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PhD in Biomedical Sciences Curriculum Biochemestry

Molecular interactions of Human Plasminogen with FnBPB, a Fibrinogen/Fibronectin-binding Protein from *Staphylococcus aureus*

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INTRODUCTION

1 Staphylococci

Staphylococcus is a genus, including fourty-five species and twenty-four sub-species, of the bacterial family of *Staphylococcaceae*. The genus name *Staphylococcus* refers to the fact that these gram-positive cocci grow in a pattern resembling a cluster of grapes (Fig. 1); however they may also appear as single cells, pairs or short chains. Most staphylococci are 0.5 to 1.5 μm in diameter, nonmotile, and able to grow in a variety of conditions: aerobically and anaerobically, in the presence of high concentration of salt and at temperature ranging from 18°C to 40°C (*Murray P.R. et al.,2009*). Staphylococci can be mainly classified in 'coagulase-positive' and 'coagulase-negative' strains depending on their ability to produce coagulase, an enzyme that interacts with prothrombin in the blood, causing plasma to coagulate by converting fibrinogen into fibrin. *Staphylococcus aureus*, the microorganism that will be considered in this thesis, is the major representative of 'coagulase-positive' staphylococci and, as it is also a 'catalase-positive' microorganism, the catalase test can be used to distinguish it from other bacteria such as Enterococci and Streptococci. The main representative of coagulase-negative microorganisms is *S. epidermidis*, a commensal bacterium of the skin that is the causative agent of severe nosocomial infections, especially in immune-suppressed patients and in those having central venous catheters (*Todar K., 2008*). These bacteria are present on the skin and mucous membranes of humans. The majority of the staphylococcal species is harmless while other are important pathogens in humans, causing a wide spectrum of life-threatening systemic diseases, including infection of the skin, soft tissues, bones, urinary tract and opportunistic infections through either toxin production or penetration into non-professional phagocytic cells (*Murray P.Ret al., 2009; Madigan M.T., & Martinko J., 2006; Ogawa S.K.et al., 1985*).

It is conceivable that a staphylococcal cell could escape phagocytosis by professional phagocytes, gain an environment rich in nutrients and be protected from certain antibiotics. Consequently these organisms have the ability to induce long-lasting chronic infections that resist convetional therapy (*Sinha B.et al., 1999*).

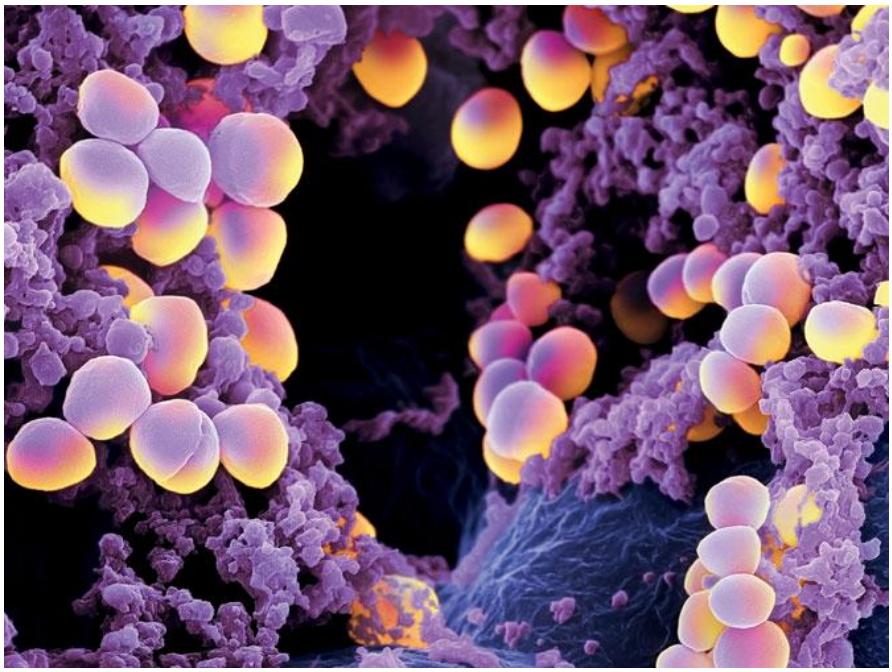


Figure 1. Scanning electron microscope image of *Staphylococcus aureus*. Magnification 20000:1. *(Oeggerli M., 2012)*.

1.1 Staphylococcal pathogenesis

The pathology of staphylococcal infections depends on the ability of the bacteria to evade phagocytosis, to produce surface proteins that mediate adherence of the bacteria to host tissues and to cause tissue destruction through the elaboration of specific toxins and hydrolytic enzymes (*Murray P.R. et al., 2009*).

1.2 *Staphylococcus aureus*

Staphylococcus aureus is a commensal bacterium that can cause both superficial and invasive, potentially life-threatening infections such as sepsis, endocarditis and pneumonia. It is a facultative anaerobic, non-motile, Gram-positive coccus. It is very tolerant to high concentrations of sodium chloride (up to 1.7 M) and can grow at a temperature range of 15°C to 45°C (*Ryan K.J.& Ray C.G., 2004*). *S. aureus* colonies can have a yellow or a gold color because of the presence of staphyloxanthin, an orange-red triterpenoid carotenoid that functions as virulence factor and also as antioxidant, protecting bacteria against oxidative stress and the host immune response (*Clauditz A. et al., 2006; Liu G.Y. et al., 2005*). Under the microscope the cells appear round and form grape-like clusters (Fig. 2) (*Ryan K.J. & Ray C.G., 2004; Todar K., 2008)*.

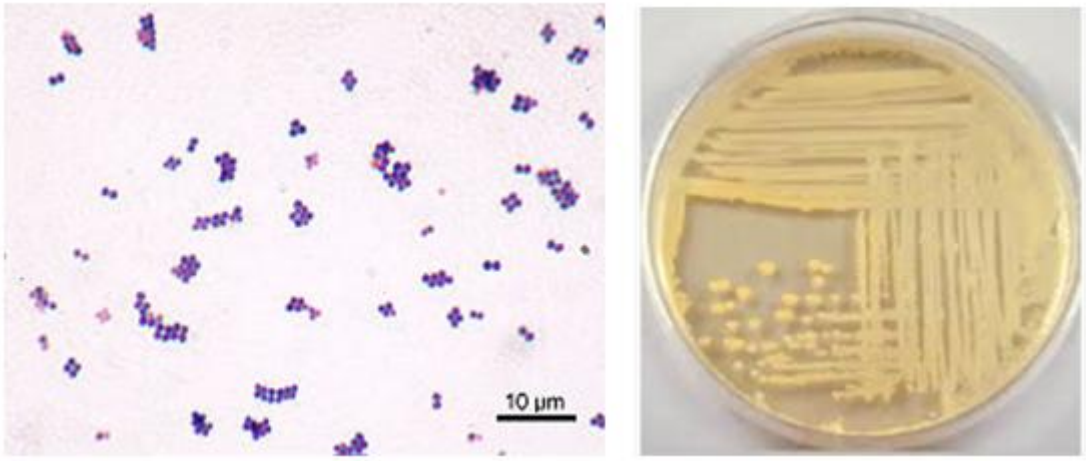


Figure 2. *Staphylococcus aureus*. Gram stain *(Y tambe., 2005)*; Tryptic Soy Agar (TSA) plate, colonies appear golden-yellow *(Kaiser G.E., 2002)*

1.3 *Staphylococcus aureus* genome

The *Staphylococcus* genome is composed of a complex mixture of genes, many of which seem to have been acquired by lateral gene transfer. Among the 3 mechanisms for horizontal gene transfer in bacteria, bacteriophage transduction appears to be the main method for gene transfer in *S aureus*. *Staphylococcus* genome typically contains housekeeping genes involved in essential functions of the vegetative life of the bacteria, such as DNA replication, protein synthesis, and carbohydrate metabolism; and accessory genes encoding a diverse range of nonessential functions ranging from virulence, drug and metal resistance, to substrate utilization and miscellaneous metabolism. Most of the antibiotic resistance genes are carried either by plasmids or by mobile genetic elements including a unique resistance island. Three classes of new pathogenicity islands have been identified in the genome: a toxic shock-syndrome toxin island family, exotoxin islands, and enterotoxin islands. In the latter two pathogenicity islands, clusters of exotoxin and enterotoxin genes were found closely linked with other gene clusters encoding putative pathogenic factors. The remarkable ability of *S. aureus* to acquire useful genes from various organisms was revealed through the observation of genome complexity and evidence of lateral gene transfer. Repeated duplication of genes encoding superantigens explains why *S. aureus* is capable of infecting humans of diverse genetic backgrounds, eliciting severe immune reactions *(Kuroda M.et al., 2001)*.

1.4 Structure and morphology

S. aureus is characterized by an optional polysaccharide capsule, a cell wall and the plasma membrane. Peptidoglycan is the basic component of the cell wall, and makes up 50% of the cell wall mass (*Waldvogel F.A.et al., 1990*). Peptidoglycan is a large polymer that is made up of glycan strands of two alternating sugar derivatives that form a disaccharide subunit: *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM).The carboxyl group of NAM is linked to a peptide subunit (or stem peptide) consisting of four alternating L- and D- aminoacids. *S. aureus* is characterized by an L-lysine in position 3 of the stem peptide that is cross-linked via a pentaglycine interpeptide bridge. These multiple glycine residues in the cross bridge cause susceptibility to lysostaphin (*Projan S.J.& Novick R.P. 1997*).

Peptidoglycan is integral in the formation of the tight multi-layered cell wall network (figure 3), capable of withstanding the high internal osmotic pressure of staphylococci (*Wilkinson B.J. & Holmes K.M., 1979*). Peptidoglycan moreover plays a role in the inflammatory response by stimulating the production of proinflammatory cytokines and chemokines (TNF-α, IL-1β, IL-6, and IL-8) in monocytes and macrophages (*Heumann D. et al., 1994; Mattsson E. et al., 1993; Timmermann C.P.et al., 1993; Wakabayashi G. et al., 1991*).

