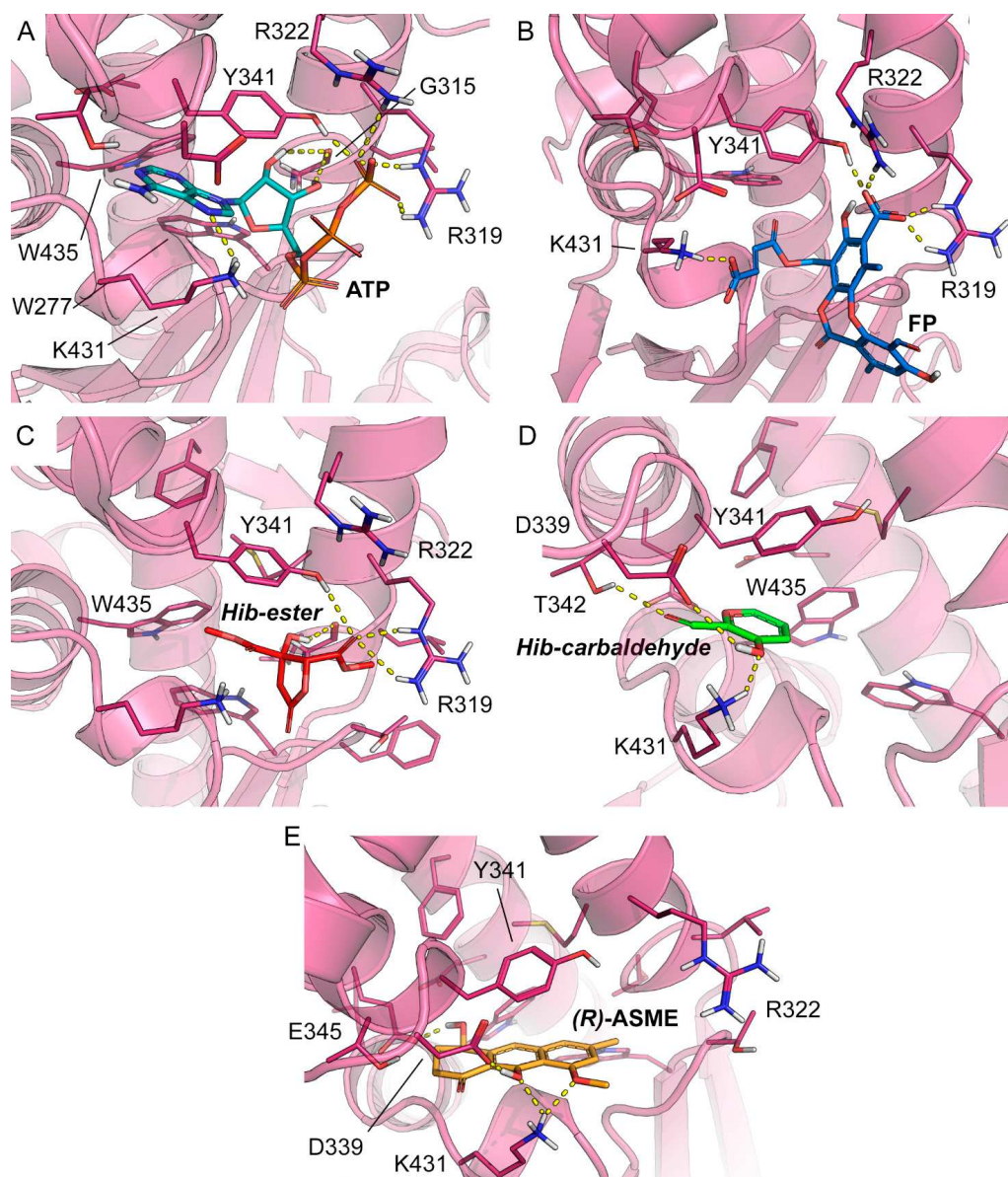


Figure 7. Co-crystallized pose of (A) ATP (teal carbon atoms) and the binding poses predicted for (B) FP (blue carbon atoms), (C) **Hib-ester** (red carbon atoms), (D) **Hib-carbaldehyde** (green carbon atoms), and (E) **(R)-ASME** (orange carbon atoms). The reference crystal structure used for the docking calculation (PDB ID: 5YA1) of LsrK is shown in the magenta cartoon; important interacting residues are visualized in sticks representation with magenta carbon atoms. Model atoms except for carbons are color-coded: oxygen (red) and nitrogen (blue). Hydrogen bonds are represented as yellow dotted lines.

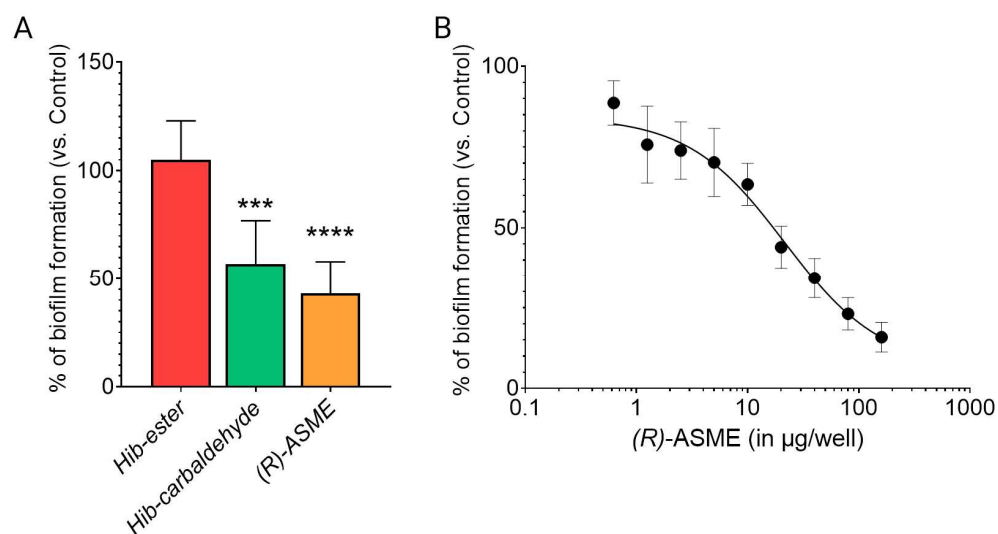


Figure 8. Effect of inhibitors on biofilm formation by staphylococci. **(A)** Staphylococcal cells were incubated with 40 µg/well of each inhibitor at 37 °C for 24 h. Biofilm was stained with crystal violet, and the absorbance was measured at 595 nm. **(B)** Staphylococcal cells were incubated with increasing concentration of ASME at 37 °C for 24 h. Biofilm formation was detected as indicated above. Values are expressed as percentage of the control (no inhibitor added). Data indicate the average of two independent experiments, each performed in quadruplicate. *p* values were determined using a two-tailed, two-sample unequal variance Student's *t*-test in Prism 4.0 (GraphPad, Prism 4.0, San Diego, CA 92121, USA) with *** *p* < 0.001 and **** *p* < 0.0001 vs. control.

3. Materials and Methods

3.1. Isolation and Purification of Hib-Ester and Hib-Carbaldehyde

The chalice of *Hibiscus sabdariffa* were finely ground using a knife mill, suspended in 80% ethanol, and subjected to microwave radiation. The mixture was cooled to room temperature, filtered, and concentrated under a reduced pressure to obtain the crude extract as a thick reddish oil. The crude was purified via a liquid–liquid extraction and the organic phase was divided into two aliquots and concentrated. The two aliquots were treated in different ways for the isolation of the two metabolites. For the purification of **Hib-ester**, the crude was dissolved in methanol and treated with the PS carbonate anionic resin (Polymer-supported carbonate) to catch all the acidic species contained in the extract. The resin was then treated with 0.1% HCl in methanol, for the recovery of the retained metabolites, thus obtaining a simplified fraction which was further purified via silica gel chromatography to give **Hib-ester** in a 0.22% yield. The yield was calculated on the weight of the vegetable drug used. The second aliquot of crude was subjected to a liquid–liquid extraction using dichloromethane and water. The organic phase was fractionated via chromatography on a silica gel, to isolate **Hib-carbaldehyde** in a 1.1% yield over the weight of the plant drug used.

3.2. Isolation and Purification of (R)-ASME

The dried roots of *E. persicus* were extracted with ethanol under microwave irradiation. The suspension was filtered, and the collected solution was concentrated. The ethanolic extract was partitioned between water and dichloromethane. The organic layer was collected, dried, and concentrated. The title compound was purified from the crude via crystallization from acetone.

3.3. Isolation and Purification of Fumarprotocetraric Acid

Thalli of *C. foliacea* were finely grinded and extracted with ethyl acetate under mw irradiation. The crude extract was initially treated with methanol to promote the crystallization of usnic acid. The filtrate was concentrated and the fumarprotocetraric acid was

recovered via precipitation from the secondary extract via treatment with a mixture of chloroform:ethanol 1:2.

3.4. Expression and Purification of LsrK

Culture was grown at 37 °C to an OD₆₀₀ of 0.3, transferred to 22 °C, and grown to an OD₆₀₀ of 0.9. Expression was subsequently induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) (Inalco, Milan, Italy) for 9 h. Cells were harvested and resuspended in 25 mM potassium phosphate, pH 7.0, 50 mM NaCl, 10 mM β-mercaptoethanol, 1 mM MgCl₂, 2.5 μg mL^{−1} DNase, and protease inhibitors and lysed via sonication (70% amplitude, 12 × 30" on/off, 1'30" interval between sonication steps). The cell debris was removed via centrifugation and proteins purified from the supernatants via Ni⁺²-affinity chromatography on a HiTrap chelating column (GE Healthcare, Buckinghamshire, UK). Protein purity was assessed via 12.5 % SDS-PAGE and Coomassie Brilliant Blue staining. A bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) was used to measure the concentration of purified proteins.

3.5. Fluorescence Binding Studies

Fluorescence quenching experiments were carried out on Jasco spectrofluorometer (FP-6200) using a 10-mm quartz cuvette. The temperature was maintained at 25 ± 0.1 °C using an external thermostatic Peltier device. The stock solution of the ligands was prepared in DMSO and the working solution at the appropriate concentration was prepared in PBS. Protein solution (0.1 μM) was titrated with increasing concentrations of ligand and the fluorescence emission spectrum was recorded in the range of 310–400 nm by keeping the excitation wavelength constant at 295 nm. The excitation and emission slit widths were kept at 5 nm. The final spectra were obtained by subtracting with the corresponding blank. For data analysis, the fluorescence intensity at λ_{max} was plotted against [ligand, μM] and then the modified Stern–Volmer equation (Equation (1)) was used to derive binding parameters viz. binding dissociation constant (K_D).

3.6. Circular Dichroism

Far-UV (195–250 nm) CD measurements were performed at 20 °C in a 0.1-cm path-length quartz cuvette. CD spectra were recorded on a Jasco J-720 spectropolarimeter. Appropriate blanks corresponding to the buffer were subtracted to correct the absorbance or fluorescence background. The results are expressed as the mean residue ellipticity (MRE in deg cm² dmol^{−1} res^{−1}), which is defined using the following equation:

$$\text{MRE} = \frac{\text{ObservedCD (mdeg)}}{C_p \times n \times l \times 10} \quad (1)$$

where n is the number of amino acid residues (530 for LsrK), l is the path length of the cell (0.1 cm), and C_p is the molar (M) concentration of the protein. All the spectroscopic measurements were performed in 20 mM phosphate buffer pH 7.4. Six scans were averaged for each spectrum, and the contribution from the buffer was subtracted in each case.

3.7. Docking

Docking with Glide in standard precision mode ("SP mode") was performed on the X-ray structure of LsrK in the complex with ATP (PDB 5YA1). The structure was imported into the Schrödinger Platform for small molecule drug discovery. Next, the X-ray structure was interpreted and processed with the Protein Preparation Wizard (part of the platform) with default settings. As part of this process, the structure was checked for completeness, hydrogens were added, hydrogen bonds optimized, solvent molecules removed, and a restrained minimization of the structure was performed with the OPLS4 force field and the default convergence RMSD tolerance of 0.3 Å. In preparation for docking with Glide, a receptor grid was generated. The enclosing box was centered onto the co-crystallized ligand, ATP. Its size was defined based on the amount of ATP (i.e., the option "dock ligands

similar in size to the workspace ligand" was used). The docking algorithm was run with default settings, i.e., the enabled sampling of nitrogen inversions, enabled sampling of ring conformations, and enabled biasing of the sampling of torsions for all predefined functional groups. The hydroxy groups of Y341 and T342 were defined as flexible.

3.8. Biofilm Formation

Overnight cultures of staphylococci were diluted 1:200 in Brain Heart Infusion broth (BHI) containing 0.5% glucose. Aliquots (200 µL) of the diluted bacterial suspensions were added to 96-well flat-bottom sterile polystyrene microplates (Costar; Corning, New York, NY, USA) and incubated statically for 24 h at 37 °C in the presence or absence of inhibitors. Biofilms formed on the plates were gently washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ [pH 7.4]) to remove planktonic and loosely adhering bacteria. Adherent cells were fixed with 25% formaldehyde for 10 min, stained with 0.1% crystal violet for 15 min, and after several washings, the wells were air-dried. For a quantitative estimation of the biofilm density, bound crystal violet was solubilized with 10% glacial acetic acid, and the absorbance of the solubilized dye was read at 595 nm in a microplate reader (model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

4. Conclusions

To sum up, in this study we apply for the first time TFS as a simple and reliable analytical method for uncovering highly promising LsrK inhibitor candidates. Accordingly, we exploited TFS to identify within an in-house library of synthetic compounds and plant secondary metabolites potential LsrK-binding hit compounds that could provide for new anti-AMR therapeutic drugs. The fluorescence results indicated that the native fluorescence of LsrK is effectively quenched in the presence of the already published LsrK inhibitor **FP** and of the secondary metabolites **Hib-ester**, **Hib-carbaldehyde**, and **(R)-ASME**, herein identified via the static quenching mechanism, resulting in a binding affinity for the four compounds to LsrK in the sub-micromolar range. The changes in LsrK conformation observed via CD spectroscopy upon treatment of the protein with the identified hits confirmed the ligand–protein binding and supported the TFS results. Moreover, molecular docking indicated that the binding of the secondary metabolites likely occurs at the ATP binding site, with hydrogen bonds playing a key role in the anchoring of the ligand. Interestingly, **Hib-carbaldehyde** and **(R)-ASME** displayed activity against *Staphylococcus* spp. biofilm formation without negatively affecting bacterial viability. **(R)-ASME** resulted in the most potent compounds with an EC₅₀ of 14 mg/well, indicating its potential as an effective inhibitor of biofilm formation. Thus, this secondary metabolite holds promise as a potential candidate to serve as an effective anti-biofilm drug that can mitigate the risk of bacterial resistance.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules28062542/s1>, Figure S1: Chemical structure, LsrK inhibitory activity and approach exploited for the identification of the LsrK inhibitors reported in literature. Figure S2: % of fluorescence quenching vs. ATP for the binding of ATP to LsrK. Table S1: Percentage of secondary structural components in LsrK.

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Catch Bond-Mediated Adhesion Drives *Staphylococcus aureus* Host Cell Invasion

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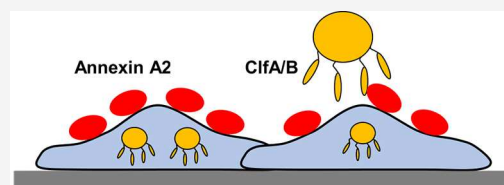
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Supporting Information

ABSTRACT: Various viruses and pathogenic bacteria interact with annexin A2 to invade mammalian cells. Here, we show that *Staphylococcus aureus* engages in extremely strong catch bonds for host cell invasion. By means of single-molecule atomic force microscopy, we find that bacterial surface-located clumping factors bind annexin A2 with extraordinary strength, indicating that these bonds are extremely resilient to mechanical tension. By determining the lifetimes of the complexes under increasing mechanical stress, we demonstrate that the adhesins form catch bonds with their ligand that are capable to sustain forces of 1500–1700 pN. The force-dependent adhesion mechanism identified here provides a molecular framework to explain how *S. aureus* pathogens tightly attach to host cells during invasion and shows promise for the design of new therapeutics against intracellular *S. aureus*.