Another cell wall constituent is a group of phosphate-containing polymers called teichoic acids (TA), which contribute about 40% of cell wall mass (*Knox K.W. & Wicken A.J., 1973*). Several crucial roles in *S. aureus* fitness and cell wall maintenance have been assigned to complex TApolymers, which are either covalently linked to peptidoglycan (wall teichoic acids, WTA) or to the cytoplasmic membrane (lipoteichoic acids, LTA) (Fig. 3). Both WTA and LTA are highly charged polymers that concentrate cations at the cell wall surface and associate with proteins to form complexes: they are involved in various biological activities such as cation balance, cell division and regulation of peptidoglycan autolysis (*Ruhland G.J. & Fiedler F., 1990*). Although crucial for bacterial life, purified WTA is not very inflammatory, but recent studies have shown that it plays a role in host tissue adhesion (*Majcherczyk P.A.et al., 2003*). Furthermore, many studies show that LTA may induce an inflammatory response (*Cleaveland M.G. et al., 1996; Kengatharan K.M.et*

al., 1998; von Aulock S.et. al., 2003) and, acting in synergy with peptidoglycan, can cause septic shock and multiple organ failure (*De Kimpe S.J. et al., 1995*).

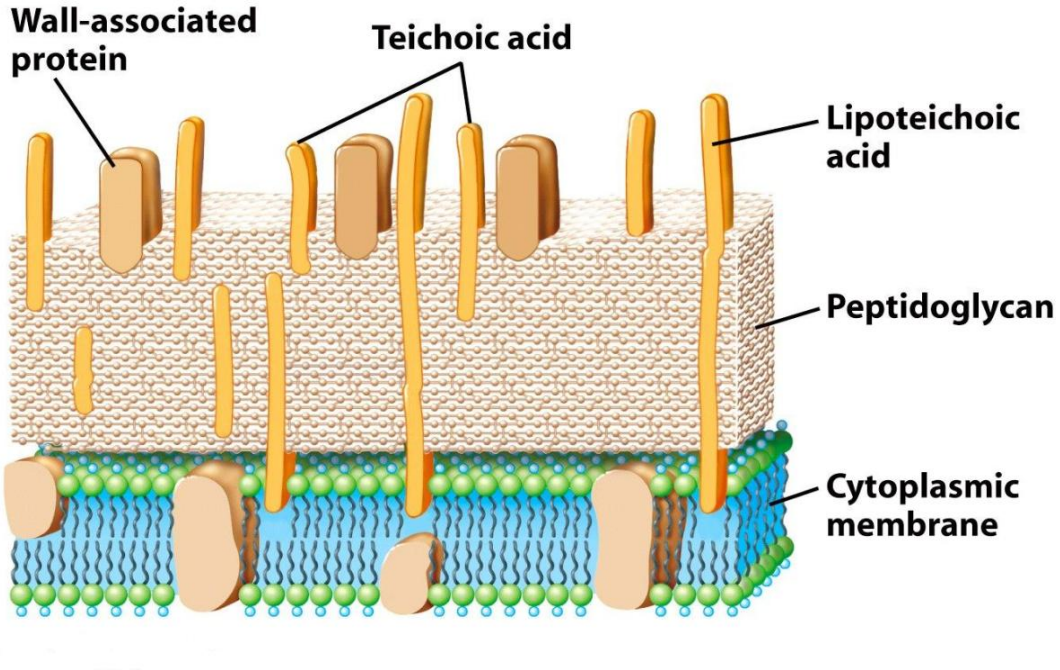


Figure3. *Staphylococcus aureus* cell wall components and organization. *(Madigan M.T. & Martinko J.M., Brock Biology of Microorganisms, 11th edition, 2006).*

1.5 *S. aureus* pathogenesis

Stahylococcus aureus often colonizes host tissues asymptomatically and lives as a commensal of the human nose. It is well known that 20% of healthy subjects are persistently colonized and 60% are intermittent carriers, whereas 20% never carry *S. aureus*, (*Kluytmans J. et al., 1997*). Nevertheless, *S. aureus* is one of the most important bacterial pathogens to affect humans both in community–acquired and nosocomial infections and other animal organisms (*Todar K., 2008*). If mucosal barriers are breached or host immunological defences are impaired, *S. aureus* can cause morbidity and mortality in humans and other animals. *S. aureus* can cause a broad spectrum of diseases such as arthritis, pneumonia (*Fluit A.C. et al., 2001*) mastitis, phlebitis, meningitis (*Aguilar J. et al., 2010*), urinary tract infections (*Fluit A.C. et al., 2001*) and also deep seated infections, such as osteomyelitis (*Corrah T.W.et al., 2011*) endocarditis (*Moreillon P. & Que Y.A. 2004*), food poisoning by releasing enterotoxin into food and toxic shock syndrome by releasing superantigens into the blood stream. Furthermore staphylococcal infection

induces an influx of neutrophils, so *S. aureus* is a pyogenic pathogen capable of tissue invasion and evasion of phagocytosis by neutrophils (*Todar K., 2008*).

Moreover although *S. aureus* is not considered an intracellular pathogen, its ability to invade, colonize, and survive inside nonprofessional phagocytic cells (epithelial cells, fibroblasts, osteoblasts and endothelial cells) (*Kintarak S. et al., 2004; Jevon M. et al., 1999; Hamill R.J. et al., 1986; Tompkins D.C.et al., 1992*) gives rise to long-lasting chronic infections that resist conventional therapy, because after internalization *S. aureus* may escape host defences and antibacterial agents, or multiply and further disseminate.

This behaviour may or may not trigger the secretion of toxins and hemolysins that lyse host cells and allow the bacteria to propagate (*Moreillon P. & Que A.Y., 2004; Novick R.P. et al., 1993; Wu S. et al., 1996*).

Infective endocarditis is a good model to study *S. aureus* pathogenesis. It implicates step wise valve colonization by circulating bacteria, local settling and invasion, and the eventual dissemination to distant organs (*Moreillon P.&Que A.Y., 2004*).

1.6 Virulence factors

S. aureus pathogenicity is even due to the repertoir of toxins, exoenzymes and proteins that it produces (*Todar K., 2008*). They are shown in figure 4, while their function is explained in table 1.

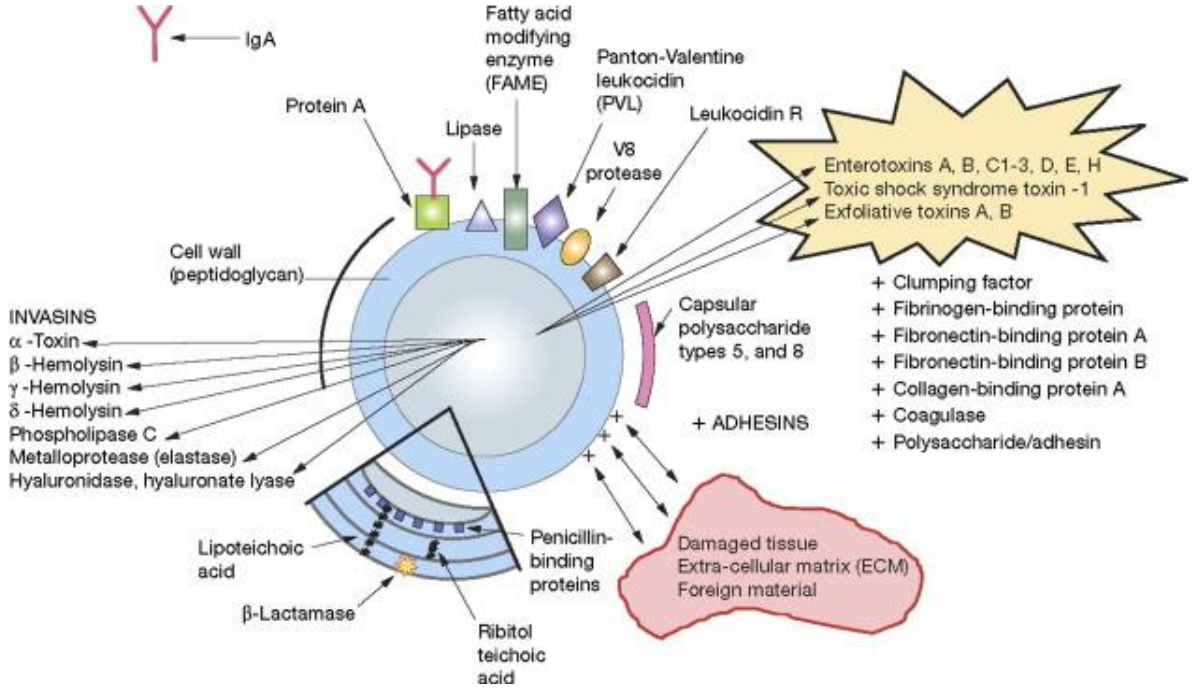


Figure4. Selected *Staphylococcus aureus* virulence factors. Abbreviation: Ig, immunoglobulin**.** (*Daum R.S. & Spellberg B., 2012)*

Structural components	
Capsule	Inhibits chemotaxis and phagocytosis; inhibits proliferation of mononuclear cells
Slime layer	Facilitates adherence to foreign bodies; inhibits Phagocytosis
Peptidoglycan	Provides osmotic stability; stimulates production of endogenous pyrogen (endotoxin-like activity); leukocyte chemoattractant (abscess formation); inhibits phagocytosis
Teichoic acid	Binds to fibronectin
Protein A	Inhibits antibody-mediated clearance by binding IgG_1, IgG_2 and IgG_4Fc receptors; leukocyte chemoattractant; anticomplementary
Toxins	
Cytotoxins	Toxic for many cells, including erythrocytes, fibroblasts, leukocytes, macrophages, and platelets
Exfoliative toxins (ETA, ETB)	Serine proteases that split the intracellular bridges in the stratum granulosum epidermis
Eterotoxins (A- R)	Superantigens (stimulate proliferation of T cells and release of cytokines); stimulate release of inflammatory mediators in mast cells, increasing intestinal peristalsis and fluid loss, as well as nausea and vomiting
Toxic shock syndrome toxin-1	Superantigen (stimulates proliferation of T cells and release of cytokines); produces leakage or cellular destruction of endothelial cells
Enzymes	
Coagulase	Converts fibrinogen to fibrin
Hyaluronidase	Hydrolyzes hyaluronic acids in connective tissue, promoting the spread of staphylococci in tissue
Fibrinolysin	Dissolves fibrin clots
Lipases	Hydrolyzes lipids
Nucleases	Hydrolyzes DNA

Table1. *S. aureus* virulence factors (*Murray P.R. et al., 2009*).