KEYWORDS: single-molecule experiments, *Staphylococcus aureus*, clumping factors, annexin A2, host invasion, catch bonds



Annexins are a group of human proteins that play multiple functional roles, such as controlling membrane scaffolding, promoting trafficking and vesicle organization, mediating cell–cell interactions and, in the extracellular environment, inducing fibrinolysis, coagulation, inflammation, and apoptosis.^{1–3} An important member of the family is annexin A2, which is found in endothelial cells, epithelial cells, monocytes, macrophages, and most cancer cells. While annexin A2 is mostly present in the cytoplasm, it can also be found associated with the plasma membrane. This protein is composed of a variable “head” at the N-terminus and of a highly conserved “core” domain located at the C-terminus⁴ (Figure 1a). The N-terminal domain is a variable, intrinsically disordered region that contains sites for post-translation modifications including acetylation, serine/tyrosine phosphorylation, and for binding to S100A10, a protein that forms heterotetramers with annexin A2. The conserved C-terminal core domain is composed of four homologous repeats of about 70 amino acids, each consisting of a right-handed superhelix of five α -helices.⁵ The core domain interacts with calcium⁶ and with many biomolecules, including RNA,^{7–9} phospholipids,^{10–12} heparin,¹³ and F-actin.^{10,14}

Viruses¹⁵ and pathogenic bacteria¹⁶ interact with surface-exposed annexin A2 to favor colonization and invasion of host cells. Annexin A2 bridges cytomegaloviruses to cell membranes to enhance infection^{17–19} and binds to human papilloma virus L2 capsid protein^{20,21} and Enterovirus type 71 for host cell entry.^{22–24} Annexin A2 colocalizes with spike glycoprotein S2 protein of SARS CoV-2 suggesting a potential role of S2 protein in viral invasion.²⁵ Intracellular annexin A2 is targeted

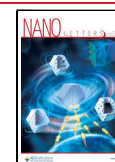
by the nonstructural protein 1 involved in the replication and pathogenesis of avian influenza virus.²⁶ Annexin A2 exposed on mammalian cells serves as a specific ligand for *Pseudomonas aeruginosa*, leading to its internalization.²⁷ Together with cellular proteins NHERF2, it forms a scaffold complex that links adjacent Tir molecules during attachment of enteropathogenic and enterohemorrhagic *Escherichia coli* to host cells.²⁸ The ligand also mediates specific binding to uropathogenic *E. coli*,²⁹ *Salmonella typhimurium*,³⁰ and *rickettsiae*,³¹ and the community-acquired respiratory distress syndrome toxin mediates annexin A2-dependent *Mycoplasma pneumoniae* interaction with eukaryotic cells and determines disseminated inflammation and tissue pathologies.³²

There is also compelling evidence that Gram-positive bacteria use annexin A2 for adherence and invasion. Pneumococcal surface protein K expressed by uncapsulated *Streptococcus pneumoniae* mediates bacterial adherence to epithelial cells via direct interaction with annexin A2.³³ *S. pneumoniae* adhesin A is involved in the annexin A2-dependent attachment and colonization of nasopharyngeal epithelial cells.³⁴ Western blotting, ELISA assays, and pull-down experiments showed that recombinant *Staphylococcus aureus* clumping factor A (ClfA; Figure 1b) interacts with annexin A2.³⁵ ClfA and

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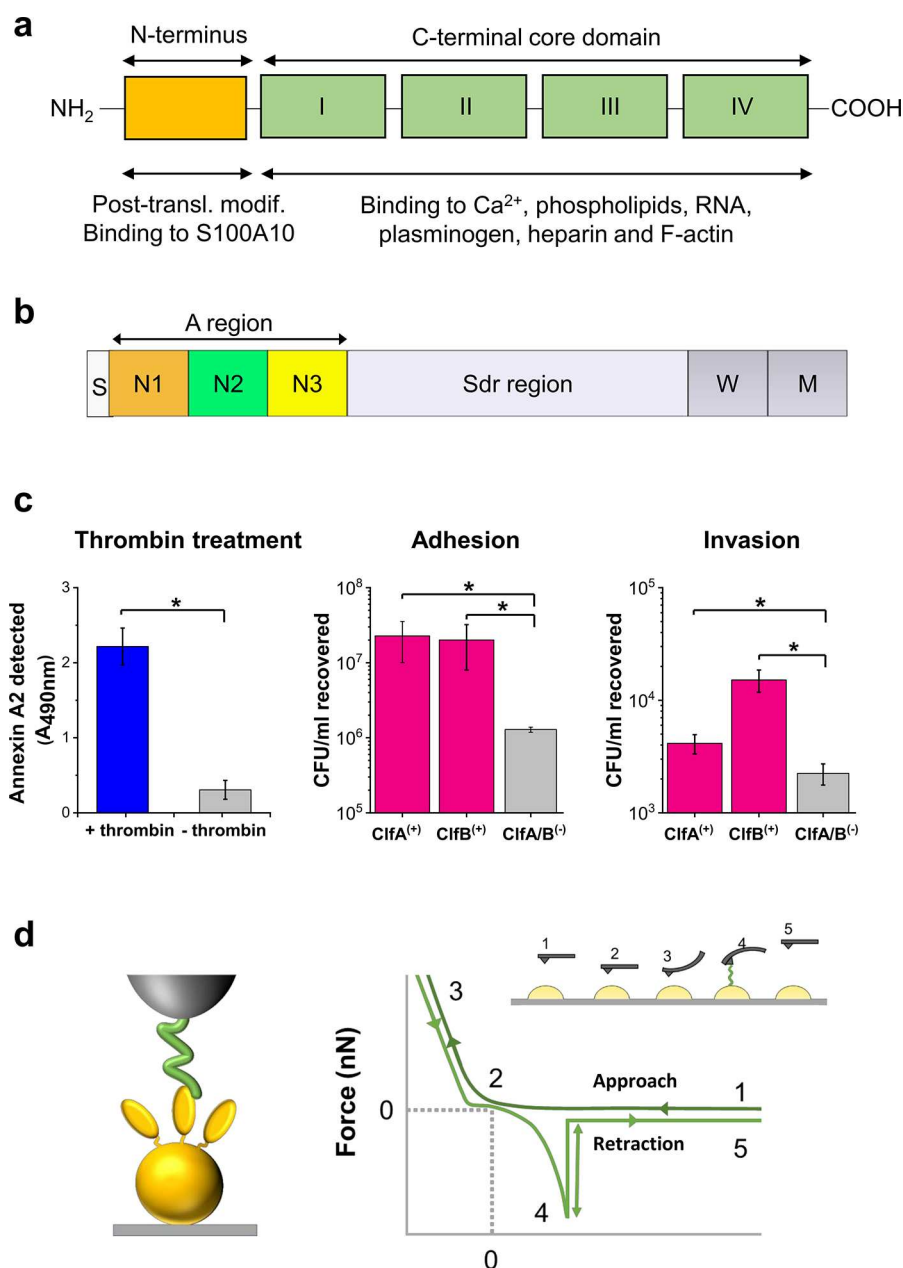


Figure 1. Studying the binding of annexin A2 by clumping factors A and B. (a) Annexin A2 (339 residues) is composed of two major domains, the amino-terminal domain or head region and the carboxyl-terminal core domain. The amino-terminal tail region is the site for post-translational modification and encompasses the redox reactive cysteine residues (Cys-8) and the nuclear export sequence (Val-3-Leu-12) and an amphipathic α -helix which binds to the S100A10 protein. The carboxyl-terminal core domain encompasses four predominantly α -helical repeats each containing 70 amino acids. Each repeat segment contains five α -helices in which four helices are oriented antiparallel and the fifth one is lying perpendicular to the other helices. The carboxyl-terminus core domain contains binding sites for heparin and RNA, calcium and phospholipids, plasminogen, and as well as for F-actin. The binding sites for viruses and bacteria remain to be determined. (b) Schematic diagram of the domain organization of ClfA (896 amino acid residues) and ClfB (933 residues) showing the ligand-binding A-region made of the N1N2N3 subdomains, the serine-aspartate repeat region (Sdr), the wall-spanning domain (W), and the membrane anchor (M). (c) Annexin A2 exposure on thrombin-treated or -untreated HUVEC cell monolayers was detected incubating the wells with an annexin A2 antibody (left). Host cell adhesion (center) and invasion (right) were tested by incubating *S. aureus* cells with thrombin-treated HUVEC cell monolayers. Inocula, adherent, and internalized bacteria were quantified by viable counting. Error bars show s.d. of the means from at least three determinations performed in triplicate (Student's test: *, $P < 0.05$). (d) The strength and dynamics of single adhesin–ligand interactions were studied by single-molecule force spectroscopy (SMFS) in which annexin A2-modified tips are used to register force–distance curves on *S. aureus* bacteria expressing exclusively ClfA or ClfB.

annexin A2 partially colocalize on the host cell plasma membrane, but essentially concentrate in the cytosol, indicating ClfA cellular uptake.³⁵ These *in vitro* observations suggest that ClfA binds to annexin A2 and that this interaction plays a role in *S. aureus* invasion. Along the same line, *S. aureus*

has recently been shown to use ClfB to bind annexin A2 for internalization by epithelial cells. Bacterial-associated annexin induced F-actin rearrangement and ClfB was identified in annexin-precipitated proteins from *S. aureus* lysates.³⁶ Pretreatment with recombinant ClfB triggered *S. aureus* internalization

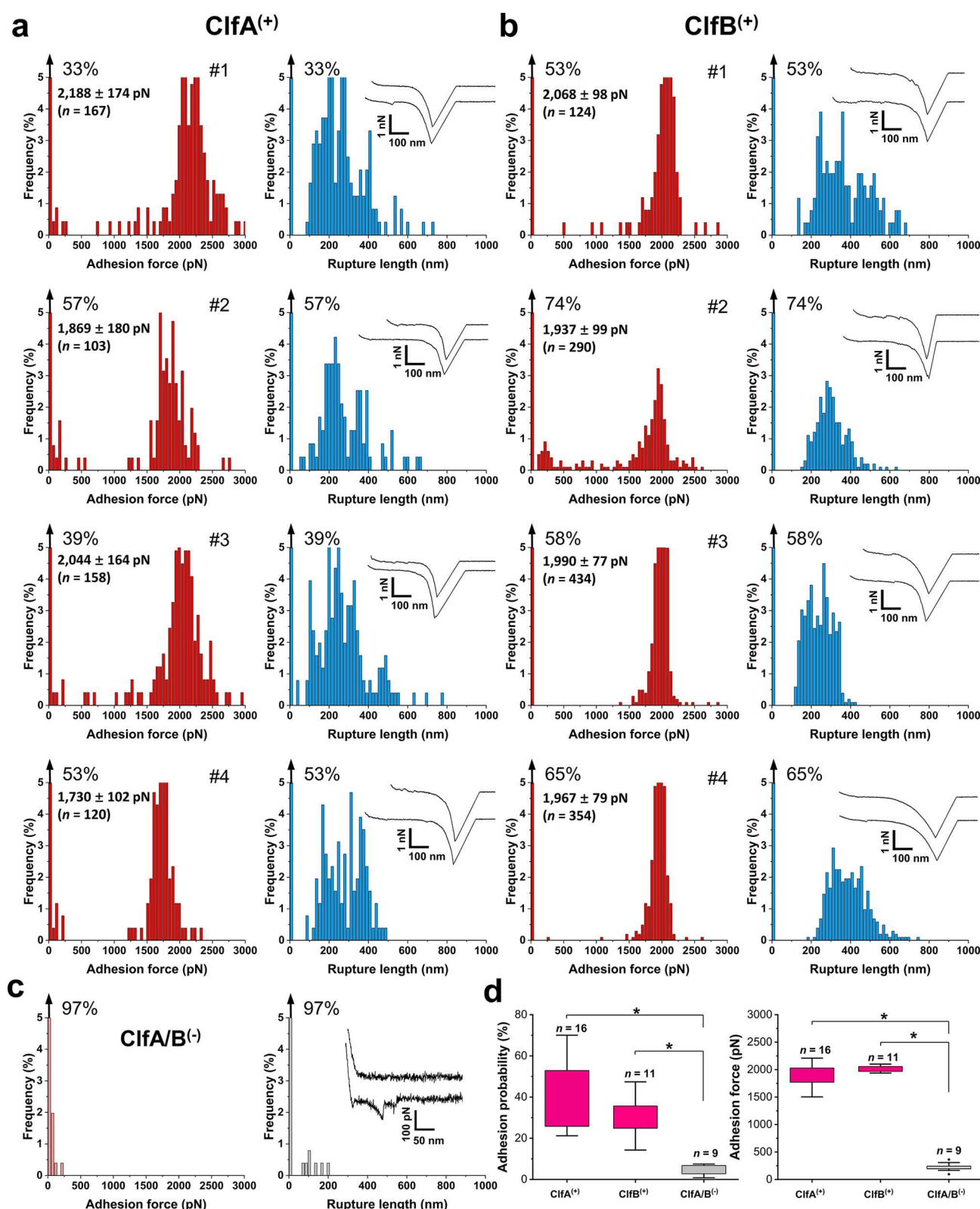


Figure 2. Strength of single clumping factor–annexin A2 interactions in living *S. aureus* bacteria. (a, b) Adhesion force (left) and rupture length (right) histograms obtained by recording force–distance curves in PBS between four different *S. aureus* ClfA⁽⁺⁾ and ClfB⁽⁺⁾ cells and AFM tips functionalized with annexin A2, at a retraction velocity of 1 $\mu\text{m s}^{-1}$. Insets show representative adhesive force profiles. (c) Representative force data obtained with *S. aureus* lacking ClfA and ClfB. (d) Box plot of adhesion probability (left) and adhesion force (right) for ClfA⁽⁺⁾ cells ($n = 16$ cells from 3 independent cultures), ClfB⁽⁺⁾ cells ($n = 11$ cells from 6 independent cultures), and ClfA/B⁽⁻⁾ cells ($n = 9$ cells from 2 independent cultures). P values were determined by Mann–Whitney test. *, $P < 0.05$ was considered as significant. If present, the arrow at the top left of a histogram stands for the nonadhesive events.

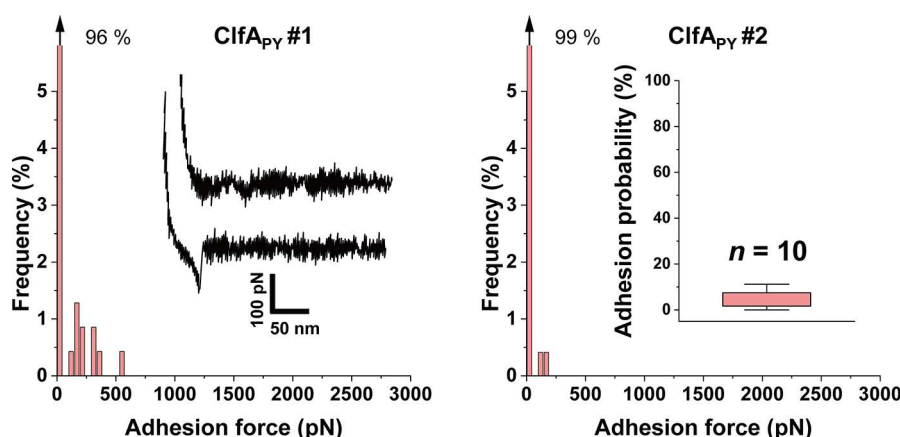


Figure 3. Substitutions within the ClfA N2N3 binding trench show that ClfA and annexin A2 engage in a dock lock and latch interaction. Representative adhesion force histograms with force curves (left inset) obtained in PBS between annexin-tips and two cells from the *S. aureus* ClfA_{PY} strain expressing ClfA carrying amino acid substitutions within the N2N3 binding trench that prevent DLL ligand-binding. Box plot (right inset) showing that the adhesion probability of ClfA_{PY} ($n = 10$ cells from 3 independent bacterial cultures) is dramatically reduced compared to the ClfA⁽⁺⁾ and ClfB⁽⁺⁾ strains.

and the action of the adhesin was specifically blocked by annexin antibodies.³⁶ While annexin A2-binding by Clf proteins appears to be important for host cell adhesion and invasion, the molecular details of this interaction are unknown. Here, we use single-molecule experiments on living bacteria to demonstrate that ClfA and ClfB form very high strength catch bonds with annexin A2, and that this force-enhanced adhesion is a key mechanism in *S. aureus* host cell invasion.

We initially tested the ability of ClfA and ClfB to bind surface-exposed annexin A2 for adhesion and invasion. Bacterial cells from the *S. aureus* SH1000 strain expressing either ClfA or ClfB (hereafter called ClfA⁽⁺⁾ and ClfB⁽⁺⁾ cells) but not the adhesins FnBPA and FnBPB, were incubated with HUVEC monolayers treated with thrombin as this molecule triggers a much higher exposure of annexin A2 on the plasma membrane (Figure 1c, left).³⁷ Hence, experimental conditions were used where the impact of adhesins such as FnBPA and B is significantly reduced and the exposure of the Clf ligand (annexin A2) optimized. Both ClfA⁽⁺⁾ and ClfB⁽⁺⁾ bacteria attached to HUVEC cells in large amounts, while attachment of bacteria lacking both adhesins was substantially lower (Figure 1c, center). In internalization assays (Figure 1c, right), we found that ClfA⁽⁺⁾ and ClfB⁽⁺⁾ bacteria invaded at substantial levels, with a more pronounced invasion for ClfB⁽⁺⁾ ones. These observations show that annexin A2 binding by ClfA and ClfB is important for *S. aureus* adhesion and invasion.

We sought to characterize the mechanical strength of the interactions of ClfA and ClfB with annexin A2 using single molecule force spectroscopy (SMFS; Figure 1d). Force–distance curves were registered between AFM tips functionalized with annexin A2 and living *S. aureus* bacteria. In Figure 2a and b we present the adhesion forces and rupture distances obtained for representative ClfA⁽⁺⁾ and ClfB⁽⁺⁾ cells (including cells from independent cultures; for data on more cells, see Figure 2d, Figure S1). Most curves featured well-defined, extremely strong adhesion forces, with a mean of 2005 ± 120 pN for ClfA (mean $\pm$ s.d., from a total of $n = 1978$ adhesive curves from 11 cells) and 1932 ± 160 pN for ClfB ($n = 1604$ adhesive curves from 16 cells). While the magnitude of strong forces slightly varied from one cell to another, their distributions were always narrow, implying they originate

from the rupture of single protein complexes. A multimodal force distribution is expected when a variable number of bonds break simultaneously, which was not observed here. Strong bonds ruptured at ~ 200 – 400 nm, reflecting extension of the intrinsically disordered C-terminal stalk domains of the adhesins (residues 543–896 in ClfA and 541–874 in ClfB) and of annexin (346 residues). The mechanically ultrastable A domains of Clfs are unlikely to unfold. Of note, adhesion force maps (Figure S2) documented heterogeneous distributions of ClfA and ClfB at the bacterial cell surfaces, with the formation of nanoclusters.

Several observations support the view that strong forces result from specific dock lock and latch (DLL) interactions: (i) ~ 2 nN forces were also observed with tips modified using an NHS surface chemistry (Figure S3) rather than a PEG-benzaldehyde linker; (ii) strong adhesion was missing on cells lacking ClfA and ClfB (Figure 2c, Figure S4); (iii) strong forces are in the range of that reported for prototypical DLL interactions;^{38–42} (iv) binding was essentially abolished when using an *S. aureus* strain (ClfA_{PY}) expressing ClfA carrying substitutions in key amino acids (specifically, Pro 336 and Tyr 338, both located in the N2 subdomain and concurring to the hydrophobic environment of the trench) involved in DLL binding (Figure 3, Figure S5).⁴¹

We next measured the strength of the Clf–annexin A2 bonds (F) while varying the rate at which force is applied (loading rate, LR , assessed from the force vs time curves). Figure 4a,b shows the dynamic force spectroscopy (DFS) data registered with retraction speeds ranging from $0.5 \mu\text{m s}^{-1}$ to $10 \mu\text{m s}^{-1}$ (data pooled from 9388 and 5312 adhesive curves on six ClfA⁽⁺⁾ and five ClfB⁽⁺⁾ cells, respectively). In line with the Bell–Evans (BE) theory,⁴³ a semilog relationship was observed between the most-probable forces and the LR s (Figures 4a,b), yielding a position of the energy barrier that separates the bound from the unbound state of $x_u = 0.063$ and $x_u = 0.077$ nm, and an off-rate constant at thermal equilibrium of $k_{\text{off}}^0 = 3.49 \times 10^{-12} \text{ s}^{-1}$ and $k_{\text{off}}^0 = 1.27 \times 10^{-14} \text{ s}^{-1}$, for ClfA and ClfB respectively. The very low k_{off}^0 values illustrate the high lifetime and mechanostability of the complexes.

The above results show that both adhesins feature similar kinetic parameters for the high forces. However, ClfA, but not ClfB, exhibited weaker intermediate forces, in the 1200–1700

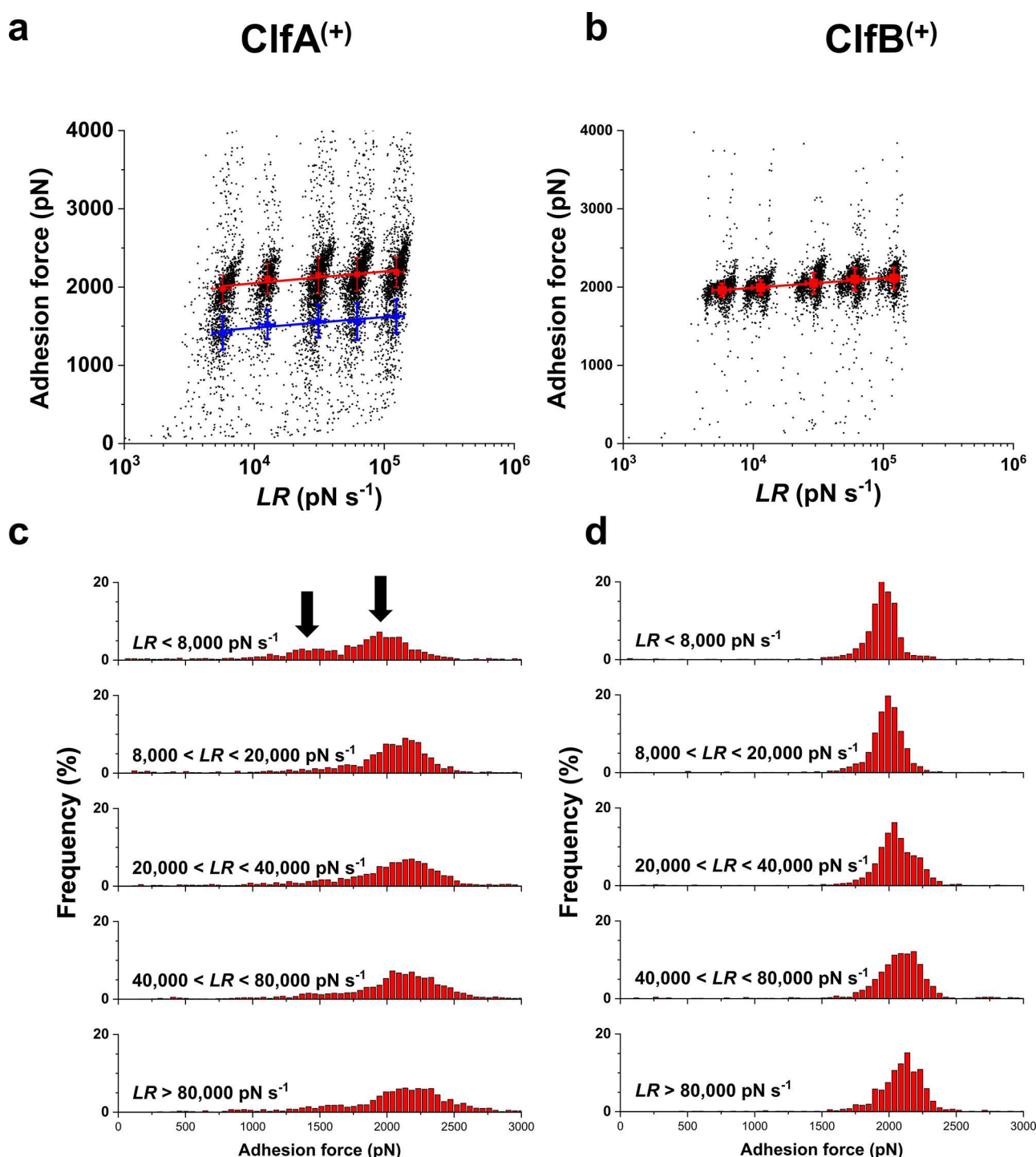


Figure 4. Dynamic force spectroscopy of the clumping factor-annexin A2 interactions. (a, b) DFS plots showing the distribution of the rupture forces vs loading rates between annexin A2-tip and ClfA⁽⁺⁾ (a) and ClfB⁽⁺⁾ cells (b). For ClfA⁽⁺⁾ cells, data were pooled from $n = 9388$ force curves from 6 cells from 4 independent bacterial cultures; ClfB⁽⁺⁾ cells, from $n = 5312$ force curves from 5 cells from 3 independent bacterial cultures. The red lines represent Bell-Evans model fit of the data, showing the expected loading-rate dependency. $x_u = 0.063$ nm and $k_{off}^0 = 3.49 \times 10^{-12}$ s⁻¹, $x_u = 0.077$ nm and $k_{off}^0 = 1.27 \times 10^{-14}$ s⁻¹, for ClfA and ClfB were extracted, respectively. For ClfA⁽⁺⁾, a second fit at lower forces is shown (blue), with $x_u = 0.068$ nm and $k_{off}^0 = 3.47 \times 10^{-9}$ s⁻¹. Error bar indicates s.d. of the mean value. (c, d) Histograms of force distribution for discrete ranges of LRs, documenting an unusual bimodal force distribution for ClfA⁽⁺⁾ (arrows).

pN range, that could also be described by the BE model, with $x_u = 0.068$ nm and $k_{off}^0 = 3.47 \times 10^{-9}$ s⁻¹, values indicating a lower bond lifetime. Sorting adhesion forces by discrete ranges over LR clearly revealed the occurrence of a bimodal force distribution for ClfA (Figure 4c). Earlier studies have shown that besides the Fg γ -peptide N2N3 binding trench of ClfA,

there is a second binding site located at the top of the N3 domain which is critical for an overall high affinity Fg-ClfA interaction.^{38,44} One could therefore argue that the intermediate forces are associated with a second distinct binding site on the adhesin. However, the lack of adhesion forces larger than ~ 500 pN observed with the *S. aureus* ClfA_{PY} strain

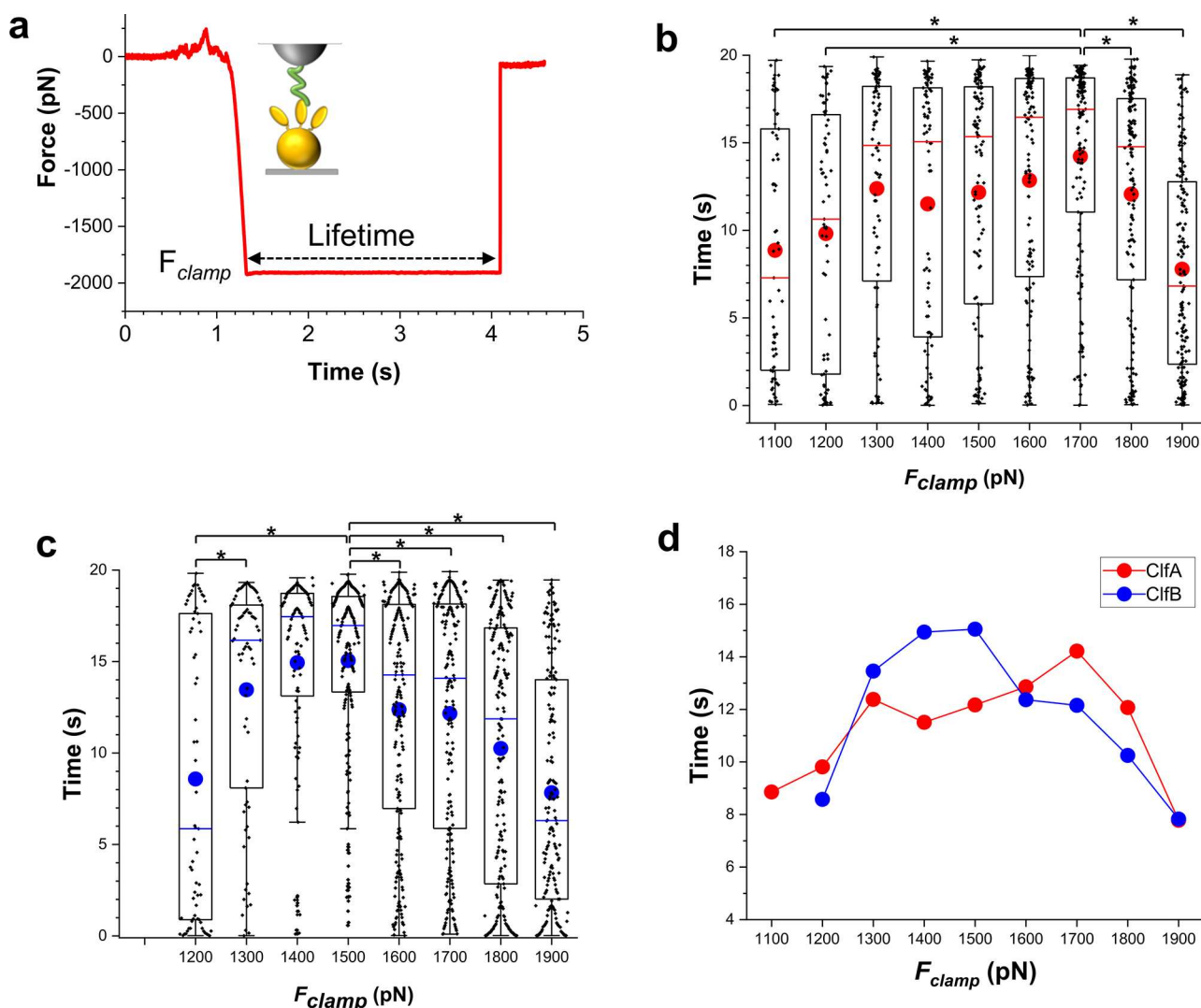


Figure 5. Clumping factor–annexin A2 interactions feature catch-slip bond transitions. (a) Representative force–clamp curve (force vs time). The bond lifetime is given by the constant force regime indicated by black dots arrow. (b, c) Box plots showing the lifetime data obtained from force vs time for ClfA (b) and ClfB (c). ClfA, data pooled from $n = 71, 71, 88, 87, 125, 140, 150, 154,$ and 167 curves for $F_{\text{clamp}} = 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800,$ and 1900 pN, respectively, recorded on a total of 6 cells from 4 independent cultures. ClfB, data pooled from $n = 67, 73, 112, 216, 225, 205, 219,$ and 186 curves for $F_{\text{clamp}} = 1200, 1300, 1400, 1500, 1600, 1700, 1800,$ and 1900 pN, respectively, recorded on a total of 5 cells from 5 independent cultures. Circles are the mean values, lines the median, boxes the 25–75% quartiles, and whiskers the 10–90% interval. Difference of distributions were determined using Dunn-Sidak multiple comparison test. *, $P < 0.05$. (d) Bond lifetimes measured at different F_{clamp} showing the catch-slip transition for ClfA and ClfB.

(Figure 3) allows us to rule out this possibility. Rather, we argue that the intermediate forces reflect the rupture of DLL complexes that are not tightly stabilized due to metastable geometries.

The high lifetimes of the ClfA and ClfB interactions estimated from the DFS data are in contrast with the moderate lifetimes obtained from bulk affinity experiments,^{13,45–47} thus indicating that unbinding occurs through a different pathway under tensile loading. We hypothesized that the force-dependent pathway may result from the formation of long-lived catch bonds, as observed for SdrG and SpsD DLL interactions.^{48,49} To test this, we used force-clamp spectroscopy (Figure 5), where the adhesin–ligand bond was clamped at forces ranging from 1100 pN to 1900 pN, while recording the time that the adhesin and ligand stay in contact before the bond ruptures spontaneously. The bond lifetime (τ) was

assessed from the persistent time extracted from the force vs time curves (Figure 5a).

For ClfA (Figure 5b), mean lifetimes of 8.9, 9.8, 12.4, 11.5, 12.2, 12.9, 14.2, 12.1, and 7.8 s were obtained at clamping forces of 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, and 1900 pN, respectively. For ClfB (Figure 5c), the lifetimes were 8.6, 13.5, 14.9, 15.1, 12.4, 12.2, 10.2, and 7.8 s at 1200, 1300, 1400, 1500, 1600, 1700, 1800, and 1900 pN. Hence, an increase in lifetimes was observed in the 1100–1700 pN and 1200–1500 pN force ranges for ClfA and ClfB (Figure 5d) reflecting a catch bond behavior. Beyond these critical forces, the lifetimes switched back to lower values, as expected for slip bonds. Intriguingly, ClfA exhibited a catch-slip transition more complex than ClfB (Figure 5d), thus correlating with the DFS plots and indicating that the ClfA–annexin A2 interaction is indeed characterized by both intermediate and high forces. Lastly, we note that survival plots (Figure S6), i.e., number of

intact bonds versus time for the different F_{clasp} values, showed that the survival probability decreased linearly (in a semilog representation) for times smaller than ~ 10 s, after which the probability dropped nonlinearly.