With the exception of diseases caused by specific toxins such as entero-toxin, exfoliative or toxic shock syndrome toxins, no single virulence factor has been shown to be sufficient to provoke a staphylococcal infection. Infection is promoted rather by the coordinated action of various virulence factors, which include **C**ell **W**all **A**nchored (**CWA**) and/or secreted proteins. Indeed, both localized infections and life-threatening systemic diseases result from the ability of this pathogen to (1) attach to cells or tissues, (2) escape the host immune system (producing factors that decrease phagocytosis or interact with anti staphylococcal antibodies); and (3) produce elaborate proteases, exotoxins and enzymes that specifically cause tissue damage allowing bacterial dissemination (*Projan S.J. & Novick R.P., 1997*).

In *S. aureus*, as well as in many other organisms, the expression of the genes that encode for these virulence factors is controlled by several regulatory systems such as Agr, SarA and Arl (*Cheung A.L. et al., 1992*): they respond to changes in bacterial surroundings during the infection process (*quorum-sensing*) and adapt the expression of the virulence genes to the benefit of bacteria. The accessory gene regulator (*agr*) is one of the best-characterized global regulatory systems involved in the regulation of virulence factor genes: it increases the expression of exoprotein genes and decreases the expression of CWA protein genes (*Morefeldt E. et al., 1988; Peng H.L. et al., 1988*). Several serious nosocomial diseases are caused by biofilm-associated *S. aureus* infections, in which the *agr* quorum-sensing system is thought to play an important role (*Shirtliff M.E. et al., 2002*).

Biofilm is a sessile community of cells that is embedded in an extracellular polymeric matrix (*Stewart P.S. & Costerton J.W., 2001; Cucarella C. et al., 2001; O'Neill E. et al., 2008*) that confers resistance to conventional therapeutic doses of antimicrobial agents (*Stewart P.S. & Costerton J.W., 2001; Donlan R.M. & Costerton J.W., 2002*) and to clearance by host response (*Vuong C.et al., 2004*) and increases the opportunity for transfer of resistance genes between bacteria, enhancing the conversion of a previously a virulent commensal organism into a highly virulent pathogen. Biofilm infections are important clinically because bacteria in biofilms exhibit recalcitrance to antimicrobial compounds and persistence in spite of sustained host defenses. The development of a bacterial biofilm is a complex, multifactorial process and can be divided into three phases which involve specific molecular factors: attachment, accumulation/maturation, and detachment/dispersal

(*Speziale P. et al., 2014)*. Many staphylococcal biofilms include, as an essential constituent, the **P**olysaccharide **I**ntercellular **A**dhesins (**PIA**s) (*Mack D. et al., 1996*), as well as proteins (*Cucarella C. et al., 2001; O'Neill E. et al., 2008*), teichoic acids (*Gross M. et al., 2001*) and extracellular DNA (*Qin Z. et al., 2007*). Staphylococci may develop biofilm on indwelling central venous catheters and on permanently implanted medical devices such as artificial heart valves, leading to a condition known as prosthetic valves endocarditis (*Karchmer A.W.et al., 1994*). As a consequence, these infections must often be resolved by removal of the contaminated devices, and thus represent a major cost burden on healthcare. Therefore growth as a biofilm makes the eradication of *Staphylococcus* infections very difficult, leading to a permanent disease status.

1.7 Emergence of methicilin/vancomycin-resistant *Staphylococcus aureus*

Staphylococcus aureus is the leading cause of bacterial infections in developed countries and produces a wide spectrum of diseases, ranging from minor skin infections to fatal necrotizing pneumonia. Although *S. aureus* infections were historically treatable with common antibiotics, emergence of drug-resistant organisms is now a major concern (*DeLeo F.R.& Chambers H.F., 2009*). Methicillin-resistant *Staphylococcus aureus* (MRSA) infections have become a major public health problem in both the community and hospitals (*Liu C. et al., 2008*).

These community-associated strains (CA-MRSA community-associated methicillin resistant *S. aureus*) combine exceptional virulence with antibacterial resistance and pose a severe threat to the public health system, second only to HIV/AIDS in scope and importance (*DeLeo F.R.& Chambers H.F, 2009*). In many infections with highly resistant strains, vancomycin is the only antibiotic of last resort. However, high-level resistance to vancomycin has been reported in several cases for *S. aureus* (*Hiramatsu K., 2001*). Significant advances have been made in recent years in our understanding of how methicillin resistance is acquired by *Staphylococcus aureus*. The mechanism of methicillin resistance was not elucidated until 1981, when Hartman and co-workers *(Hartman B. et al, 1981*) discovered altered penicillin-binding proteins (PBP2') in MRSA that had markedly reduced affinity for all currently available beta-lactam antibiotics while maintaining effective cell wall-building activity. PBP2' is encoded by the mecA gene which is carried on a mobile DNA element, the staphylococcal cassette chromosome mec (SCCmec). The

other important component of SCCmec, the chromosome cassette recombinase (ccr) genes, encodes for proteins that enable precise integration into and excision from a specific site of the *S. aureus* chromosome (attBscc) *(Wijaya L. et al, 2006)*.

Integration of a staphylococcal cassette chromosome mec (SCCmec) element into the chromosome converts drug-sensitive *S. aureus* into the notorious hospital pathogen methicilin-resistant *S. aureus*, which is resistant to practically all beta-lactam antibiotics. SCCmec is a novel class of mobile genetic element that is composed of the mec gene complex encoding methicillin resistance and the ccr gene complex that encodes recombinases responsible for its mobility. These elements also carry various resistance genes for non-beta-lactam antibiotics. After acquiring an SCCmec element, MRSA undergoes several mutational events and evolves into the most difficult-to-treat pathogen in hospitals, against which all extant antibiotics including vancomycin are ineffective (*Hiramatsu K., 2001*).

A major class of proteins responsible for the survival of the microorganism in the host, is the one of surface proteins that mediate adherence of *S. aureus* during initiation of tissue colonization: the CWA proteins, or adhesins, and the main family that represents them is the one of **MSCRAMM**s (**M**icrobial **S**urface **C**omponents **R**ecognizing **A**dhesive **M**atrix **M**olecules).

1.8 Cell Wall-Anchored proteins

S. aureus can express a broad range of virulence factors, including surface proteins that are covalently attached to peptidoglycan, which are known as Cell Wall Anchored proteins (CWA). These surface proteins are crucial to the success of the organism as commensal bacterium and as a pathogen. This is due to the fact that they are involved in several function like adhesion to and invasion of the host cells, iron acquisition, evasion of the immune response, and biofilm formation (Fig. 5) (*Foster T.J. et al., 2014*). The precise repertoire of CWA proteins on the surface varies among strains: *S. aureus* can express up to 24 different CWA proteins, whereas coagulase-negative staphylococci such as *Staphylococcus epidermidis* and *Staphylococcus lugdunensis* express a smaller number (*Heilbronner S. et al., 2011; Bowden M.G. et al., 2005*). Moreover, the expression of CWA proteins can be altered by growth conditions; for example, some proteins are expressed only under iron-limited conditions (*Hammer N.D. & Skaar E.P, 2011; Mazmanian S.K. et*

al., *2003*), whereas others are found predominantly on cells in the exponential (*McAleese F.M*. *et al*., *2001*) or stationary phases of growth (*Bischoff M*. *et al*., *2004*). Secretory signal sequences that are located at the amino termini direct the translated proteins to the secretory (Sec) apparatus in the membrane and are cleaved during secretion. At their carboxyl termini, each of these proteins has a characteristic sorting signal, which facilitates their covalent anchorage to peptidoglycan.

Many CWA proteins of *S*. *aureus* have evolved to have multiple roles. Moreover, these proteins show functional redundancy; for example, at least five CWA proteins bind the plasma glycoprotein fibrinogen and several promote adhesion to squamous epithelial cells. One consequence of this redundancy is that a null mutant that affects one CWA protein might only be partially defective in the studied function (*Foster T.J et al*., *2014*).

CWA proteins can be classified into four groups on the basis of the presence of motifs that have been defined by structure–function analysis. The most prevalent group is the **M**icrobial **S**urface **C**omponent **R**ecognizing **A**dhesive **M**atrix **M**olecule (**MSCRAMM**) family, which is defined by tandemly linked IgG-like fold domains. The other groups comprise: **NEAT** (NEAr Iron transporter) **motif family**, proteins involved in the capture of haem from haemoglobin to survive in iron-deficient environments, as the case of Iron-regulated Surface Determinant (Isd) proteins; **Protein A**, which is characterized by tandem repeated three-helical bundles; **G5-E repeat protein** (SasG) containing G5 (five conserved glycine) domains in tandem array that is separated by 50-residues sequences that are known as E-region (*Conrady D.G.et al*., *2013; Gruszka D.T*. *et al*., *2012*).