Collectively, our results demonstrate high force catch bonds between ClfA/B adhesins and annexin A2. A variety of bacterial pathogens, including *P. aeruginosa*, *E. coli*, *S. typhimurium*, *rickettsiae*, and *S. aureus*, interact with annexin A2 on the surface of mammalian cells,^{27,28,30,31,35,36} yet the molecular details of these interactions and their role in host cell invasion have remained largely unexplored. This work represents the first identification of a catch bond mechanism used by a bacterial pathogen for host cell invasion, thus highlighting the role of catch bonding in governing *S. aureus* pathogenicity.

ClfA/B-annexin A2 complexes have a force resilience similar to that of covalent bonds, and in the range of that reported for staphylococcal DLL-interactions.^{38–40,42,48,49} Analysis of the ClfA_{py} mutant strain demonstrates that the ~ 2 nN binding forces are associated with a DLL mechanism, in which the adhesin N2N3 binding domains undergo conformational changes under stress, enabling the formation of long-lived bonds with its ligand. Adhesins are generally heterogeneously distributed across the cell surface and localize in nanoclusters that are likely to strengthen bacterial adhesion *in vivo* through multivalent binding.⁵⁰

ClfA/B-annexin A2 interactions exhibit catch–slip bond transitions at critical forces of 1.5–1.7 nN. Below this transition force, the bond is longer-lived with increasing mechanical force (catch-bond behavior) while above it, the bond weakens with further increasing force (slip-bond behavior). The critical force is orders of magnitude larger than that of all other cellular receptor–ligand bonds investigated so far, e.g., FimH, PSGL-1 ligand, actin/myosin, and T cell receptors (transition forces smaller than 10 pN).^{51–54} The bond lifetime and critical force of the catch–slip transition are substantially larger than those of the DLL SpsD-Fg interaction,^{48,55} which is indicative of a higher mechanical stability. Notably, ClfA, but not ClfB, exhibit both intermediate and high binding forces, indicating that the adhesin forms DLL complexes that are not fully stabilized. Perhaps, this unusual binding behavior explains the lower capacity of ClfA to support host cell invasion.

Lifetime measurements generally follow an exponential decay, which is not observed here. While this deviates from classical catch-bond behaviors, we recently found that in the case of the very strong interaction between the *S. aureus* adhesin SdrE and complement factor H, lifetime values follow exponential distributions for all clamping forces studied, except for those matching the critical force at which the catch–slip transition occurs.⁵⁶ We believe this atypical behavior, similar to what we observe in the present study, is related to the unique binding strength and complexity of the mechanisms at play, which are associated with a much higher mechanostability than in typical receptor–ligand bonds. This is supported by the fact that not only the critical forces we measured, but also the bond lifetimes, are much larger than those reported for the DLL interaction between the staphylococcal adhesin SpsD and its Fg ligand.⁴⁸ Formation of long-lived catch bonds is of relevance in the early stage of invasion as surface-attached bacteria experience mechanical cues induced by fluid flow and cellular contacts.^{57–60} *In vivo*, annexin A2 is exposed to shear stress associated with blood flow under specific physiological

conditions, e.g., when coagulation starts and prothrombin is activated into thrombin.

Molecular dynamics (MD) simulations of staphylococcal adhesins engaged in a DLL mechanism^{41,42,61,62} have shown that the target ligand peptide is confined in a screw-like manner in the adhesin binding pocket. Rupture of the complex involves the simultaneous breakage of numerous hydrogen bonds in a cooperative shear geometry, yielding an extreme mechanical strength. We argue that the high mechanical stability of the Clf-annexin complexes results from an intricate hydrogen bond network between the ligand peptide backbone and the adhesin. For SdrG, MD simulations revealed that a catch bond mechanism explains the force-enhanced mechanical strength and stability of the complex.⁴⁹ While allowing for thermal dissociation in a low-force regime,⁶³ an entirely different mechanical dissociation path emerged in a high-force regime, revealing a complex mechanism that does not depend on the peptide amino acid sequence.

An interesting question is how do our measured ligand-binding forces compare with the anchoring strengths of the adhesin and annexin proteins? ClfA and ClfB belong to a family of adhesion proteins that are covalently attached to the bacterial cell wall, the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs), and which target host extracellular proteins.⁶⁴ Annexin A2 tightly associates with the host plasma membrane via Ca^{2+} -dependent ionic binding to negative charged phospholipids.⁶⁵ Notably, this annexin can bind up to 12 Ca^{2+} ions, suggesting it engages in multivalent interactions with the cell membrane. Other interactions may play a role in membrane association, as some annexins, like annexin A5, bind hydrophobic chains of membrane lipids via insertion of a tryptophan residue into the membrane,⁶⁶ while annexin A13 can insert into membranes through the N-terminal myristoylated domain.⁶⁷ Therefore, it is possible that similar hydrophobic bonds are involved in binding annexin A2 to the host membrane. Membrane anchoring of annexin A2 could also be reinforced by the formation of heterotetrameric complexes with S100A10 and by interaction with specific proteins and cholesterol-rich lipid raft microdomains.⁶⁸ In addition, self-association of the annexin into oligomers results in stable clusters on plasma membranes.⁶⁹ These putative interactions are not mutually exclusive, but may cooperate in the stabilization of the annexin A2-membrane association.

Finally, it is tempting to speculate on the mechanism of bacterial internalization triggered by the adhesin–annexin interaction. The main *S. aureus* invasion process involves a ternary bridge between bacterial FnBP adhesins, fibronectin (Fn), and integrin $\alpha 5 \beta 1$ on human cells.^{70,71} Fn-binding by FnBPs disrupts specific intermolecular contacts in the Fn N-terminal domain resulting in structural rearrangements at sites involved in integrin recognition.⁷² Then, endocytosis occurs through clathrin-coated pits through rearrangement of the cytoskeleton⁷³ and F-actin polymerization.^{74,75} Aside from this classical pathway, there are other secondary mechanisms of invasion⁷⁶ including the ClfA/B-annexin A2 system investigated here. Currently, the molecular details of annexin A2-mediated invasion remain essentially unknown. An important open question is whether binding of ClfA/B elicits molecular changes in annexin A2, activating the internalization process, as for the FnBP-Fn complex. Moreover, as annexin A2 participates in the organization of lipid rafts at sites of actin recruitment,⁶⁵ adhesin–annexin raft complexes could trigger a

raft-dependent biochemical pathway in host cells. It is also worth noting that *E. coli* strains infecting epithelial cells can reorganize the cytoskeleton via annexin A2, which is known to regulate actin dynamics at the bacterium contact.^{77,78} Annexin A2 is recruited to the membrane adhesion site by *E. coli*, where F-actin bundling activity is enhanced.^{79,80} Annexin A2 also promotes cytoskeletal rearrangements during host cell invasion by *Salmonella*,³⁰ which is reminiscent of the FnBP-dependent internalization mechanism. We therefore suggest that a similar scenario may occur in which ClfA/B-annexin A2 complexes induce cytoskeletal rearrangement and F-actin polymerization, and the subsequent events leading in *S. aureus* internalization.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.nanolett.3c01387>.

Detailed information about methods. Figure S1, adhesion forces between annexin A2-modified tips and *S. aureus* bacteria expressing ClfA or ClfB. Figure S2, localization of ClfA and ClfB adhesins on *S. aureus* bacteria. Figure S3, influence of tip functionalization procedures. Figure S4, adhesion forces of bacteria lacking both ClfA and ClfB. Figure S5, adhesion forces of *S. aureus* ClfA_{py} bacteria. Figure S6, survival plots obtained for different clamping forces for ClfA and ClfB (PDF)

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C.W., T.O.P., C.M., P.S., G.P., and Y.F.D. designed the experiments, analyzed the data and wrote the article. C.W. and C.M. collected the data.

Notes

The authors declare no competing financial interest.

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RESEARCH

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The serine-rich repeat glycoprotein Srr2 mediates *Streptococcus agalactiae* interaction with host fibronectin

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Abstract

Background Group B Streptococcus (GBS) is a commensal of healthy adults and an important pathogen in newborns, the elderly and immunocompromised individuals. GBS displays several virulence factors that promote colonisation and host infection, including the ST-17 strain-specific adhesin Srr2, previously characterised for its binding to fibrinogen. Another common target for bacterial adhesins and for host colonization is fibronectin, a multi-domain glycoprotein found ubiquitously in body fluids, in the extracellular matrix and on the surface of cells.

Results In this study, fibronectin was identified as a novel ligand for the Srr2 adhesin of GBS. A derivative of the ST-17 strain BM110 overexpressing the *srr2* gene showed an increased ability to bind fibrinogen and fibronectin, compared to the isogenic wild-type strain. Conversely, the deletion of *srr2* impaired bacterial adhesion to both ligands. ELISA assays and surface plasmon resonance studies using the recombinant binding region (BR) form of Srr2 confirmed a direct interaction with fibronectin with an estimated K_d of 92 nM. Srr2-BR variants defective in fibrinogen binding also exhibited no interaction with fibronectin, suggesting that Srr2 binds this ligand through the dock-lock-latch mechanism, previously described for fibrinogen binding. The fibronectin site responsible for recombinant Srr2-BR binding was identified and localised in the central cell-binding domain of the protein. Finally, in the presence of fibronectin, the ability of a $\Delta srr2$ mutant to adhere to human cervico-vaginal epithelial cells was significantly lower than that of the wild-type strain.

Conclusion By combining genetic and biochemical approaches, we demonstrate a new role for Srr2, namely interacting with fibronectin. We characterised the molecular mechanism of this interaction and demonstrated that it plays a role in promoting the adhesion of GBS to human cervico-vaginal epithelial cells, further substantiating the role of Srr2 as a factor responsible for the hypervirulence of GBS ST-17 strains. The discovery of the previously undescribed interaction between Srr2 and fibronectin establishes this adhesin as a key factor for GBS colonisation of host tissues.

Keywords Fibronectin, Bacterial adhesins, Host-pathogen interaction, Srr2 adhesin, Streptococcus agalactiae, DLL binding mechanism

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Introduction

Streptococcus agalactiae, commonly known as Group B *Streptococcus* (GBS), is frequently found as an asymptomatic colonizer of the human gastrointestinal and genital tract. However, under certain conditions, it can act as an opportunistic pathogen, leading to severe infections in newborns, the elderly, and immunocompromised individuals [1]. The most common clinical manifestations of GBS infections in infants include meningitis and sepsis, whereas in adults, they manifest as bacteremia, soft tissue infections, and pneumonia [2].

The ability of GBS to breach host barriers is given by virulence factors exposed on its bacterial surface and conferring the ability to evade the immune system, colonize, invade and damage host's tissues [3]. In this context, adhesins play a key role in mediating the interaction of GBS with host cells, plasma proteins (such as fibrinogen, Fbg, and plasminogen, Plg), and components of the extracellular matrix (ECM), such as laminin and fibronectin (Fn) [3].

Notably, the expression of two GBS surface proteins, HvgA and Srr2, encoded exclusively in strains belonging to the sequence-type 17 (ST-17) [4, 5], was demonstrated to contribute to the hyper-virulence of these isolates, which strongly associate with the development of Late-Onset Disease (LOD) and meningitis in newborns [6, 7]. Non-ST-17 strains express other allelic variants of these proteins [8].

Serine-rich repeat (Srr) proteins, such as Srr1 in non-ST-17 strains and Srr2 in hypervirulent ST-17 strains, have a similar structure, comprising a signal sequence, a serine-rich region (SRR), a binding region (BR) for ligand attachment, a second SRR region, and an LPXTG motif anchoring the C terminus to the cell wall [9]. Both proteins display the ability to bind the plasma glycoprotein Fbg through their BR domain [9, 10]. The binding mechanism employed is defined as “dock-lock-latch” (DLL), whereby Fbg binds between the N2 and N3 domains of the BR, inducing a conformational change in

the C-terminal portion of the N3 domain (latch), which folds to form a β -sheet and interacts with the N2 domain (Fig. 1). The ligand is thus locked at the BR [9, 11].

Besides binding to Fbg, Srr1 facilitates GBS interaction with cytokeratin 4, contributing to colonization of the female genital tract [12].

In contrast, Srr2 enhances GBS virulence by binding to Fbg, plasmin, and Plg, disrupting the host coagulation system and promoting bacterial spread [11].

Srr2 also interacts with eukaryotic receptors $\alpha 5\beta 1$ and $\alpha \nu \beta 3$, overexpressed in cerebral vessels, enhancing adhesion of ST-17 strains to brain endothelial cells and facilitating blood brain barrier (BBB) invasion, especially in newborns [13]. Crossing the BBB can occur transcellularly or paracellularly, modifying endothelial cell permeability via integrins binding [13].

Srr2 also enables GBS ST-17 translocation across the intestinal epithelial barrier via transcytosis of Peyer's patch M cells, leading to neonatal LODs [7]. Hormones estradiol (E2) and progesterone (P4), which accumulate in the fetus during pregnancy and are abundant in the first months of newborns' lives, increase barrier permeability, promote M cell maturation and enhance Srr2-mediated GBS crossing of the intestinal barrier, thereby favouring concentrations, its access to the lymphatic system and dissemination throughout the body [7].

The expression of *srr2* is controlled by two regulatory systems: the two component-system CovRS and the global transcriptional regulator CodY [14, 15]. The response regulator CovR is a global repressor of virulence-associated genes whose activity is dependent on its cognate histidine kinase CovS and on at least two associated regulators, Abx1 and Stk1 [16, 17]. CodY is a branched-chain amino acids dependent regulator which acts mainly as a repressor of GBS gene expression, controlling almost 13% of the genome of the bacterium. Its activity is required for GBS virulence in vivo, as a *codY*-null strain displays a reduced ability to cause infections in neonatal and adult mice [15]. Deletion of *codY* or *covR* in

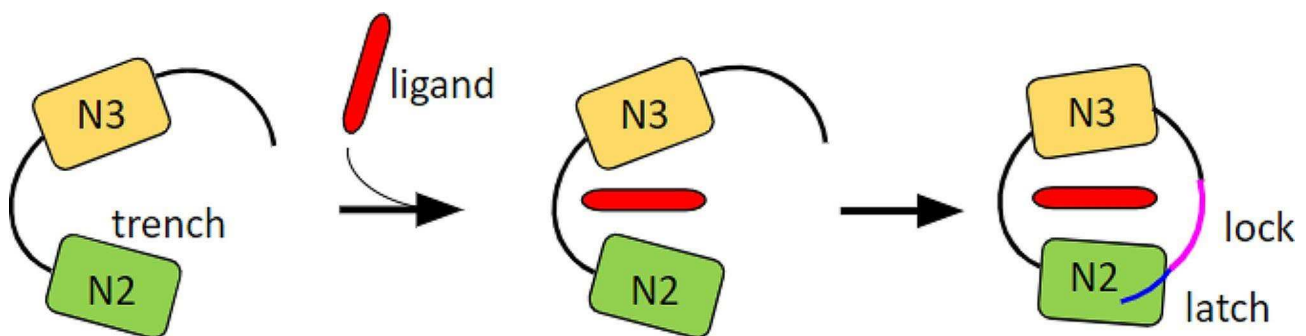


Fig. 1 Schematic representation of the dock, lock, and latch (DLL) mechanism of binding. According to the DLL mechanism, the ligand binding takes place in multiple steps: the ligand first docks into the trench (N2 subdomain), followed by conformational change and redirection of the carboxy-terminal extension of the N3 subdomain (latch), so that amino acid residues in this extension crossover the binding trench and lock the bound ligand in place

Table 1 List of bacterial strains used in this study

Bacterial strain	Relevant properties	Reference
<i>S. agalactiae</i>		
BM110	Serotype III, ST-17, human hypervirulent clinical isolate	[4]
BM110 $\Delta codY$	BM110 carrying an in-frame <i>codY</i> deletion	[15]
BM110 $\Delta srr2$	BM110 carrying an in-frame <i>srr2</i> deletion	This study
BM110 $\Delta srr2$ (<i>srr2</i> BTWT)	BM110 $\Delta srr2$ with genome complemented to wild-type	This study
BM110 $\Delta codY \Delta srr2$	BM110 carrying in-frame deletions of <i>srr2</i> and <i>codY</i> genes	This study
BM110 $\Delta codY \Delta srr2$ (<i>srr2</i> BTWT)	BM110 $\Delta codY \Delta srr2$ with genome complemented to wild-type for <i>srr2</i> gene	This study
NEM316	Serotype III, ST-23, human clinical isolate	[21]
A909	Serotype Ia, ST-7, human isolate	[8]
COH1	Serotype III, ST-17, human hypervirulent clinical isolate	[8]
<i>E. coli</i>		
XL-1blue	<i>E. coli</i> cloning host	Agilent Technologies, CA, USA
BL21 (DE3)	<i>E. coli</i> cloning host	Agilent Technologies, CA, USA

Table 2 List of primers used in this study

Primer	5' – 3' sequence
srr2192F	GTGGACAGCAAATGGGTCGCGGATCCGAAGCGGCAACGACCGCTAGAGTTTC
srr2BRRev	AAGCTTGTGCGACGGAGCTCGAATTCTCATTGAGCATTTACATCTGAATATCCCGATTTCAG
srr2BR_latchR	AAGCTTGTGCGACGGAGCTCGAATTCTCAAGTATGATTATGTTCTACACCATTTGTTTC
trenchR	GGCAGCGCCGGGTGCTGCCGCTTGAGAATCATAAATCCAGTCGC
trenchF	GCGGCAGACCCGGGCGTGCCGCGGCTTCAAAATAATATT
pG1srr2UpF	CTGAAGCCGAGTTATCACCTGTCATAACACTTTTGACCCAGTG
pG1srr2UpR	GGAAACAGCTATGACCATGATTACGAATTCGAGAGCGGCTATTATTTTATG
pG1srr2DwF	GCATGCCTGCAGGTCGACTCTAGAGGATCCGAACACCATATGCTTGATAAC
pG1srr2DwR	CACTGGGTCAAAAAGTGTATGACAGGTGATAACTCGGCTTCAG
pG1F	GAGCTCGGTACCCGGGGA
pG1R	GAATTCGAATCATGGTCATAG
secY2F	CAGTGGATAATAAACACGTTGTCC
srr2Up1	GATAGGAGGGGTCTTTATAGGC
Srr2-BR_N2For	TCACGGATCCGAAGCGCAACGACC
Srr2-BR_N2Rev	ACGTCGACTCATTTCACTGTTCTTTAATTGTAGC
Srr2-BR_N3For	TCACGGATCCGATCCACCGTAAGAATTGATTTTG
Srr2-BR_N3Rev	ACGTCGACTCATTGAGCATTACATCTGAATATCC

the hypervirulent GBS strain BM110 (ST-17) resulted in *srr2* overexpression, demonstrating that both regulators act as repressors of this gene [14, 15].

With the objective of elucidating the mechanisms by which CodY influences GBS virulence and considering the important contribution of interactions with plasma and ECM proteins to the infection process, in the present study we compared the adhesive properties of a *codY*-null and wild-type ST-17 strain (BM110) to fibronectin and fibrinogen and investigated the role played by Srr2 in such interactions. Furthermore, we report a new ligand for this multivalent adhesin and describe for the first time the interaction of an adhesin with fibronectin via the DLL mechanism. We also investigate the functional implications of this interaction concerning the adhesive properties of GBS to host cells.

Materials and methods

Bacterial strains and culture conditions

All strains used in this study are listed in Table 1. GBS cells were grown overnight in Todd-Hewitt (TH) medium supplemented with yeast extract (THY) (SIGMA) at 37 °C. For ELISA assays involving bacterial adhesion to immobilized proteins, bacteria were harvested from the cultures by centrifugation, washed, and suspended in phosphate-buffered saline (PBS, NaCl 130 mM, NaH₂HPO₄ 7 mM, NaH₂PO₄ 3 mM, pH 7.4) at an optical

density at 600 nm (OD₆₀₀) of 1.0. *Escherichia coli* XL1-Blue (Agilent Technologies, CA, USA) or BL21 (DE3) (Agilent Technologies, CA, USA) strains transformed with vector pET28a (Stratagene, La Jolla, CA) or derivatives were grown in Luria agar and Luria broth (VWR) containing 50 µg/ml kanamycin at 37 °C with shaking. Erythromycin (Sigma-Aldrich) was used at 10 µg/ml for selection and maintenance of plasmid pG1 and its derivatives in GBS.

Plasmids and strains construction

Oligonucleotides and plasmids used in this work are listed in Tables 2 and 3, respectively.

To prepare plasmid pET28a-*srr2*-BR, a 1056 bp fragment encoding amino acids 192–543 of Srr2 was PCR amplified with primers srr2192F and srr2BRRev using the BM110 chromosomal DNA as template. The obtained amplicon was inserted by Gibson assembly (NEBuilder HiFi DNA Assembly Cloning Kit, New England Biolabs) between the *Bam*HI and *Eco*RI restriction sites of plasmid pET28a. The Δ latch variant (amino acids 192–530) was obtained by the same approach, using srr2BR_latchR as reverse primer. Plasmid pET28a-*srr2*-BR- Δ trench, containing substitution and deletion mutations in the trench sequence [11], was constructed by producing two PCR fragments: an amplicon containing the 5' part of the sequence encoding the Srr2-BR was synthesized by using

Table 3 List of plasmids used in this study

Plasmid	Resistance	Relevant properties	Reference
pG1	Erythromycin	Plasmid containing a temperature sensitive origin of replication for GBS and a ColE1 origin of replication for <i>E. coli</i> .	[22]
pG1- Δ srr2	Erythromycin	pG1 carrying the <i>srr2</i> deletion cassette	This study
pET28a	Kanamycin		Qiagen
pET28a-srr2-BR	Kanamycin	pET28a carrying the wild-type <i>Srr2</i> binding region ORF	This study
pET28a-srr2-BR Δ latch	Kanamycin	pET28a carrying a mutated <i>Srr2</i> binding region	This study
pET28a-srr2-BR Δ trench	Kanamycin	pET28a carrying a mutated <i>Srr2</i> binding region	This study
pET28a-srr2-BR_N2	Kanamycin	pET28a carrying the N2 subdomain of <i>Srr2</i> -BR	This study
pET28a-srr2-BR_N3	Kanamycin	pET28a carrying the N3 subdomain of <i>Srr2</i> -BR	This study

oligonucleotide srr2192F as the forward primer and mutagenic oligonucleotide trenchR as the reverse primer; a fragment containing the 3' part of the sequence encoding the *Srr2*BR was synthesized by using the mutagenic forward oligonucleotide trenchF and the reverse primer srr2BRRev. The PCR products were fused and cloned into pET28a by Gibson assembly. The DNA fragments encoding the N2 and N3 subdomains of *Srr2*-BR were PCR amplified from the genome of the GBS strain BM110 using the *Srr2*-BR_N2For - *Srr2*-BR_N2Rev primers for N2 region and *Srr2*-BR_N3For - *Srr2*-BR_N3Rev primers for N3 region. Amplified N2 and N3 fragments were cloned into the expression plasmid pET28a between the BamHI and SalI restriction sites, generating constructs pET28a-srr2-BR_N2 and pET28a-srr2-BR_N3, respectively. All constructs were verified by sequencing.

The BM110 Δ srr2 mutant was constructed by allelic replacement using the thermosensitive pG1 vector (kind gift from Arnaud Firon, Institut Pasteur, France). An in-frame deletion of 3429 nucleotides was created within

the *srr2* gene, corresponding to the sequence encoding amino acids 34 -1176. The upstream (717 bp) and downstream (567 bp) genomic regions flanking the target sequence were PCR amplified using the primers pairs pG1srr2UpF/pG1srr2UpR and pG1srr2DwF/pG1srr2DwR, respectively. The two PCR fragments were fused and cloned by Gibson assembly into plasmid pG1, previously linearized by PCR using the divergent primers pG1F and pG1R. The obtained pG1- Δ srr2 plasmid was used to electroporate BM110 and BM110 Δ codY electrocompetent cells and transformants were selected at permissive temperature (30 °C) with erythromycin. After chromosomal integration at the target locus at restrictive temperature (37 °C), de-recombination and loss of the deletion vector were obtained by growth at 30 °C without selective pressure. The final step can result in a back to WT (BTWT) allele (*srr2* BTWT) or in a strain carrying the desired deletion (BM110 Δ srr2; BM110 Δ codY Δ srr2). The BTWT or deletion genotypes were discriminated by PCR (with external primers secY2F and srr2Up1) and Sanger sequencing.

Expression and purification of recombinant proteins

Recombinant proteins *Srr2*-BR, *Srr2*-BR Δ latch, *Srr2*-BR Δ trench, *Srr2*-BR-N2 or N3 subdomain were expressed with His₆ C-terminal tags using pET28a vector (Qiagen, Hilden, Germany) in *E. coli* BL21 DE3 cells (Agilent Technologies, CA, USA). Overnight starter cultures were diluted 1:100 in Luria broth containing kanamycin (50 µg/ml) and incubated with shaking until the culture reached the exponential phase (OD₆₀₀ of 0.6). Recombinant protein expression was induced by the addition of 1 mM (final concentration) isopropyl 1-thio- β -D-galactopyranoside (IPTG) (Thermo, Italy) to the culture. After overnight shaking at 20 °C, bacterial cells were harvested by centrifugation and resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 7.4, containing 1 mM Phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich) as the protease inhibitor, 20 µg/ml DNase (Sigma-Aldrich), MgCl₂ (1 mM)), and lysed by sonication (70% amplitude, 12×30" on/off, 1'30" interval between sonication steps). The cell debris was removed by centrifugation, and proteins were purified from the supernatants by Ni²⁺-affinity chromatography on a HiTrap chelating column (GE Healthcare, Buckinghamshire, UK). Protein purity was assessed by 12.5% SDS-PAGE and Bio-Safe Coomassie staining (BioRad, Hercules, CA, USA). A bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) was used to measure the concentration of purified proteins.

Reagents, proteins, and antibodies

Bovine serum albumin (BSA), human Fbg, human collagen, protease-free DNase I, skim milk, trypsin, and

HRP-conjugated secondary anti-mouse and anti-rabbit IgG were purchased from Sigma-Aldrich. The anti-Fn antibody, anti-Fbg antibody and anti-Srr2 antibody were raised in mice by a routine immunization procedure using purified Fn or Fbg or Srr2-BR as antigens, respectively. Briefly, the polyclonal antibodies were produced by immunizing 6-week-old specific pathogen-free CD1 mice (Charles River Laboratories, Italia) by the intraperitoneal injection of 50 µg of the antigen in alum on days 0, 14, and 28. Sera were collected 15 days after the last immunization. The anti-GBS antibody was raised in rabbit using 50 µg of heat-inactivated GBS bacterial cells as antigen as reported above. Animals were sacrificed by cervical dislocation after sedation with carbon dioxide. The polyclonal antibodies production method was carried out in accordance with relevant guidelines and regulations; all experimental protocols were approved by the Ethics Committee of the University of Pavia (OPBA permit n° 1,909,213) and of the University of Messina (OPBA permit n° 1,805,210) and by the Ministero della Salute of Italy (permit no. 665/2015). The study is reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>). Rabbit anti-mouse and goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Dako Cytomation (Glostrup, Denmark). OPD tablets (o-phenylenediamine dihydrochloride) were purchased by ThermoScientific (Rockford, IL, USA). Fn was purified from plasma through gelatin-Sepharose affinity chromatography as previously described [18]. Freshly drawn blood to prepare human plasma was obtained from healthy volunteers with informed consent and permission of the ethical board of the University of Pavia (permit no. 19,092,019). The research abides by the Declaration of Helsinki principles. Plasma fractions were frozen in aliquots after blood centrifugation and stored at -20 °C. Fn fragments were previously obtained by fractionation of thermolysin-digested plasma Fn on ion-exchange/gel filtration chromatography column [18].

ELISA-type solid-phase binding assays

Fn or Fbg binding by GBS strains

The ability of bacteria to interact with immobilized human Fn or Fbg was evaluated by ELISA-based assay. Microtiter wells were coated overnight at 4 °C at the indicated concentrations of Fn or Fbg in 0.1 M sodium carbonate, pH 9.5. The plates were washed three times with 0.5% (v/v) Tween 20 in PBS (PBST). To block additional protein-binding sites, wells were treated for 1 h at room temperature (RT) with 200 µl of 2% BSA in PBS. Bacterial adhesion was evaluated by incubation of 100 µl/well bacterial cells ($OD_{600nm}=1.0$) in PBS for 1 h at 37 °C. Attached bacteria were detected through rabbit anti-GBS antibody (1 µg/well) followed by peroxidase-conjugated secondary goat anti-rabbit IgG diluted 1:1000. After

washing, OPD was added and the absorbance at 490 nm was measured by using an ELISA plate reader (BMG Labtech).

Binding of rSrr2-BR, rSrr2-BR Δ latch, rSrr2-BR Δ trench, rSrr2-N2 or rSrr2-N3 subdomains to Fn and Fbg

The ability of soluble human Fn or Fbg to bind to immobilized recombinant proteins (rSrr2-BR, rSrr2-BR Δ latch, rSrr2-BR Δ trench, rSrr2-N2 or rSrr2-N3 subdomains) was determined using ELISA assay. Microtiter wells were coated overnight at 4 °C with 1 µg/well of each recombinant protein in 0.1 M sodium carbonate, pH 9.5. After 1 h treatment at RT with BSA, the plates were incubated for 1 h with increasing amounts of Fn (up to 240 nM) or Fbg (up to 7.5 nM). Mouse anti-Fn IgG (1:4000) or anti-Fbg (1:1000) in 1% BSA was added to the wells, followed by incubation for 60 min. After washing, the plates were incubated for 45' with peroxidase-conjugated secondary rabbit anti-mouse IgG diluted 1:1000. After washing, OPD was added and the absorbance at 490 nm was determined.

The ability of soluble rSrr2-BR to bind to immobilized Fn was also determined. Microtiter wells were coated overnight at 4 °C with 1 µg/well of Fn in 0.1 M sodium carbonate, pH 9.5. After 1 h treatment at RT with BSA, the plates were incubated for 1 h with increasing amounts of rSrr2-BR (up to 2.5 µM). Mouse anti-Srr2 IgG (1:4000) in 1% BSA was added to the wells, followed by incubation for 60 min. After washing, the plates were incubated for 45' with peroxidase-conjugated secondary rabbit anti-mouse IgG diluted 1:1000. After washing, OPD was added and the absorbance at 490 nm was determined.

Inhibition - competition assays

To assess the effect of sodium chloride on rSrr2-BR / Fn or rSrr2-BR / Fbg interaction, microtiter wells coated with 1 µg of rSrr2-BR were incubated with (5 µg) of Fn or (0.25 µg) of Fbg in presence of increasing concentrations of salt (from 0 to 1 M). After washing, complex formation was detected by incubation of the wells with a mouse anti-Fn (1:4000) or anti-Fbg (1:1000) IgG followed by peroxidase-conjugated secondary rabbit anti-mouse IgG diluted 1:1000. After washing, OPD was added and the absorbance at 490 nm was determined.

The binding of 1 µg of Fn to surface coated rSrr2-BR (1 µg/well) in presence of equimolar increasing concentrations (up to 300 nM) of Fbg or collagen was detected with anti-Fn antibody as reported above.

To determine the effect of soluble CBD fragment on the rSrr2-BR / Fn interaction, microtiter wells, coated with 1 µg of Fn, were incubated with 1 µg of rSrr2-BR in the presence of increasing concentration of CBD fragment (up to 10 µg/well). rSrr2-BR binding to Fn was detected with mouse anti-Srr2 antibody (1:4000) followed by

peroxidase-conjugated secondary rabbit anti-mouse IgG diluted 1:1000. After washing, OPD was added and the absorbance at 490 nm was determined.

Detection of Srr2-expression on BM110 cells during bacterial growth phases

The expression of Srr2 on the surface of BM110 cells was assessed through ELISA-based assay. Evaluation was performed by harvesting bacteria by centrifugation at different time points of growth and immobilizing 100 µl of cells resuspended in PBS at OD₆₀₀=1 per well (10⁷ cells/well), overnight at 37 °C. Protein expression was detected using the mouse anti-Srr2 antibody (1:4000), followed by HRP conjugated rabbit anti-mouse, OPD and absorbance reading at 490 nm. To confirm that the same amount of BM110 cells were immobilised at the different time points, control wells were seeded and incubated with the anti-GBS antibody. The complex was detected as reported above (Fig. S1). To exclude the possibility that the reactivity could be due to the expression of Ig-binding proteins on the bacterial surface, the assay was conducted in parallel by incubating the wells with an isotype control antibody (data not shown).