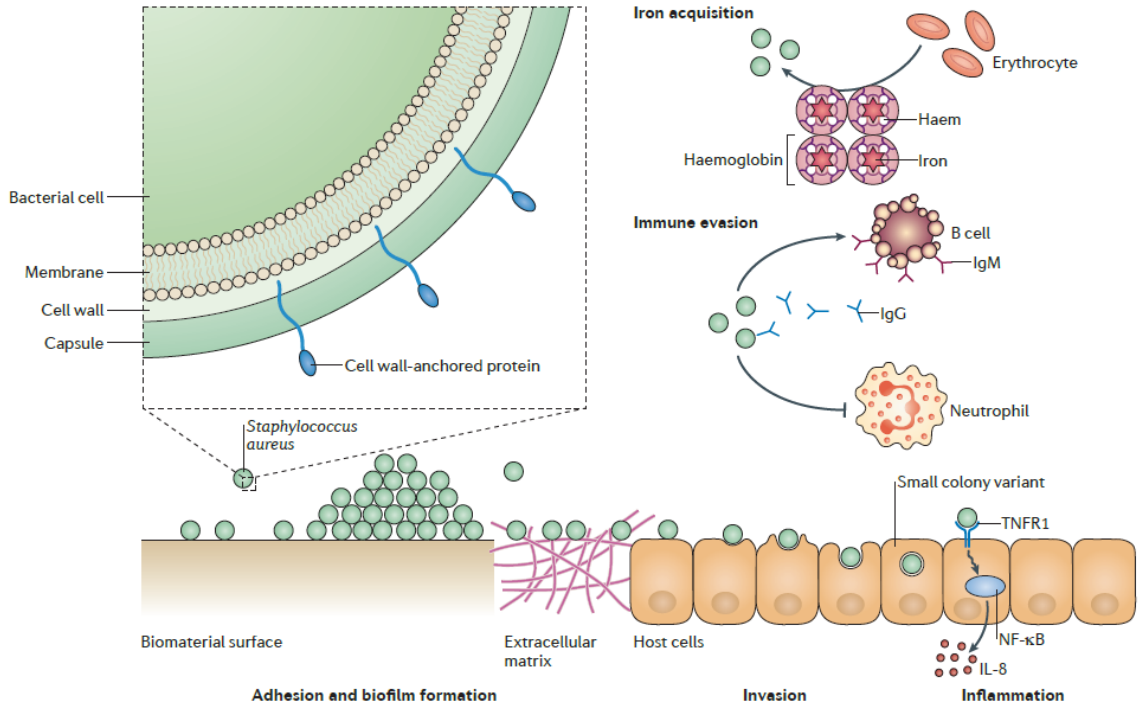


Figure 5. Key functions of CWA proteins in *S. aureus*. (1) The cell wall-anchored (CWA) protein iron-regulated surface determinant (Isd) binds haemoglobin and extracts and transports haem across the cell wall and membrane into the cytoplasm, where iron is released. (2) Protein A acts as a superantigen for B lymphocytes and disrupts adaptive immune responses and immunological memory. (3) Phagocytosis by neutrophils is inhibited by the binding of CWA proteins to IgG and other plasma proteins, by reducing the level of — or the access by — neutrophil receptors to the complement opsonin C3b and, if engulfed, by inhibiting the oxidative burst. (4) CWA proteins promote adhesion of *Staphylococcus aureus* to the extracellular matrix, to the surface of host cells and to biomaterial surfaces that are conditioned by the deposition of plasma proteins. (5) Interactions between CWA proteins on adjacent cells contribute to the accumulation phase of biofilm formation. (6) CWA proteins directly or indirectly interact with integrins and promote the invasion of non-phagocytic host cells. Intracellular bacteria can cause host cell apoptosis or necrosis, or they can enter a non-disruptive semi-dormant state known as small colony variants. (7) By binding to and activating tumour necrosis factor receptor 1 (TNFR1) on host epithelial cells, protein A triggers the synthesis of cytokines (for example, interleukin-6 (IL-6)) and causes disruptive inflammation, which contributes to pathogenesis. NF-κB, nuclear factor-κB (*Foster T.J. et al., 2014*).

1.8.1 NEAT motif family

Near iron transporter (NEAT) motif proteins are involved in haem capture from haemoglobin and help bacteria to survive in the host, where iron is restricted. Haem is transported via several CWA proteins, called iron-regulated surface (Isd) proteins, to a membrane transporter and then to the cytoplasm, where haemoxygenases release free iron(*Hammer N.D.& Skaar E.P., 2011; Cassat J.E.& Skaar E.P., 2012*).The defining characteristic of Isd CWA proteins is the presence of one or more NEAT motifs, which bind either haemoglobin or haem (Fig. 6).

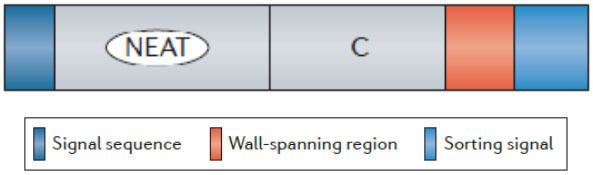


Figure6. NEAT motif family (*Foster T.J. et al., 2014*)

1.8.2 The G5–E repeat family

S. aureus surface protein G (SasG) is closely related to the accumulation-associated protein (Aap), which is needed for biofilm formation in *S. epidermidis*. Notably, both proteins contain identical G5 domains in a tandem array that are separated by 50-residue sequences that are known as E regions (*Conrady D.G. et al., 2013; Gruszka D.T. et al., 2012*). G5 domains are characterized by five conserved glycine residues, and they adopt a β–triple helix–β-like fold that has no known ligand-binding function (Fig.7). In general, proteins that comprise highly similar domains in a tandem arrangement are prone to misfolding (*Borgia M.B. et al., 2011*). As the amino acid sequence of each G5 domain is identical, it is thought that alternating individually folded G5 and E regions is a mechanism to prevent protein misfolding. The G5–E domains of Aap and SasG become exposed on the surface of the bacteria either by proteolytic removal of the N-terminalA domain (in the case of Aap) (*Rohde H. et al., 2005*) or by limited cleavage within the G5–E domains (in the case of SasG) (*Geoghegan J.A. et al., 2010*).

Figure 7. G5–E repeat family (*Foster T.J. et al., 2014*)

1.8.3 Protein A and Sbi: three helical bundle motif

Protein A is a multifunctional CWA protein that is ubiquitous in *S. aureus* and is often used in strain typing on the basis of variation in the DNA sequence-encoding region Xr. At the N terminus, protein A contains five homologous modules (known as EABCD; Fig. 8A), each of which consists of single separately folded three-helical bundles (*Deisenhofer J., 1981; Cedergren L. et al.,1993*) that can bind to several distinct ligands.

Protein A is the only CWA protein of *S. aureus* that has the repeated three-helical bundles; however, the *S. aureus* binder of IgG protein (Sbi), which is non covalently associated with lipoteichoic acid in the cell wall (*Smith E.J. et al., 2012*), contains four three-helical bundles, two of which have sequence similarity to protein A (*Burman J.D. et al., 2008*). Sbi protein at C-terminus comprises the proline-rich region Wr and the tyrosine-rich region Y that is assumed to be involved in Sbi association with the cell envelope. The first two N-terminal repeats (D1 and D2) have sequence similarity to the IgG-binding domains of protein A. Indeed the predicted structures show that residues on the faces of helices I and II of protein A and Sbi are conserved, allowing Sbi to bind to IgG in a similar fashion to the archetypal LPXTG-anchored immune evasion protein. Domains D3 and D4 are also separately folded and contribute to the elongated structure of the protein (*Burman J.D. et al., 2008; Upadhyay A. et al., 2008*). They bind to complement protein C3. It has been argued that binding to and promoting the conversion of C3 to C3b would only be an effective immune evasion mechanism if the protein were extracellular (*Smith E.J. et al., 2012*) (Fig. 8B). In Fig. 8C is shown the hypothesis formulated by E. J. Smith et al. (Smith E.J. et al., 2012), according to which Sbi would be associated to the surface of bacterium through a bond with lipoteichoic acid and exposing the N-domain D1D2 terminals that interact with the IgG. The extracellular part of Sbi not bind to the wall and is available and likely triggers the metabolism of C3 as described by J. D. Burman et al., 2008. The protein, according to E. J. Smith et al., is present in extracellular and associated form, both forms are needed for full protection from host defense systems. The binding of IgG to D1D2 domains would be biologically active when Sbi is associated with the cell surface, while the D3D4 domains would fold at C3 only in the case the protein is secreted.