Surface plasmon resonance

The binding of Fn to Srr2 proteins was evaluated by Surface Plasmon Resonance (SPR). SPR analyses were performed on a Biacore X-100 dual flow-cell instrument (GE Healthcare, Chicago, IL, USA). Purified rSrr2 proteins (50 µg/mL) were covalently immobilized at pH 4.75 on a carboxymethylated-dextran chip (CM5) using the amine coupling chemistry. All measurements were carried out at 25 °C in HEPES-EP+buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 50 mM EDTA, 0.005% v/v polyoxyethylene sorbitan) at a flow rate of 30 µL/min. Each sensogram was subtracted for the corresponding baseline, obtained on the reference flow cell and accounting for nonspecific binding. The dissociation constants (K_d) of the Fn-Srr2 interaction were obtained as a fitting parameter by plotting the RU values at the steady state (RU_{eq}) versus [Fn] and interpolating the data points with Eq. 1, describing a 1:1 binding model [19, 20]

$$RU_{eq} = RU_{max} \cdot \frac{[L]}{K_d + [L]} \quad (1)$$

where L is the concentration of Fn, while RU_{eq} and RU_{max} are the RU values measured at the steady state with intermediate or saturating [L], respectively.

Dot-blot and western-blot analyses

Binding of rSrr2-BR to Fn or its fragments was evaluated through Dot-blot and Western-blot analyses. For dot-blot assay, equimolar concentrations of Fn or its fragments

were dotted onto a PVDF membrane (BioRad). After overnight incubation at 4 °C with 5% skim milk (w/v) in 0.5% (v/v) Tween 20 in PBS (PBST), the membrane was treated for 1 h at 22 °C with 1 µg/mL of rSrr2-BR. The membrane was incubated with a mouse polyclonal anti-Srr2 antibody (1:2000) in 2% (w/v) skim milk for 1 h at 22 °C. Following several washings with PBST, the membrane was treated with 45 min at 22 °C with an HRP-conjugated rabbit anti-mouse IgG (1:10000) in 2% (w/v) skim milk. Dot-blot was developed using the Westar Supernova detection kit (Cyanagen srl, Bologna, Italy), and ImageQuant™ LAS 4000 mini-biomolecular imager (GE Healthcare) was used to capture images of the spots. For Western-blot analysis, rSrr2-BR was covalently coupled to Carboxyl (COOH) microparticles through the PolyLink Protein Coupling Kit for COOH Microspheres (Polysciences, Germany). Srr2-coupled particles were incubated for 1 h with equimolar concentrations of Fn or its fragments to allow binding. After washings with PBS, proteins bound to microparticles were dissociated through incubation with SDS-containing loading buffer and the samples were subjected to SDS-PAGE and transferred to a PDVF membrane. After overnight incubation at 4 °C with 5% skim milk in PBST, the membrane was treated for 1 h at 22 °C with mouse polyclonal anti-Fn antibody (1:4000) in 2% (w/v) skim milk for 1 h at 22 °C. After washing, the membrane was treated with 45 min at 22 °C with an HRP-conjugated rabbit anti-mouse IgG (1:10000) in 2% (w/v) skim milk and bands developed using the Westar Supernova detection kit (Cyanagen srl, Bologna, Italy). Signal was detected through an ImageQuant™ LAS 4000 mini-biomolecular imager (GE Healthcare).

Epithelial cell culture and cell adhesion assays

Epithelial cervico-vaginal HeLa cells were seeded at 1.5×10⁵ cell density per well in 24-well tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fn- depleted fetal bovine serum (FBS) and allowed to attach for 24 h at 37°C in 5% CO₂. The effect of Fn on bacterial adhesion/invasion to monolayers was tested by pre-incubating the cells with 20 µg/ml of Fn for 1 h prior to infection. GBS cells grown to the late exponential phase and resuspended in PBS were used to infect the cell monolayers with a multiplicity of infection (MOI) of 10. After a 30' incubation, monolayers were washed three times with PBS to remove the non-adherent bacteria, lysed using 500 µl of cold 1% Triton X-100, and serial dilutions of the cell lysates were plated, and CFU were counted after incubation. Percent of adhesion of each strain was calculated as follows (number of CFUs on plate)/(number of CFUs of initial inoculum) × 100. To enumerate internalized bacteria, 30' after infection the monolayers were further incubated for 1 h in medium

supplemented with 100 µg/ml penicillin/streptomycin to kill adhered extracellular bacteria. Following incubation, monolayers were washed three times with PBS, lysed and plated to enumerate cell-internalised bacteria.

Statistical methods

Analyses were performed using Prism 9.0 (GraphPad). A minimum of three biological replicates (three independent experiments) were conducted for each experiment. Comparison of two groups was conducted through the two-tailed Student's t-test. The one-way ANOVA test was used to compare more than two groups, in combination with a post-hoc test (Dunnett's multiple comparison test or Tukey's honest significant test). *P* values < 0.05 were considered statistically significant.

Results

Srr2 promotes specific binding of GBS ST-17 strains to Fn

We have recently shown that deletion of the gene encoding the global transcriptional regulator CodY in the hypervirulent GBS strain BM110 ($\Delta codY$) results in a twelve-fold increase in *srr2* transcript abundance during exponential growth in liquid TH medium, compared to the wild-type strain [15]. As the ability of CodY to bind DNA is increased by its interaction with branched-chain amino acids, the regulatory capacity of this protein is dependent on the abundance of these cofactors.

To assess whether the levels of this adhesin vary in response to the bacterial growth conditions, Srr2 protein levels were evaluated by an ELISA assay performed using an anti-Srr2 antibody on immobilized BM110 wild-type cells collected at 30-min intervals during growth in TH

medium. A sharp increase in protein levels was observed at the beginning of the stationary phase, starting at T0 (transition point from the exponential to stationary growth phase, estimated at 150 min) (Fig. 2).

To investigate whether *codY* deletion affected the adhesive properties of GBS, an ELISA-based assay was performed to evaluate the adhesion of wild-type and $\Delta codY$ BM110 cells to the immobilised ECM proteins Fn and Fbg, well-known targets of GBS during the colonisation and infection processes [23]. The data shown in Fig. 3A–B clearly indicate that the $\Delta codY$ mutant strain displayed a higher binding capacity to both ligands. As Srr2 is already known for its high affinity for Fbg [9], we hypothesized that the enhanced ability of the $\Delta codY$ strain to bind to this ligand could be due to the overexpression of *srr2* gene, as a result of the removal of the repression exerted by CodY. To confirm this hypothesis and investigate whether Srr2 is involved also in binding to Fn, a marker-less in-frame deletion of *srr2* was introduced in the BM110 strain. As expected, the resulting $\Delta srr2$ strain showed a significant decrease in GBS binding to Fbg compared to the wild-type strain (Fig. 3B, D). Noteworthy, $\Delta srr2$ strain also showed a remarkable decrease in GBS binding to Fn (Fig. 3A, C). The latter result is unprecedented and further emphasises the role of Srr2 in driving GBS interaction with ECM proteins. Furthermore, a double deletion mutant ($\Delta codY/\Delta srr2$) lost its capacity to bind both ligands (Fig. 3A, B, E, F). This confirms that the increased adhesion to Fn and Fbg observed in the $\Delta codY$ mutant, in comparison to the wild-type strain, can be solely attributed to the overexpression of *srr2* and is not a result of differential expression of alternative factors controlled by the global regulator CodY. As an additional experimental evidence of the importance of Srr2 in both Fbg and Fn binding by GBS, *srr2* complementation successfully restored GBS binding to both ligands in the $\Delta srr2$ and $\Delta codY/\Delta srr2$ mutants, bringing the binding levels back to those observed in the wild-type and $\Delta codY$ strains, respectively (Fig. 3C–F).

Altogether, our data concurrently indicate that Srr2 is required for GBS interaction with both Fbg and Fn and that the increased ability of the $\Delta codY$ mutant to bind ECM proteins (i.e. Fbg and Fn) is caused by Srr2 overexpression in this strain. Accordingly, while the ST-17 strains BM110 [4] and COH1 [8] efficiently bind Fn in a dose-dependent manner, the non ST-17 strains lacking the *srr2* gene (NEM316 and A909) [8, 21] display a significantly lower ability to bind the same ligand (Fig. 3G).

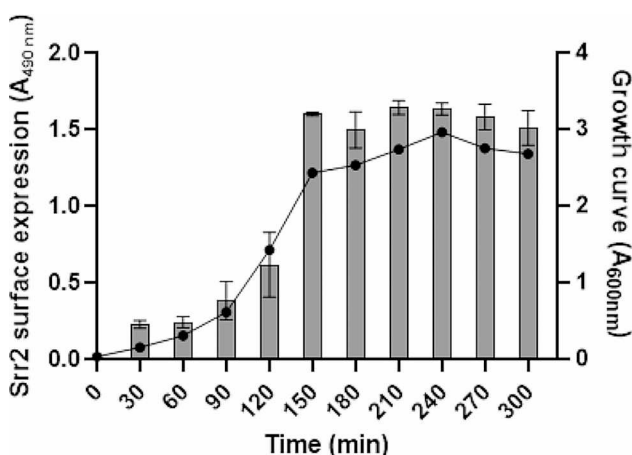


Fig. 2 Srr2 expression on GBS cell-surface increases during bacterial growth. At the indicated times, bacterial cells were collected, washed and then immobilized onto microtiter wells. The presence of Srr2 on cell surface was revealed using anti-Srr2 IgG followed by secondary HRP-conjugated IgG (left axis). Bacterial growth curve is also reported (right axis). The data points are the means $\pm$ SD from three independent experiments, each performed in triplicate

Characterization of Fn-binding activity of Srr2

To investigate the specificity of Srr2 binding to Fn, the recombinant Srr2 binding region (rSrr2-BR) was produced (Fig. 4A), immobilised onto microtiter wells and tested for binding to increasing concentrations of Fn or

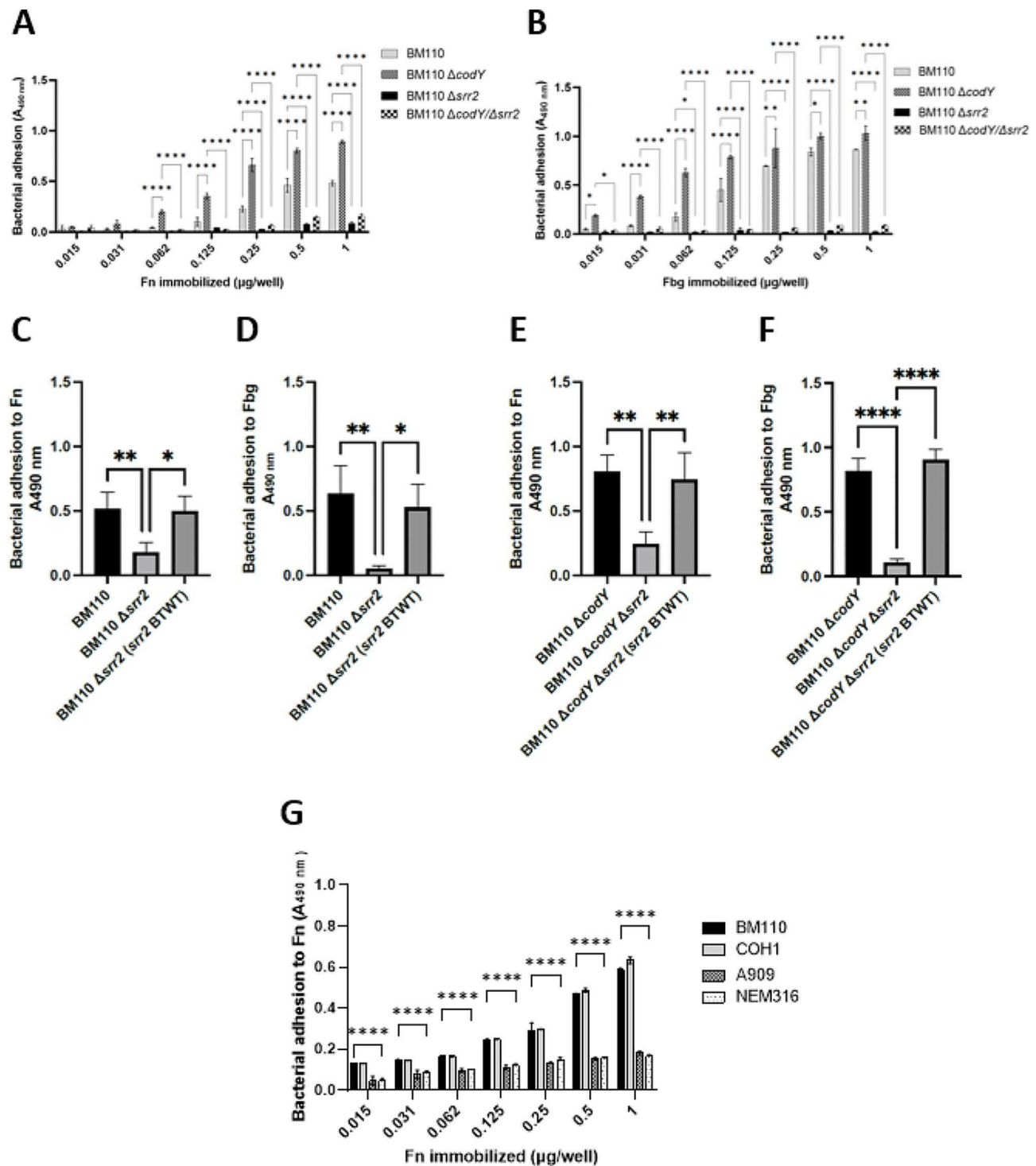


Fig. 3 Srr2 promotes specific binding of GBS BM110 ST-17 to Fn. Panels **A–B**. Adhesion of the indicated bacterial strains to immobilized Fn (**A**) or Fbg (**B**). Bound bacteria were detected by the addition of polyclonal anti-GBS IgG and secondary HRP-conjugated IgG to the wells. The ligand concentration given on the x-axis is the concentration of the solution used to coat the wells. Statistically significant difference is indicated (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$). The data points are the means $\pm$ SD from three independent experiments, each performed in triplicate. Panels **C–F**. Adhesion of the indicated bacterial strains to immobilized Fn (**C**, **E**) or Fbg (**D**, **F**). Bound bacteria were detected as reported for panels **A** and **B**. Statistically significant difference is indicated (*, $P < 0.05$; **, $P < 0.01$). The data points are the means $\pm$ SD from three independent experiments, each performed in triplicate. **G** Adhesion of GBS strains to increasing amounts of immobilized Fn. Bound bacteria were detected as reported for panels **A** and **B**. The ligand concentration given on the x-axis is the concentration of the solution used to coat the wells. Statistically significant difference is indicated (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$). The data points are the means $\pm$ SD from three independent experiments, each performed in triplicate

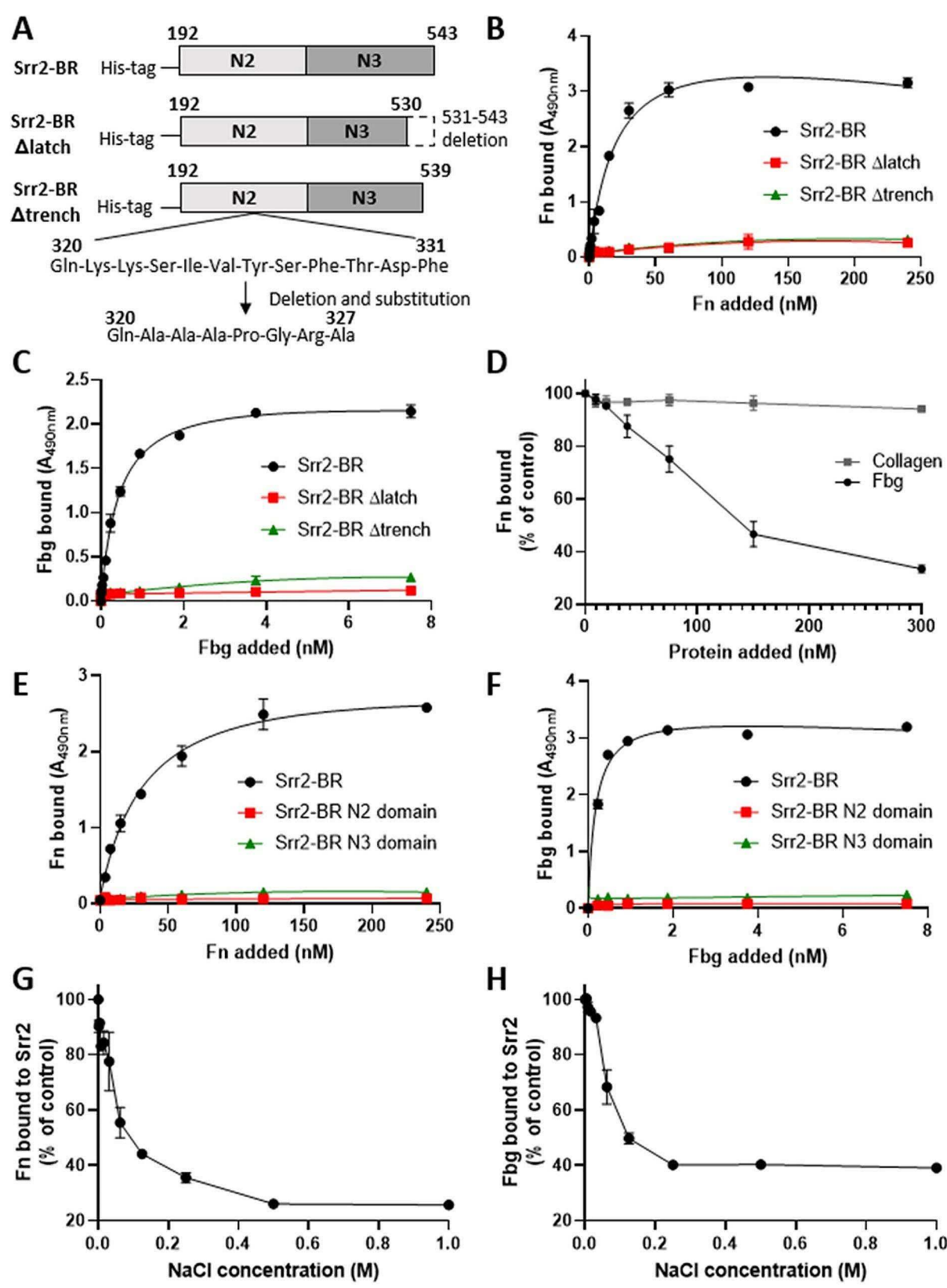


Fig. 4 (See legend on next page.)

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Fig. 4 Srr2 binds Fn through the dock-lock and latch mechanism. **A**) Schematic representation of wild-type or mutated recombinant Srr2 binding regions. Srr2-BR is composed of two domains (N2-N3) spanning residues 192 (amino-terminal domain) – 543 (carboxyl-terminal domain). Srr2-BR Δ latch is composed of two domains (N2-N3) spanning residues 192–530 lacking the latching sequence (dotted white box residues 531–543) as previously reported [9]. Srr2-BR Δ trench is composed of two domains (N2-N3) spanning residues 192–539 where the sequence known to form the ligand-binding trench (residues 320–331 in the N2 domain) was replaced with the sequence Gln-Ala-Ala-Pro-Gly-Arg-Ala (residues 320–327) as previously reported [11]. Panel **B–C**. Concentration dependent-binding of Fn (**B**) or Fbg (**C**) to immobilized Srr2-BR (black line), Srr2-BR Δ latch (red line) or Srr2- Δ trench (green line). Complex formation was detected by addition of a polyclonal anti-Fn IgG or a polyclonal anti-Fbg IgG to the wells, followed by secondary HRP-conjugated anti-IgG. **D**) Binding of Fn to immobilized Srr2-BR in the presence of equimolar concentration of Fbg or collagen. A saturating amount of Fn was added along with increasing concentrations of the indicated proteins on immobilised Srr2-BR. Complex formation was detected by addition of a polyclonal anti-Fn IgG followed by secondary HRP-conjugated anti-IgG. Data are expressed as a percentage of the control, i.e. incubation performed in the absence of any potential Fn competitor. Panels **E–F**. Concentration dependent-binding of Fn (**E**) or Fbg (**F**) to immobilized Srr2-BR (black line) or Srr2-BR N2 (red line) or Srr2-BR N3 (green line). Complex formation was detected as for panels **B–C**. Panels **G–H**. Binding of Fn (**G**) or Fbg (**H**) to immobilized Srr2-BR was analyzed under increasing concentrations of NaCl. Complex formation was detected as for panels **B–C**. Binding data are expressed as a percentage of the control, i.e. incubation performed in the absence of NaCl. Data points are the means $\pm$ SD from three independent experiments, each performed in triplicate

Fbg (used as control). The amount of Fn or Fbg bound to immobilized rSrr2-BR was detected using in-house produced polyclonal anti-Fn or anti-Fbg antibodies and a secondary HRP-conjugated antibody. As reported in Fig. 4B, soluble Fn bound the surface-coated rSrr2-BR in a concentration-saturable manner with an apparent K_d of 30 ± 1.3 nM. Soluble Fbg bound rSrr2-BR with a higher affinity compared to Fn, with an apparent K_d of 0.45 ± 0.02 nM (Fig. 4C), comparable to the affinity previously estimated by others [9]. Of note, rSrr2-BR is also able to bind immobilised Fn although with a 20-fold higher apparent K_d (Fig. S2).

To test whether Fbg and Fn share the same binding site on Srr2, rSrr2-BR was immobilised on microtiter wells and the binding of Fn was studied in the presence of increasing concentrations of Fbg (up to 10 μ g). Under these conditions, Fbg was able to inhibit Fn binding to rSrr2-BR in a dose-dependent manner up to 60% (Fig. 4D). No inhibitory effect was observed when the assay was conducted in the presence of collagen, used as control. These results suggest that Fn and Fbg share at least in part the same binding site on rSRR2-BR.

Seo et al. demonstrated that Fbg binds Srr2 at the level of the BR exploiting the so-called dock, lock and latch (DLL) mechanism [9]. To the best of our knowledge, the DLL mechanism has never been described for the binding of Fn to bacterial adhesins. Considering the inhibitory effect of Fbg in Fn binding to rSrr2-BR, we investigated whether the inhibition could be due to a steric hindrance or physical binding competition at the same binding site on rSrr2-BR. To study the Fn binding mechanism, we prepared two mutant versions of rSrr2-BR. The rSrr2-BR Δ latch version carries a deletion of the last 12 amino acids of the N3 subdomain, corresponding to the latch fragment involved in host ligand blocking. The rSrr2-BR Δ trench version has a mutation at the level of the docking cleft on the N2 subdomain, where the host ligand is supposed to bind as the first step of the DLL mechanism (Fig. 4A) [11]. Both mutated versions of rSrr2-BR were unable to bind Fn and Fbg (Fig. 4B, C), supporting the hypothesis of a DLL binding mechanism

for Fn as well. To further prove the binding mode of Srr2 to Fn, the subdomains N2 and N3 of the Srr2-BR were produced individually, immobilised onto microtiter wells and tested for Fn and Fbg binding. As reported in Fig. 4E, F, the two subdomains lost the ability to bind Fn and Fbg when tested separately. These results indicate that the binding of Fn to Srr2 requires the presence of both N2N3 domains as predicted by the DLL binding model.

To investigate whether ionic forces play a role in the interaction of Srr2 with Fn, the effect of sodium chloride (NaCl) on Fn binding to rSrr2-BR was investigated using the same immunochemical methods highlighted above. The addition of increasing concentrations of NaCl strongly impaired the binding of Fn (Fig. 4G) and Fbg (Fig. 4H) to immobilised rSrr2-BR, further supporting the hypothesis that Fn and Fbg share a similar binding mechanism to Srr2.

Quantitative assessment of Srr2 binding to Fn by SPR

A more quantitative estimate of Fn interaction to Srr2 proteins was obtained using a label-free biophysical technique, such as surface plasmon resonance (SPR), yielding a direct measure of the affinity between two interacting partners [19]. For this analysis, each rSrr2 protein was covalently immobilized onto a carboxymethylated-dextran sensor chip (CM5) and increasing Fn concentrations were injected into the mobile phase (Fig. 5). Data analysis was carried out in the affinity mode [24], whereby the SPR signal at the steady state in each sensogram was plotted as a function of Fn concentrations. The resulting data points were interpolated with Eq. 1, describing a one-site binding model [25], to yield an equilibrium dissociation constant (K_d) of 92 ± 15 nM for the binding of Fn to immobilized rSrr2-BR, which is comparable with the affinity estimated by ELISA ($K_d = 30 \pm 1.3$ nM), as reported in Fig. 4B. Furthermore, as already observed with ELISA measurements (Fig. 4B), the binding of Fn to Srr2 Δ latch and Δ trench mutants was almost entirely abolished (Fig. 5B), thus further supporting the hypothesis that Srr2 follows a DLL mechanism in Fn binding.

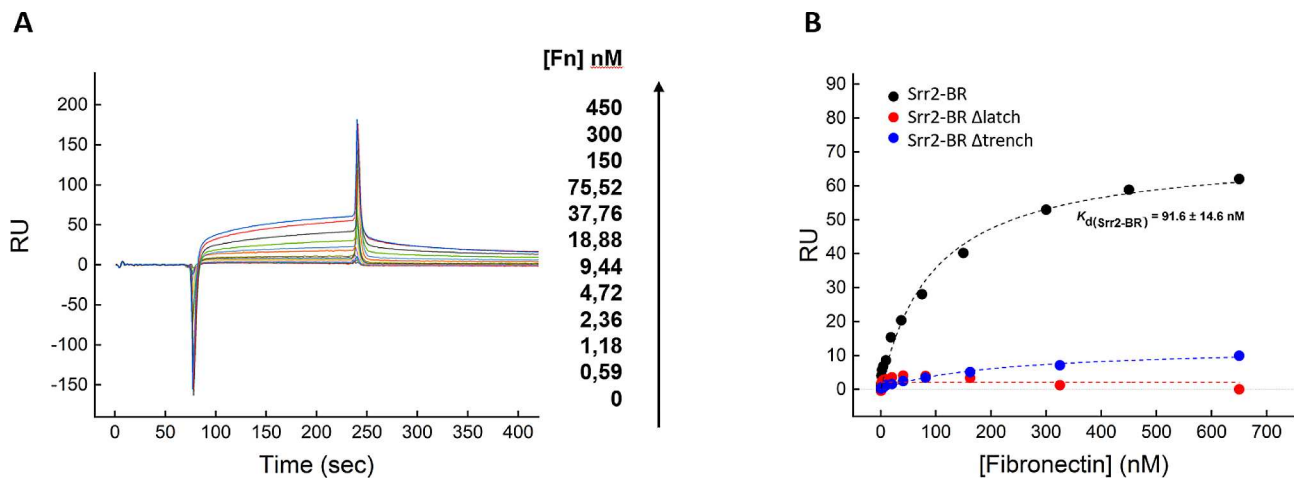


Fig. 5 SPR analysis of Fn binding to immobilized rSrr2. Recombinant Srr2 proteins were immobilized onto a CM5 sensor chip and increasing concentrations of fibronectin were injected in the mobile phase. **(A)** Representative sensograms relative to the binding of Fn to Srr2-BR. **(B)** Plot of RUmax vs. Fn concentration relative to the binding to Srr2-BR (●), Srr2-BR Δ latch (●), and Srr2-BR Δ trench (●). Fitting of data points with Eq. 1 yielded the K_d value for the Srr2-BR-Fn complex, as reported. All SPR measurements were carried out at 25 °C in HBS-EP+

Identification of the Fn region bound by Srr2-BR

Fn is a 440 kDa dimeric protein composed of several domains involved in different functions (Fig. 6A) [26]. To localize the Srr2 binding site in Fn, carboxylate microspheres were covalently coated with rSrr2-BR and then incubated with equimolar concentrations of soluble Fn or Fn fragments, i.e. the N-terminal domain (NTD, 30 kDa), the gelatin-binding domain (GBD, 40 kDa), and the cell-binding domain (CBD, 110 kDa). After washing, the beads were incubated with SDS-containing gel loading buffer, to dissociate the Srr2-Fn complex that might have been formed, and the supernatant samples were subjected to SDS-PAGE. Immunoblotting with anti-FN antibody revealed that rSrr2-BR is able to capture soluble Fn and its CBD fragment (Fig. 6B). The results obtained were also confirmed by a dot-blot assay in which Fn and its fragments were immobilised on a nitrocellulose membrane, incubated with rSrr2-BR, and binding finally revealed with an anti-Srr2 antibody (Fig. 6C). Both assays suggest that the binding site of rSrr2-BR on Fn is located in its CBD. Furthermore, the addition of increasing CBD fragments concentrations dose-dependently decreases the binding of rSrr2-BR to immobilised Fn, such that at 0.5 μ M CBD, rSrr2-BR binding is completely abolished (Fig. 6D).

Srr2 mediates GBS adhesion to epithelial cells via fn binding

To understand the biological significance of the in vitro results obtained so far in this study, we investigated the possibility that Srr2-Fn interaction could be involved in promoting GBS adhesion to cervico-vaginal epithelial cells. For this purpose, BM110 wild-type or Δ srr2 cells were used to infect HeLa cell monolayers. The cells

were seeded in DMEM medium supplemented with Fn-depleted FBS. The absence of Fn in the monolayer was confirmed by ELISA assay (data not shown). Under these conditions, after infection, no differences in adhesion levels were observed between the wild-type and Δ srr2 strains (Fig. 7). The addition of exogenous Fn to the cell monolayers prior to infection promoted a significant increase in adhesion of the wild-type but not of the Δ srr2 strain (Fig. 7). As additional experimental evidence of the importance of Srr2-Fn interaction in mediating GBS adhesion to epithelial cells, srr2 complementation restored the adhesion levels back to those observed in the wild-type strain (Fig. 7). These results suggest that the interaction of GBS with HeLa cells is promoted by the presence of Fn and that this interaction is mediated by Srr2. We also investigated whether the Srr2-Fn interaction could be involved in mediating the invasion of HeLa cells by GBS. Contrary to what was observed for adhesion, no increase in invasion levels was observed in presence of Fn in both wild-type and Δ srr2 strains, suggesting that the interaction between Srr2 and Fn does not affect BM100 invasion of HeLa cells (data not shown).

Discussion

Fn is widely abundant in animal tissues and fluids and potentially serves as a substrate for bacterial adhesion and host cell infection [27]. Bacteria can express several receptors on their surface, which exhibit recognition specificity towards distinct Fn domains, mainly the N-terminal (NTD) and central cell-binding domains (CBD) [27]. In many cases, Fn receptors engage in functions beyond simple adhesion. In fact, adhesion is often the prerequisite for the invasion and internalisation of microorganisms into the cells of colonised