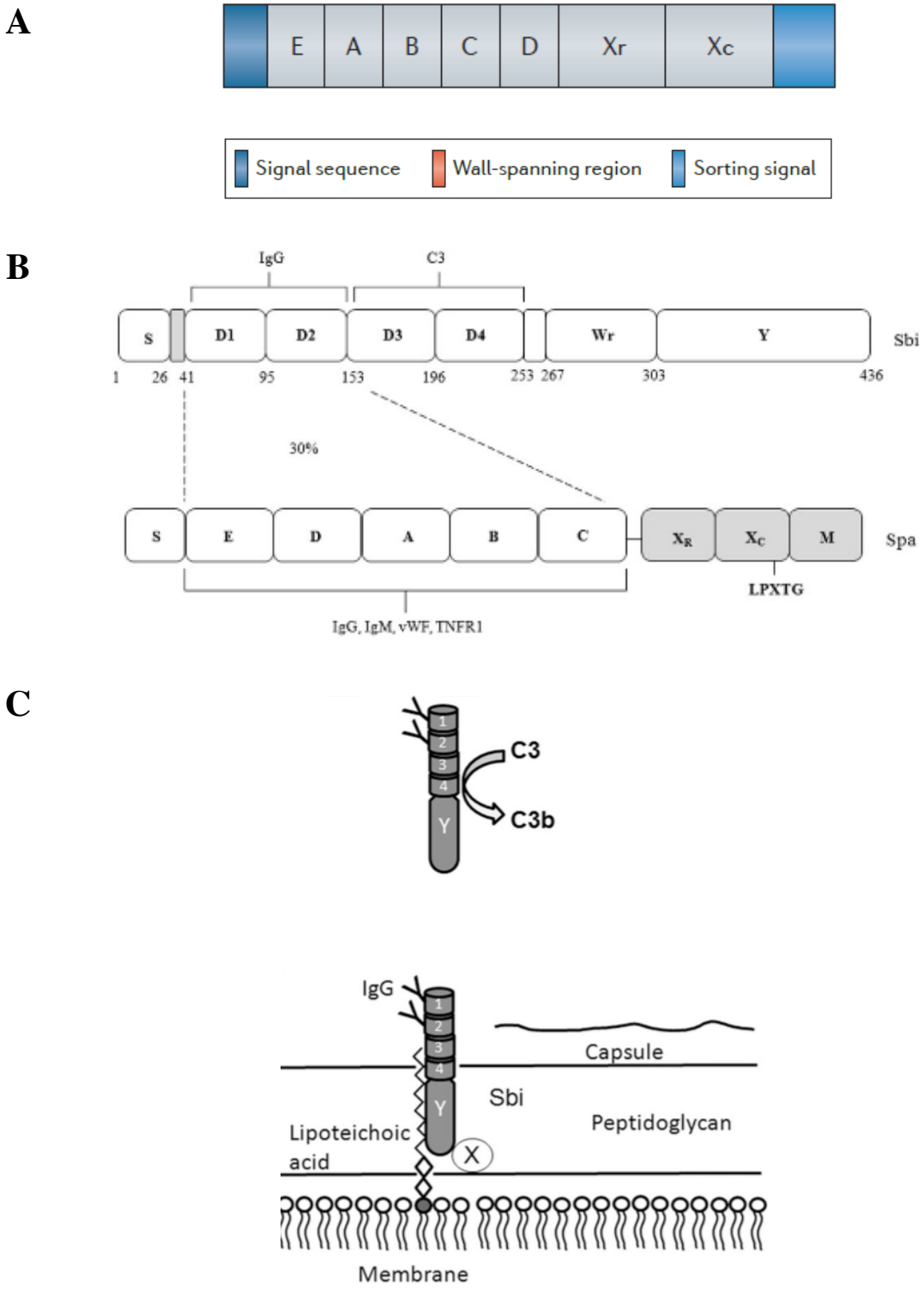


Figure 8.A) Schematic organization of three-helical bundle protein family (*Foster T.J. et al., 2014*).
B) Schematic diagrams of Sbi and Spa. The upper figure is Sbi and the lower is Spa. S, signal sequence; D1D2, Sbi IgG-binding domains that have sequence similarity to the IgG-binding domains of Spa (E, D, A, B, C); D3D4, Sbi complement factor C3-binding domains; Wr and Xr, proline-rich C-terminal domains; Y, C-terminal domain; LPXTG, wall-anchoring motif; M, transmembrane domain and positively charged C-terminus. Spa ligands are indicated. (*Smith E.J. et al., 2012)*
C) Proposed model for Sbi cellular localization and surface expression. The diagram shows Sbi binding to lipoteichoic acid (LTA) and to a putative second membrane component (X). Only one face of the lipid

bilayer is shown. The IgG-binding domains D1D2 are biologically active when Sbi is associated with the cell membrane. Whereas D3D4 are biologically active only when secreted. The wall-anchored proteins Spa and ClfA are shown for context (*Smith E.J. et al., 2012*).

1.8.4 The MSCRAMM family

Staphylococcus aureus can express up to twenty-one different surface proteins (*Roche F.M. et al., 2003*), mainly MSCRAMM, which are addressed to discrete locations in the bacterial envelope by a mechanism requiring recognition of specific signal peptides and are then covalently anchored to the cell wall (*DeDent A. et al., 2008*). Covalent attachment of surface proteins is catalyzed by sortase transpeptidase enzymes, which joins proteins bearing a highly conserved Leu-Pro-X-Thr-Gly (LPXTG, where X is any amino acid) sorting signal to the peptidoglycan scaffold. The sortase A enzyme of *S. aureus*, the prototypical member of the sortase enzyme family, cleaves the LPTXG motif between the threonin and glycine residues, forming a thioacyl- linked acyl- enzyme intermediate, and catalyzes the formation of an amide bond between the carboxyl group of the threonine and the amino group of a glycine residue in peptidoglycan cross bridges (*Marraffini L.A. et al., 2006; Maresso A.W. & Schneewind O., 2008)*. The acronym MSCRAMM was originally reported to surface proteins of *S. aureus* that mediate binding to extracellular matrix components (ECM) of the host cell such as fibrinogen, fibronectin and collagen. However, many bacterial surface proteins are not MSCRAMMs and also some MSCRAMMs have additional functions other than promoting the adhesion. For this reason it has been proposed to use the term MSCRAMM to define a family of proteins on the basis of structural similarities and a common mechanism for ligand binding, which is mediated by two adjacent subdomains containing IgG-like folds in the N-terminal A region (*Deivanayagam C.C. et al.,2002*).

The A regions were shown by X-ray structural and biophysical analysis to be composed of three separately folded subdomains: N1, N2 and N3. N2 and N3 comprise the IgG-like folds (Fig. 9). Other proteins in this family can be modelled with a high degree of certainty and are predicted to have A domains with a similar structure and to also bind ligands using the **DLL** (**D**ock- **L**ock- **L**atch) mechanism.

The major MSCRAMM proteins and their functions are listed in table 2.

MSCRAMMs	Ligand binding and mechanism	Function
Clumping factor A (ClfA)	– Fibrinogen γ- chain carboxyl terminus: DLL – Complement factor I	– Adhesion to immobilized 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 colonization
Serine-aspartate repeat protein C (SdrC)	– B- neurexin; DLL – Desquamated epithelial cells	– Unknown
Serine-aspartate repeat protein D (SdrD)	– Desquamated epithelial cells	– Nasal colonization ?
Serine-aspartate repeat protein E (SdrE)	– Complement factor H	– Immune evasion; degradation of C3b
Bone sialoprotein-binding protein (isoform of SdrE)	– Fibrinogen α- cahin; 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	– Collagen triple helix; collagen hug – Complement protein C1q	– Adhesion to collagen-rich tissue – Prevention of classical pathway of complement activation

Table2. The main *Staphylococcal* MSCRAMM proteins, their ligand, binding mechanism and function (*Foster T.J. et al., 2014*).

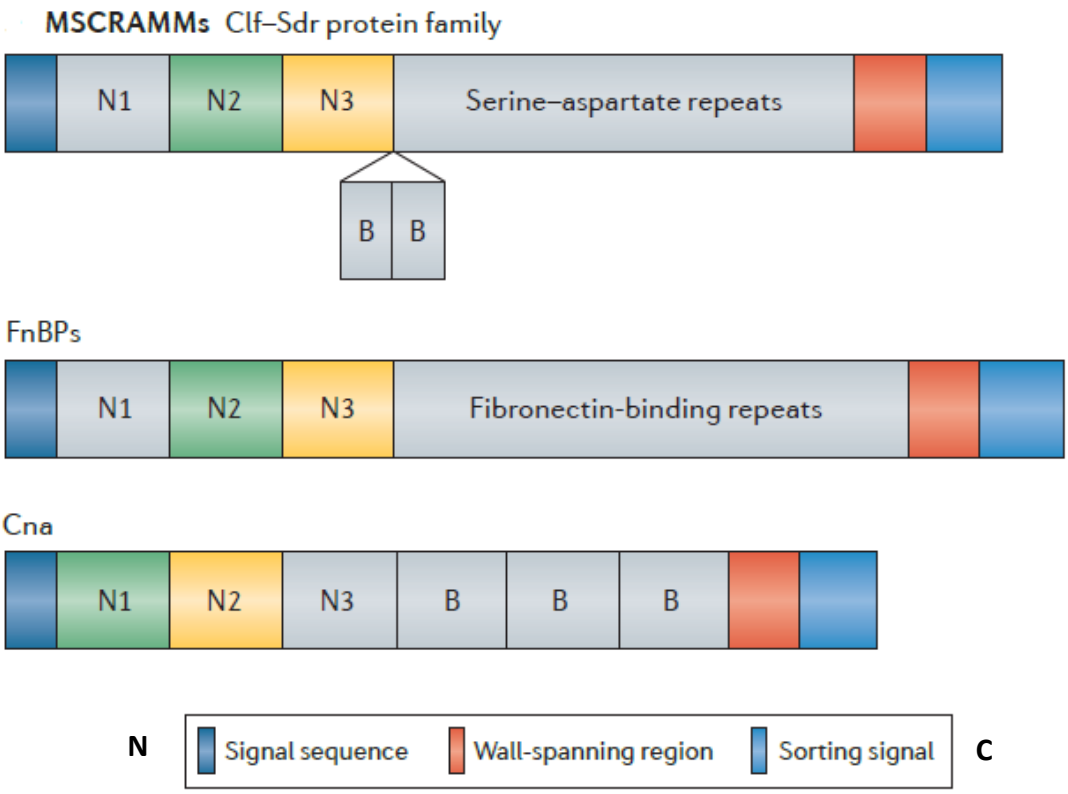


Figure 9. Schematic structure of MSCRAMMs. The N-terminal A region contains three separately folded domains known as N1, N2 and N3 subdomains. The clumping factor (Clf)-serine-aspartate repeat (Sdr) group comprises proteins that are closely related to ClfA. ClfA and ClfB have similar domain organization, whereas SdrC, SdrD and SdrE of *S. aureus* and SdrF of *S.epidermidis* contains additional BSDR repeats that are located between the A domain e the serine-aspartate repeat R region. Fibronectin binding protein A and B (FnBPA and FnBPB) have A domains that are structurally and functionally similar to the A domain of Clf-Sdr group. Located in place of the serine-aspartate repeat region there are tandemly repeated fibronectin-binding domains (11 in FnBPA, 10 in FnBPB). The A region of collagen adhesin (Cna) protein is organized differently to other MSCRAMMS, with N1 and N2 comprising IgGlike folds that binds ligands using the "collagen hug" mechanism. The A region is linked to the wall spanning and anchorage domains by variable numbers of B_{CNA} repeats (*Foster T.J. et al., 2014*).

1.8.4.1 Fibronectin binding protein family

The fibronectin-binding proteins (FnBPs) A and B of *S. aureus* are multifunctional MSCRAMMs which recognize fibronectin (Fn), fibrinogen (FBG) and elastin (El) (Jonsson K, *et al*. 1991, Roche FM *et al*. 2004, Signas C. *et al*. 1989, Wann E.R. *et al*.2000). FnBPA and FnBPB have considerable organizational and sequence similarity and are composed of a number of distinct domains as show in figure 10. Both proteins contain a secretory signal sequence at the N-terminus and a C-terminal LPETG motif required for

sortase-mediated anchoring to cell wall peptidoglycan. The N-terminal A domains of FnBPA and FnBPB are exposed on the cell surface and promote binding to FBG and El (Wann E.R. *et al*.2000, Keane FM *et al*. 2007). On the basis of their sequence similarity to the FBG-binding A domain of clumping factor A (ClfA) (McDevitt D.*et al*. 1995), both FnBP A domains are predicted to fold into three subdomains: N1, N2 and N3. (Deivanayagam CC *et al*. 2002).Seven isotypes of FnBPA and FnBPB have been identified on the basis of sequence variation in the N2 and N3 subdomains. Each recombinant isotype retains ligand-binding function, but is antigenically distinct (Loughman A.*et al*. 2008, *Buke F.M. et al., 2011)*.

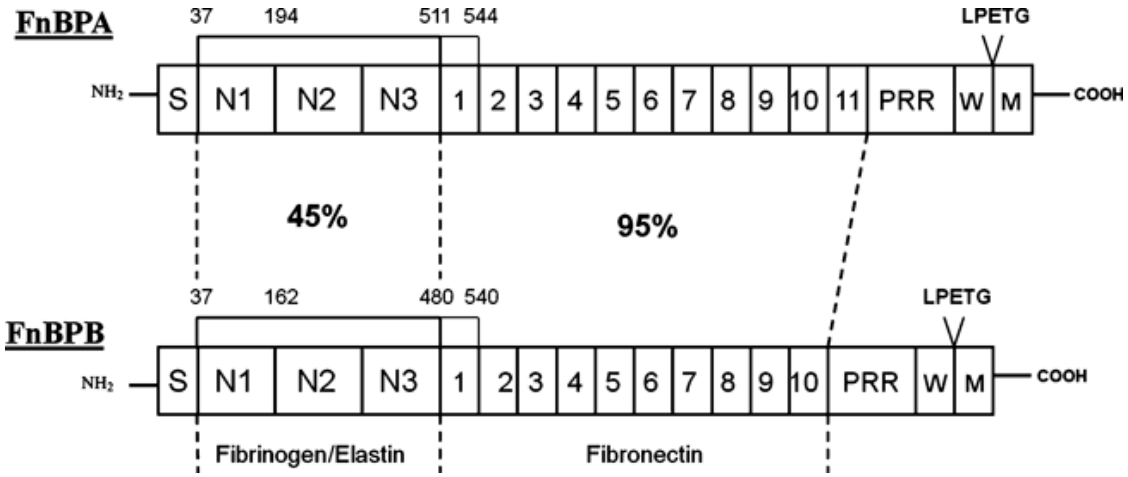


Figure10. Structural organization of fibronectin-binding proteins FnBPA and FnBPB from *Staphylococcus aureus*. The N-terminus of FnBPA and FnBPB contain a signal sequence (S) followed by a fibrinogen and elastin binding A domain consisting of subdomains N1, N2 and N3. Following the A domains there are tandemly repeated fibronectin-binding motifs. The A domains as they were originally defined contain a single fibronectin-binding motif. The true A domains of FnBPA and FnBPB are now considered to include residues 37- 511 and residues 37-480, respectively. At the C-termini are proline-rich repeats (PRR), wall (W) and membrane (M)-spanning domains, and the sortase recognition motif LPETG. The percentage amino acid identities between the binding domains of FnBPA and FnBPB from *S. aureus* are shown (*Burke F.M. et al., 2010*).

Mutants defective in either of the two genes (*fnbA*/*fnbB*) adhered equally well to fibronectin-coated surfaces in vitro, while a double mutant was completely unable to adhere, indicating that both genes are expressed and contribute to fibronectin binding (*Greene C.et al., 1995*).

Located distal to the A domains of FnBPs are multiple tandemly arranged Fn-binding repeats (FnBRs) which mediate binding to the N-terminal type I modules of Fn by a tandem b-zipper mechanism. Presumably, FnBPs are thereby capable of associating with

several Fn molecules at once (*Schwarz-Linek U. et al., 2003*). The major binding site for *S. aureus* has been localized to the N-terminal fragment of fibronectin, whereas the RDG motif, which is important for the binding of integrins such as integrin $\alpha_5\beta_1$, is found on a central part of molecule. Furthermore, the FnBP binding site is located at some distance from the central integrin binding RGD motif in Fn. Therefore, Fn can form a bridge between FnBPs and integrins (*Sinha B. et al.,1999 ; Fowler T.* et *al., 2000*). Apparently through these features, FnBPs are capable of inducing integrin clustering and signaling in cells. This leads to invasion of staphylococci via src tyrosine kinase activation and reorganization of the actin cytoskeleton (*Agerer* F. *et al., 2003; Fowler T. et al., 2003*).

1.8.4.2 MSCRAMM ligand-binding mechanism: dock, lock and latch

(Foster T.J. et al., 2014)

The ligand binding mechanism of MSCRAMMs has been investigated by X-ray structural analysis of the proteins with and without bound ligands. Analysis of the crystal structures of the minimum ligand-binding subdomains (N2–N3) of ClfA and ClfB from *S. aureus*, and SdrG from *S. epidermidis*, both in the apo form and in complex with ligand peptides (*Deivanayagam C.C. et al., 2002; Ponnuraj K.et al., 2003; Ganesh V.K. et al., 2011*), enabled the definition of a ligand-binding mechanism that is known as 'Dock, Lock and Latch' (DLL) (*Ponnuraj K. et al., 2003*). Ligands can dock to the open apo form and conformational changes create a closed form, in which the ligands are locked into place. The DLL mechanism was originally described for SdrG of *S. epidermidis*. The N2 and N3 subdomains of this protein (and of ClfA and ClfB) are folded into a variant of the IgG fold (known as DEv-IgG), which is predominantly composed of two β-sheets that contain, in total, nine β-strands (Fig. 11). The two subdomains are arranged in a specific orientation, which creates a trench between N2 and N3, in which ligands dock. The disordered ligand peptide aligns via β-strand complementation with the G' strand in the N3 subdomain. Ligand docking results in a redirection of the disordered extension of the N3 subdomain so that residues in this extension can interact with the ligand and lock it in place. Further propagation of the conformational change enables C-terminal residues of the N3 extension to be inserted into a trench in the N2 subdomain via β-strand complementation by forming an additional β-strand (known as the G"β-strand) that aligns with the E β-strand in the N2 subdomain. This 'latch' further stabilizes the DEv-IgG–ligand complex. This event does

not involve direct interaction with the ligand. Ligand–peptide interactions with the G′ β-strand are mainly backbone–backbone interactions that involve hydrogen bonds. The nature of the side-chain interactions in the binding trench depends on the ligand sequence and the binding residues in the MSCRAMM; for example, binding of SdrG to fibrinogen involves a stretch of hydrophobic residues in the ligand peptide that is complemented by hydrophobic pockets in the 'locked' binding trench. The precise nature of these interactions determines binding specificity.

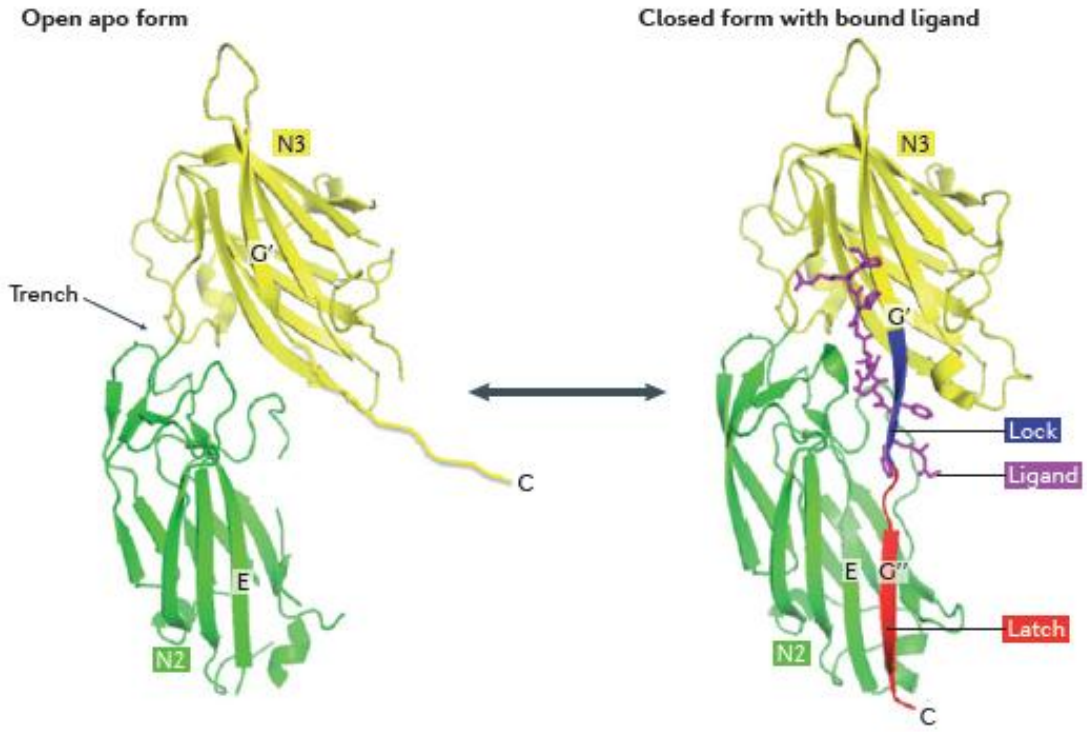


Figure 11. Mechanisms of ligand binding by MSCRAMM proteins: DLL mechanism. The A region of the microbial surface component recognizing adhesive matrix molecule (MSCRAMM) protein in its open apo form has a wide trench between the N2 and N3 subdomains (apo serine–aspartate repeat-containing protein (SdrG). The ligand peptide (purple) inserts into this trench and the MSCRAMM protein undergoes conformational changes to a closed form and locks the ligand in place (SdrG–ligand complex; PDB reference: 1R17). In the apo form, the disordered carboxy-terminal extension of the N3 subdomain is not part of the crystal structure. After ligand binding, this region forms the lock (blue) and the latch (red), thus locking the ligand in place (*Foster T.J. et al., 2014*).

ClfA and ClfB bind to their peptide ligands using subtle variations of the DLL mechanism,(Deivanayagam, C. C. *et al* 2002, Ganesh V.K *et al*.2011) in which, unlike the

mechanism of SdrG, ligand binding involves parallel (rather than antiparallel) β-sheet complementation. As a result, the orientation of the ligand in the trench is inverted.