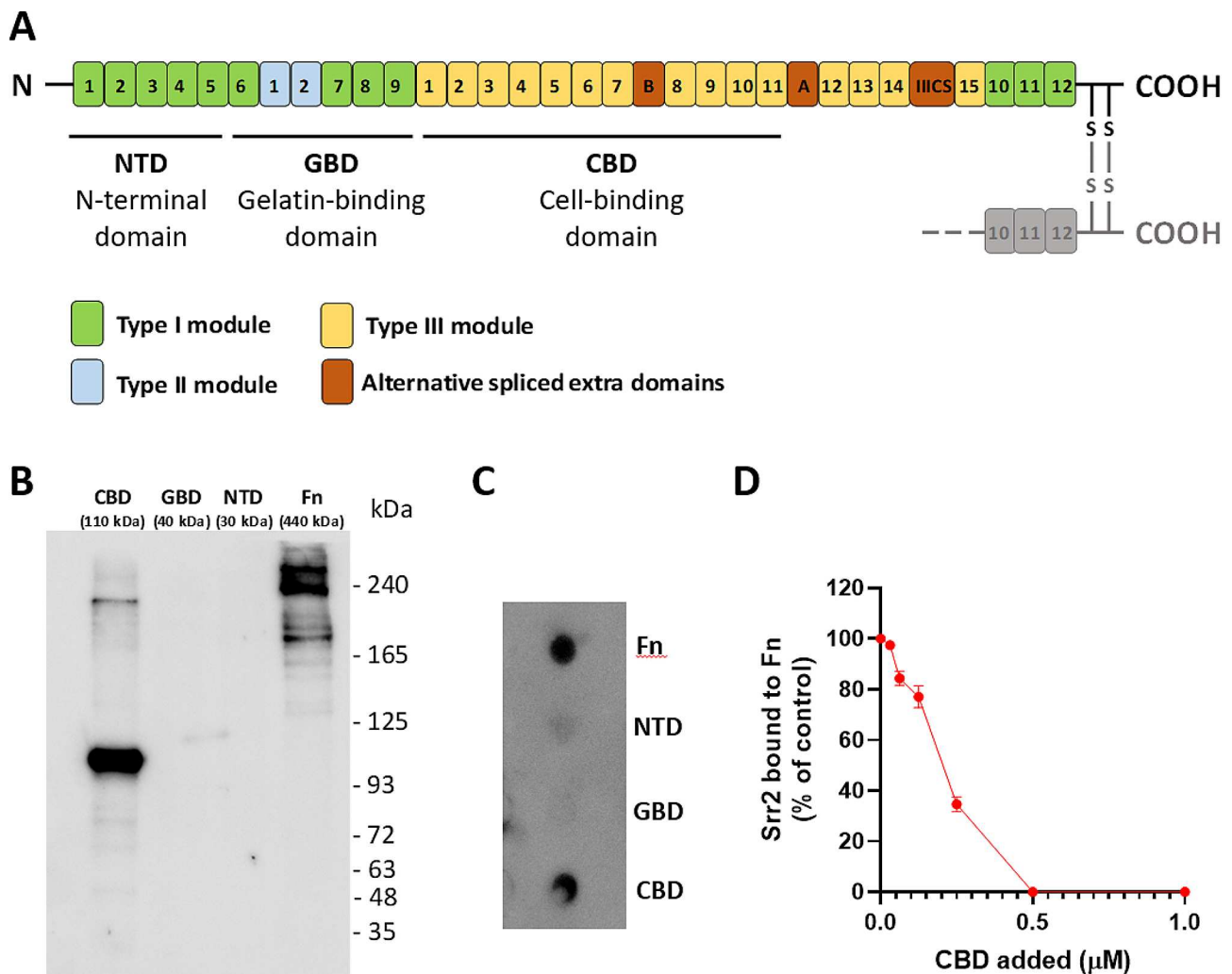


Fig. 6 Srr2-BR binds Fn at the level of CBD domain. **(A)** Schematic representation of Fn. Type I, II, and III modules are denoted by green square, light blue square, and yellow square, respectively. Alternative spliced extra domains (B-A and III-CS) are shown in brown square. N-terminal domain (NTD), the gelatin-binding domain (GBD) and the cell-binding domain (CBD) are also indicated. **(B)** Western-blot analysis of the binding of Fn or its fragments (CBD, 110 kDa; GBD, 40 kDa, and NTD, 30 kDa) to Srr2-BR-conjugated microspheres. After incubation of equimolar concentrations of Fn or its fragments with Srr2-BR-conjugated microspheres, the beads were washed and the complex dissociated using SDS-containing buffer. Samples were then subjected to SDS-PAGE and Western Immunoblotting. The membrane was probed with a polyclonal anti-Fn antibody followed by secondary HRP-conjugated anti-IgG. Molecular masses of standard proteins are indicated on the right. Original blot is presented in Supplementary Fig. 3. **(C)** Dot-blot analysis of the Srr2-BR binding to Fn or its fragments dotted onto a nitrocellulose membrane. The membrane was probed with Srr2-BR followed by incubation with polyclonal anti-Srr2 antibody and secondary HRP-conjugated anti-IgG. The figures **B** and **C** are representative of three independent experiments. **(D)** Inhibitory effect of CBD fragment on the binding of Srr2-BR to immobilized Fn. Srr2-BR was added along with increasing concentrations of CBD to Fn. Complex formation was detected by addition of a polyclonal anti-Srr2 IgG to the wells, followed by secondary HRP-conjugated anti-IgG. Binding data are expressed as a percentage of the control, i.e. incubation performed in the absence of CBD. The data points are the means $\pm$ SD from three independent experiments, each performed in triplicate

tissues. Furthermore, the presence of Fn on the surface of microbes may enable them to evade recognition by host defence mechanisms [27]. Consequently, Fn-binding proteins on bacterial surfaces can serve both as adhesion/invasion and anti-opsonic factors, and therefore have good potential as virulence determinants.

Among the best-characterised Fn adhesins are two proteins encoded by closely related genes, Fn-Binding Protein A and B (FnBPA and FnBPB), expressed by the

majority of *Staphylococcus aureus* strains [28]. FnBPA and FnBPB have the ability to bind the NTD of Fn through an intricately disordered region consisting of several repeating units (11 repeats for FnBPA and 10 for FnBPB) located at the C-terminus of the proteins [29]. Each repeat unit binds Fn with varying affinities, adopting an ordered secondary structure upon binding. At least six repeats bind to the NTD of Fn with high affinity through the β -zipper model [30–32].

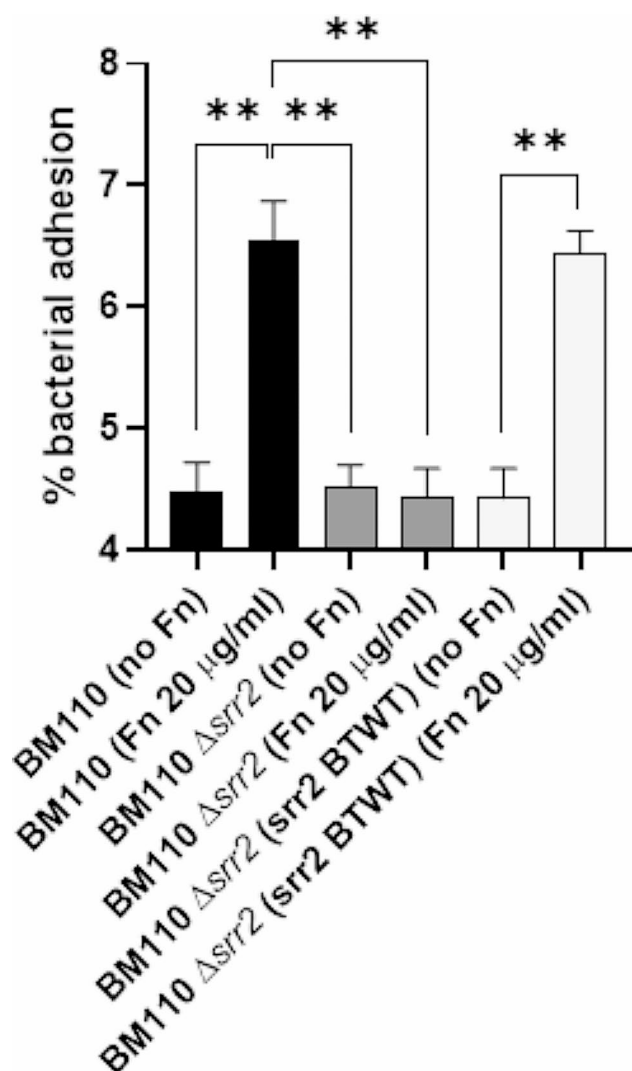


Fig. 7 Srr2 mediates GBS adhesion to epithelial cells through Fn binding. Confluent HeLa cells' monolayers grown in Fn-free medium preincubated or not with exogenously added Fn were infected with the indicated bacterial strains. The percent of adhesion of each strain was calculated relative to the initial inoculum. Statistically significant difference is indicated (** $P < 0.01$). The data points are the means $\pm$ SD from three independent experiments, each performed in triplicate

Several Fn-binding adhesins have been described in streptococcal species, e.g. *S. pyogenes* [33] and *S. pneumoniae* [34–36]. The Fn binding proteins identified in GBS so far include: (i) the streptococcal C5a peptidase B (ScpB), which is able to interact with adsorbed Fn [37], (ii) the streptococcal Fn binding protein A (SfbA), which facilitates the invasion of GBS into human brain microvascular endothelial cells (hBMECs) in vitro and contributes to meningitis development in vivo [38], and (iii) the GBS bacterial surface adhesin (BsaB), that promotes GBS attachment to epithelial vaginal ME-180 cells [39].

In this work we show that deletion of the global transcriptional regulator CodY in the hypervirulent ST-17

GBS strain BM110 causes a significant increase in bacterial interaction with Fn and Fbg. We have recently shown that deletion of *codY* increases the expression of the *srr2* gene [15]. Considering that this adhesin is a well-known ligand of Fbg [9], we postulated its potential involvement also in Fn binding. The results reported in this study concurrently support this hypothesis, as the deletion of *srr2* resulted in a remarkable decrease in the ability of strain BM110 to bind not only Fbg (used as control) but also Fn. Conversely, complementation of the *srr2* mutation restored binding to wild-type levels, strongly suggesting that the protein can indeed play a role in this interaction. As deletion of *srr2* in the *codY*-null background significantly impaired bacterial binding to Fn, we ruled out the possibility that other proteins, whose expression is controlled by the global regulator CodY, could account for the increase in Fn binding observed in the *codY* mutant strain when compared to the wild-type. Nonetheless, bacterial interaction with Fn was restored in the back to wild-type strain ($\Delta codY \Delta srr2$ (*srr2* BTWT)). Noteworthy, a residual binding to Fn was still detectable upon deletion of *srr2*, either in the single or double $\Delta srr2/codY$ deletion mutants. This could be due to the other Fn-binding adhesins of GBS (e.g. ScpB, SfbA, BsaB), whose expression might be regulated by different mechanisms independent from the regulator CodY [37–39]. Redundancy of bacterial adhesins known to bind the same ligand is common among bacterial pathogens. The extensive redundancy of GBS surface adhesins that bind Fn could be exploited by the bacterium to colonise different host niches, whose environmental signals could influence the expression of an adhesin ahead of another.

The hypervirulent ST-17 strains exhibit a significantly higher interaction with Fn compared to the non-ST-17 strains. Therefore, the capacity of Srr2 to interact with Fn, abundantly found in both plasma and the extracellular matrix, may substantially contribute to the enhanced virulence of ST-17 strains [8].

GBS colonisation of the vaginal mucosa is the very first essential step for bacterial pathogenesis [40]. In this work we observed that in the presence of Fn, GBS shows significantly greater adhesion to cervico-vaginal epithelial (HeLa) cell monolayers. Conversely, the *srr2* deletion mutant exhibited comparable adhesion levels to epithelial cell monolayers regardless of the presence or absence of Fn. Our results indicate that the interaction between Srr2 and Fn contributes to cell adhesion but not to the invasion process for the cell line under our investigation. However, we cannot exclude that it is involved in invasion of other cell lines. It is likely that Srr2 binding to Fn is important for GBS colonisation and niche establishment in the vaginal tract. As the most common route of GBS dissemination to the newborn is by vertical transmission at the moment of labour from a vaginally colonised

mother, we can speculate that Srr2 interaction with Fn at the level of the vaginal epithelium could additionally contribute to the increased pathogenic potential of GBS ST-17 strains. Furthermore, the Srr2-Fn interaction could provide additional insight into the greater adhesive properties of ST-17 strains compared to non-ST-17 isolates [4].

These isolates are able to translocate across the intestinal epithelial barrier via transcytosis of Peyer's patch M cells through Srr2 [7]. It is interesting to note that Fn is abundantly expressed in the intestinal epithelium [41]. Consequently, the interaction between Srr2 and Fn might facilitate gut colonisation by GBS ST-17 strains, serving as an initial step that promotes subsequent stages of bacterial invasion in the host. In a future study, it would be worth investigating the role of Srr2-Fn interaction in the colonisation of the intestinal epithelium by strain ST-17.

In order to deepen our understanding of Srr2-Fn interaction at the molecular level, we produced by recombinant DNA techniques the BR domain of Srr2, which has been already known to interact with Fbg [9]. The results obtained provide solid experimental evidence that the BR region is involved in Fn binding as well. The apparent K_d value of the Srr2-BR-Fn interaction obtained by ELISA was approximately 60-fold higher than that measured for Fbg. It is conceivable that the interaction between Srr2 and Fn plays a greater role in Fn-rich tissues. Srr2-Fn interaction was also confirmed by surface plasmon resonance, obtaining a K_d value comparable to that determined by ELISA. Interestingly, we observed that rSrr2-BR is able to bind both soluble and immobilised Fn. This property differs from other GBS Fn-binding proteins, such as rScpB, able to bind only immobilised Fn [37]. This ability could influence the colonisation and pathogenesis of GBS strains expressing Srr2.

The strong inhibition exerted by Fbg on the binding of Fn to the Srr2-BR region suggests that the binding sites for the two proteins on the Srr2-BR region are partially overlapped. Furthermore, increasing ionic strength had a detrimental effect on the binding of both Fn and Fbg to Srr2, indicating that electrostatic interactions play a significant role in the formation of these complexes. These results led us to assume that both ligands exploit a similar mechanism to bind the BR region. Since the binding of Fbg to the BR region has previously been shown to occur *via* the dock, lock, and latch (DLL) [9] mechanism, here we decided to verify whether this mechanism was also involved in the interaction with Fn. We found that Fn binds to the BR region of Srr2, most likely by the DLL mechanism used to bind Fbg, because variants lacking the ability to bind Fbg were also defective in Fn binding. To localise the binding site(s) of Srr2-BR on Fn, we tested its interaction with the main Fn fragments. The results obtained indicate that Srr-BR binds Fn at the level of the

cell-binding domain (CBD). Similar conclusions have been also reported for other Fn-binding bacterial adhesins, such as BBK32 from *Borrelia burgdorferi* [42] and Scl1 from *S. pyogenes* [43]. In our laboratory, structural biology studies are underway to locate which of the type III modules, forming the Fn CBD domain, are involved in the interaction with Srr2-BR. Whereas the results of this study concurrently indicate that Srr2-BR is the hot spot for both Fbg and Fn binding, the structural and physicochemical properties of the putative Srr2 binding regions on the two proteins remain elusive.

Intriguingly, Fbg and Fn display remarkably different structural properties and organization, but nevertheless, our results concurrently indicate that they are both able to interact with the same target adhesin (i.e., Srr2), at roughly the same binding site (i.e., Srr2-BR), and exploiting the same interaction mechanism (i.e., DLL).

More specifically, Fbg consists of three non-identical polypeptide chains ($A\alpha$ -, 610 residues; $B\beta$ -, 461 residues; γ -, 411 residues in the human species) and circulates in the plasma as a rod-shaped dimer of the three chains ($A\alpha B\beta\gamma$)₂, held together by disulfide bridges and assembled in a three-nodule architecture, with a central "E" nodule formed by the N-termini of the six chains while the C-termini of the $B\beta$ and γ chains extend outward to form two distal "D" nodules [44]. The region between the E and D nodules is mainly a three-stranded α -helical coiled-coil connector, fixed by disulfide bonds, while the $A\alpha$ chain extends with the flexible αC connector region (amino acids 221–391) and the two structured/globular αC domains (amino acids 392–610). The flexible αC connector contains ten 13-residues tandem repeats, all rich in proline and glycine [45]. Noteworthy, earlier work has localized the binding site for Srr2 on Fbg structure at the level of the tandem repeats 6–8 of the conformationally flexible αC connector [9]. Conversely, Fn is a dimeric protein made of two 250-kDa subunits, which are linked by a pair of disulfide bonds at their C-terminal ends [46]. Two forms of Fn are known, i.e. a plasma form, predominantly synthesized by liver hepatocytes, and a cellular form, which is produced by a wide variety of cells including fibroblasts, chondrocytes, myocytes, and synovial cells. Plasma Fn circulates in the blood, while cellular Fn is locally secreted in the ECM. The molecule is composed of globular modules of type I, II and III, each containing two sandwiched β -sheets and connected in series to provide a very long contour length of 120–160 nm and the sites for Fn-binding proteins [47]. Whereas type-I and type-II modules each contain two disulfide bonds that structurally reinforce the domain fold, type-III modules do not have any internal disulfide bonds, highlighting the possibility that these domains could be stretched by mechanical forces for interacting with other proteins [48]. The binding data reported in this study show that

Srr2 interacts with the CBD, mainly formed by type-III modules, puts forward the promiscuity of Srr2 in protein binding, and emphasizes the structural plasticity that Srr2-BR should have to recognize different protein entities, such as Fbg and Fn. Structural biology studies are awaited to address this key feature of Srr2 interaction mechanism.

In conclusion, the results of this work highlight a new role for the multivalent adhesin Srr2 expressed by the hypervirulent strain BM110. Noteworthy, the role of Srr2 interaction with Fn in promoting GBS adhesion to epithelial cells could represent a novel pathogenic route that could explain the increased virulence potential of this strain in the development of GBS-related diseases. Further experiments, however, are awaited to validate Srr2-Fn binding complex as a druggable interface for the development of novel therapeutic strategies aimed at interfering with GBS adhesion to target tissues and, finally, in the treatment of GBS infections.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12866-024-03374-6>.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

G.B. and G.P. conceived and coordinated the study. A.P. from University of Pavia, G.B., S.V., C.B., G.P. designed the experiments, analyzed the data, and wrote the article. A.P. from University of Pavia, C.M., E.B.M., F.C., collected the data and prepared all figures except Fig. 4. A.P. from University of Padua performed SPR analysis and prepared the Fig. 4. V.D.F. supervised SPR tasks and contribute to wrote the article. All authors reviewed the manuscript.

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Data availability

Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval

The polyclonal antibodies production protocols were approved by the Ethics Committee of the University of Pavia (OPBA permit n° 1909213) and of the University of Messina (OPBA permit n° 1805210) and by the Ministero della Salute of Italy (permit no. 665/2015).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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