The SdrG and ClfB ligand-binding sequences are flanked by additional residues. As a consequence, only the open apo form of the proteins can accept the ligand. By contrast, both the closed and open forms of ClfA bind to its ligand. ClfA recognizes the extreme C terminus of the γ-chain of fibrinogen, which contains residues that can penetrate the hole that is formed by the lock region of the closed form.

Several MSCRAMMs can bind to two or more ligands; for example, in addition to binding to fibrinogen, ClfA also binds to complement factor I (*Hair P.S. et al., 2008*). However it is not clear whether binding to factor I involves DLL.

FnBPA binds to fibrinogen and elastin by DLL, but structural models of the two ligands in complex are not available, therefore a consensus binding motif has not been defined (*Keane F.M. et al., 2007*).

1.8.5 CWA as virulence factors

CWA are involved in a number of different diseases (Table 3). The functional redundancy of CWA proteins made difficult to experimentally show that a surface protein can act as a virulence factor and to detect a reduction in virulence in a mutant that is defective for a single factor. Most strains of *S. aureus* have two Fibronectin Binding Proteins (FnBPs) and up to five proteins that can bind to fibrinogen. To overcome the problem of redundancy and to better define the role as virulence factor, a single CWA protein can be expressed alone in a surrogate host, such as *Lactococcus lactis* (*Mulcahy M.E. et al., 2012; O'Brien L.M. et al., 2002; Que Y.A.et al., 2005*) or *Staphylococcus carnosus* (*Sinha B. et al., 2000*).

The first CWA proteins that were experimentally shown to be virulence factors were protein A (in mouse sepsis and skin abscess) (*Patel A.H. et al., 1987*), FnBPA (in endocarditis) (*Kuypers J.M. & Proctor R.A., 1989*) and Cna (in mouse septic arthritis) (*Patti J.M. et al., 1994*).

Role in colonization or infection	CWA proteins	Mechanism
Nasal or skin colonization	ClfB	Adhesion to loricrin or squames
	IsdA	Adhesion to squames
	SasX	Adhesion to squames
Endocarditis	ClfA	Adhesion to squames
	FnBPA	Adhesion to thrombus: invasion of adjacent endothelium
	ClfB	Adhesion to thrombus
	Srap	Adhesion to platelets: colonization of thrombus
Mastitis	FnBPs	Invasion of epithelial cells in mammary gland
Pneumonia	Protein A	Increased inflammation of lung epithelium
Foreign body infection	FnBPs	Promotion of MRSA biofilm: adhesion to intra- aortic patch
Ocular keratitis	Cna	Enhanced colonization and infection
Septic death: survival in blood	ClfA, Protein A, IsdH, AdsA, SasX	Reduced opsonophgocytosis
Kidney abscess: following survival in blood	ClfA, IsdA, IsdB, SdrD, ClfB, IsdC (Spa and SasG: modest effects)	Increased survival in the bloodstream prior to kidneys infection
Septic arthritis	ClfA, Protein A	Enhanced survival in bloodstream prior to invasion of joint
	Cna	Enhanced survival in bloodstream: adhesion to cartilage within joint

Table 3. Cell wall-anchored proteins as virulence factors (*Foster T.J. et al., 2014*).

2 *S. aureus* and the complement system

2.1 The complement system

The complement system consists of a tightly regulated network of more than 30 proteins that play an important role in host defense and inflammation. Activation of complement, by antibodies (classical pathway, CP) by mannan (lectin pathway, LP) or by foreign surfaces (alternative pathway, AP), results in opsonization of pathogens and their removal by phagocytes, as well as cell lysis (Fig. 12). The classical pathway plays a role in both innate and adaptive immunity. The first component of this pathway, C1q, links the adaptive humoral immune response to the complement system by binding to antibodies complexed with antigens. C1q can, however, also bind directly to the surface of certain pathogens and thus trigger complement activation in the absence of antibody. C1q is part of the C1 complex, which comprises a single C1q molecule bound to two molecules each of the zymogens C1r and C1s. C1q is a calcium-dependent sugar-binding protein, a lectin. The MB-lectin pathway uses a protein very similar to C1q to trigger the complement cascade. This protein, called the mannan-binding lectin (MBL), is a collectin, like C1q. Mannan-binding lectin binds specifically to mannose residues, and to certain other sugars, which are accessible and arranged in a pattern that allows binding on many pathogens. On vertebrate cells, however, these are covered by other sugar groups, especially sialic acid. Thus, mannan-binding lectin is able to initiate complement activation by binding to pathogen surfaces. The third pathway of complement activation is called the alternative pathway because it was discovered as a second, or 'alternative,' pathway for complement activation after the classical pathway had been defined. This pathway can proceed on many microbial surfaces in the absence of specific antibody, and it leads to the generation of a distinct C3 convertase designated C3b,Bb. In contrast to the classical and MB-lectin pathways of complement activation, the alternative pathway does not depend on a pathogen-binding protein for its initiation; instead it is initiated through the spontaneous hydrolysis of C3. The formation of C3 convertases is the point at which the three pathways of complement activation converge, because both the classical pathway and MB-lectin pathway convertases C4b,2b, and the alternative pathwayconvertaseC3b,Bb have the same activity, and they initiate the same subsequent events. They both cleave C3 to C3b and C3a *(Janeway et al., Immunobiology., 2001)*. C3a is anaphylatoxin and C3b is opsonin, C3a

attracts and activates granulocytes, whereas C3b attaches covalently to the bacterial surface, amplifies complement activation, and thereby labels cells for phagocytosis (*Walport M.J., 2001; Zipfel P.F. & Skerka C. 2009*). Moreover, C3b also binds the C3 convertase to form a C5 convertase that produces the most important small peptide mediator of inflammation, C5a, as well as a large active fragment, C5b, that initiates the 'late' events of complement activation. These comprise a sequence of polymerization reactions in which the terminal complement components interact to form a membrane-attack complex, which creates a pore in the cell membranes of some pathogens that can lead to their death. (*Dunkelberger J.R. et al., 2010*; *Janeway et al., Immunobiology, 2001*). The complement system is one of the innate immune responses that recognizes and targets *S. aureus* as soon as the bacterial infection occurs.

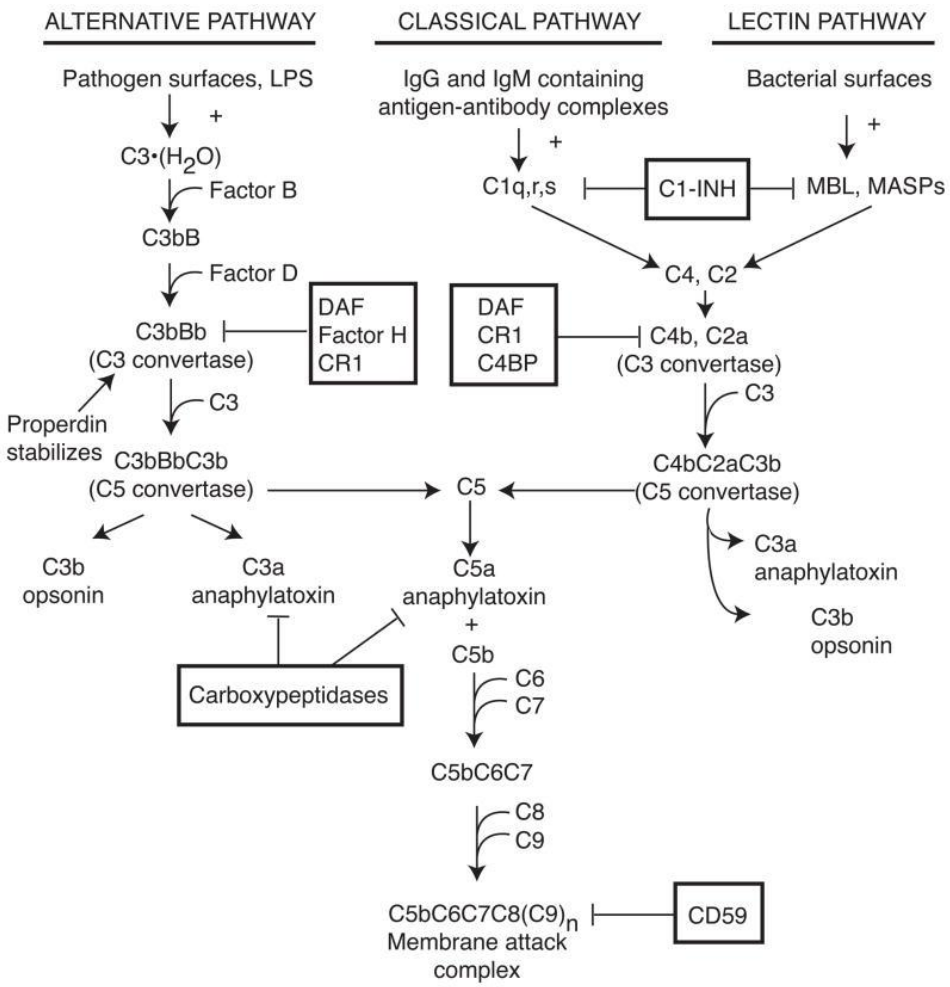


Figure 12. The three different pathways of complement activation: alternative, classical and lectin pathways; factors that can inhibit the pathways are indicated in boxes. *(Sarma J.V. & Ward P.A., 2011)*

2.2 Interactions *S. aureus*/complement system

Staphylococcus aureus expresses two proteins on its surface: Staphylococcal protein A (SPA) and staphylococcal immunoglobulin-binding protein (Sbi) that can bind to the Fc portion of IgG and thus prevent complement activation and Fc receptor-mediated phagocytosis (*Zhang L.et al., 1998*). Moreover Sbi can block binding of C1q preventing its activation and can bind C3 complement component. Similarly, group C and G streptococcal bacteria express protein G that can bind the Fc portion of IgG. Furthermore, *Staphylococcus aureus* expresses receptors that bind plasminogen and Staphylokinase secreted by *Staphylococcus aureus* cleaves bound plasminogen to plasmin. Bacterial surface bound plasmin can degrade IgG and the opsonin C3b, and thus evade the complement system. Further, *Staphylococcus aureus* secreted staphylococcal complement inhibitors (SCINs) inhibit complement activation. These SCINs bind to C3 convertases and prevent subsequent steps involved in complement activation and thus prevent opsonization, phagocytosis and cell lysis *(Rooijakkers S.H. et al., 2005, Rooijakkers S.H. etal., 2007)*. Moreover *S. aureus* can prevent the recruitment of phagocytes inhibiting the interaction between C5a and C5aR (C5a Receptor). In fact Chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS), an exoprotein produced by several strains of *S. aureus* is a potent inhibitor of neutrophil and monocyte chemotaxis toward C5a. CHIPS acts on its target cells by binding and activating the C5aR preventing C5a mediated signalling (*Postma B. et al., 2004)*.

Another *Staphylococcus aureus* product SSL-7 can bind C5 and inhibit its cleavage preventingthe formation of the lytic Membrane Attack Complex (MAC) (*Laursen N.S. et al., 2010*).

3 Plasminogen

Plasminogen (PLG) is a secreted blood zymogen that is activated by proteolysis and converted to plasmin, the primary fibrinoilytic protease (*Law R.H. et al., 2012*). Plasminogen circulates in blood at approximately 200 mg/L *(Gebbink M.F., 2011)*.While human PLG is synthesized as a 810-amino acid polypeptide protein, the mature form of this protein is 791-amino acids, due to cleavage of a 19-amino acid leader peptide during secretion. Plasminogen (PLG) is a modular protein that comprises sequentially from the amino-terminus to the carboxyl-terminus, pre-activation peptide (PAP), followed by five kringle domains (K1-K5) , which are triple-disulfide linked peptides of approximately 80 aminoacids, and a catalytic C-terminal serine proteinase domain (SP) (Fig. 13).

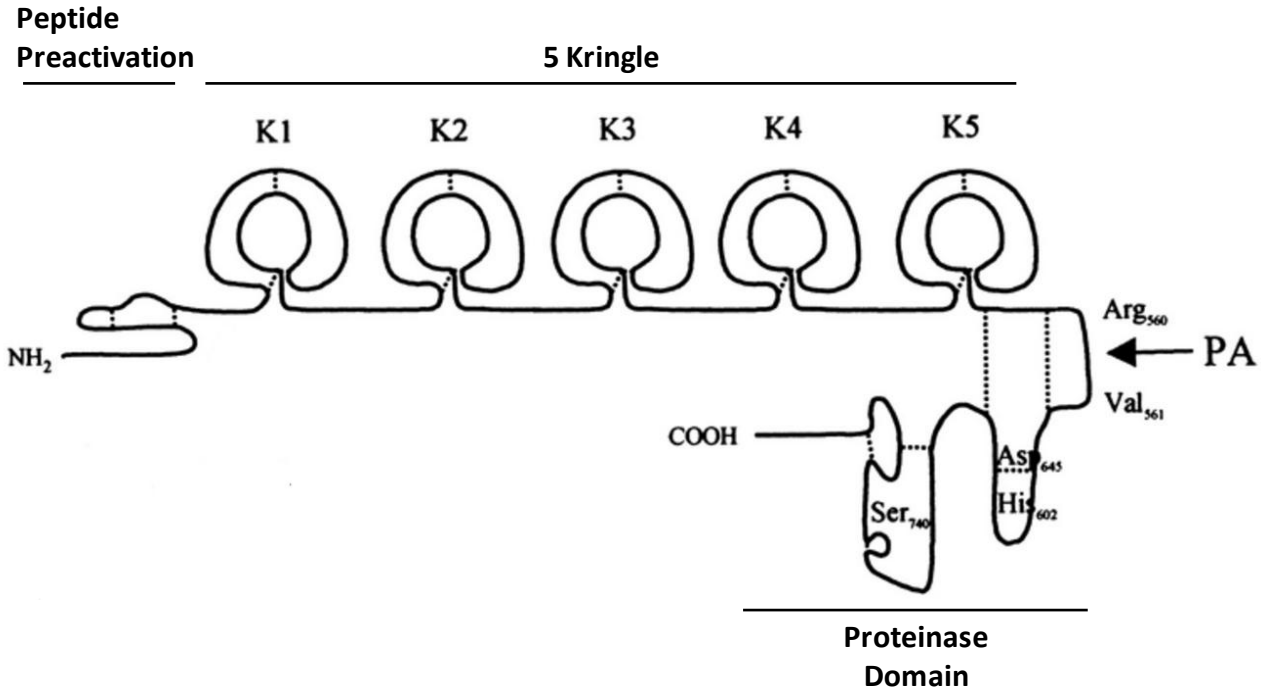


Figure 13. Domain structure of the human plasminogen molecule. *(Lähteenmäki K. et al., 2001)*.

The kringle domains of PLG contain lysine-binding sites that mediate mainly its localization to fibrin and cellular surfaces. The kringle domains facilitate PLG binding to large substrates, e.g., fibrinogen, bacterial proteins, and mammalian cell surfaces, as well as small molecule ligands, Cl^- and α, ω-amino acids, interactions that also regulate the activation of this protein. Interactions of human PLG or its isolated kringles with lysine-type ligands takes place with all kringles, except for K3. The K1 and K4 modules exhibit the strongest affinities for this class of ligands, and K2 possesses the weakest affinity. The nature of the ligand is critical to its binding strength.. On the other hand, a strong

specificity for K2 exists in the binding of PLG to aendo-polypeptide (VEK-30) that is derived from a bacterial cell wall PLG binding M-protein (PAM) *(Castellino F.J. et al., 2005).*

The circulating form of PLG, amino-terminal glutamic acid Glu-PLG (commonly indicated as PLG), is readily converted by limited proteolysis to several modified forms, known collectively as amino-terminal lysine Lys-PLG. Hydrolysis of the Lys77-Lys78 peptide bond gives rise to a conformationally modified form of the zymogen that more readily binds fibrin, displays two- to threefold higher avidity for cellular receptors, and is activated 10–20 times more rapidly than Glu-PLG. Lys-PLG does not normally circulate in plasma but it has been identified on cell surfaces *(Cesarman-Maus G. & Hajjar C.A., 2005)*.

Circulating plasminogen, adopts a closed, activation resistant conformation. The kringle domains mediate interactions with fibrin clots and cell-surface receptors. These interactions trigger plasminogen to adopt an open form that can be cleaved and converted to plasmin by tissue-type and urokinase-type plasminogen activators. The structure of closed plasminogen reveals that the PAP and Serine protease domains, together with chloride ions, maintain the closed conformation through interactions with the kringle array. Differences in glycosylation alter the position of K3, although in all structures the loop cleaved by plasminogen activators is inaccessible. In particular the ions chloride plays an important role in stabilizing closed plasminogen. A single chloride ion bound at both the K4/PAP and the SP/K2 interfaces. A third and fourth Cl^- are coordinated by K2 and the SP domain, respectively, but these do not mediate interdomain interactions. In contrast to the other kringle domains, K3 contains a mutation in its LBS (Lysine Binding Sites) (DXD/E motif to DXK) and does not bind lysine in vitro *(Law R.H.P et al., 2012)*.

The conversion of plasminogen into plasmin occurs by a variety of enzymes including tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), kallicrein, factor XII, each of which hydrolyzes the Arg561-Val562 peptide bond of plasminogen and yield two chains (N-terminal A-chain and C-terminal proteotically active, B-chain) that remain covalently associated by a disulfide bond. (Fig. 14) Elastase truncates the A-chain of plasmin(ogen) and yields two fragments: angiostatin 9, formed by kringles 1–4, and mini-plasmin(ogen), composed of kringle 5 and the C-terminal serine proteinase domain. *(Parry M.A.A et al., 2000).* Microplasmin (mPl) is truncated forms of Plasmin, consisting of a short 31-residue A-chain connected by two disulphide bridges to the catalytically active 230- residue B-chain *(Wu H.L et al., 1987)*.

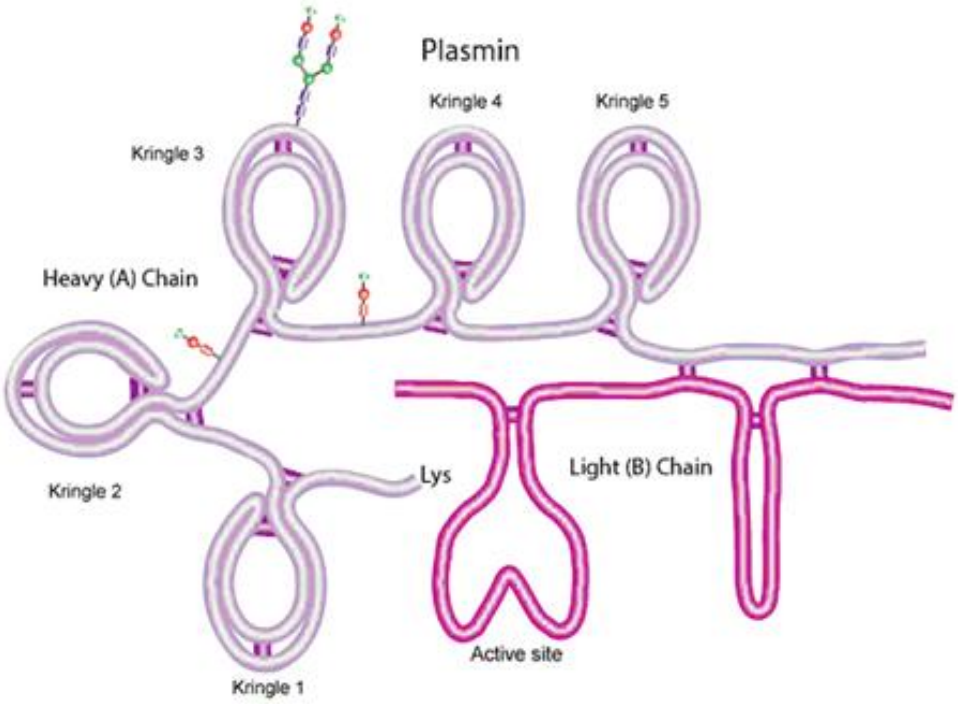


Figure 14. Plasmin structure. It consists of two disulfide-linked polypeptide chains The plasmin heavy chain (MW 60 kDa) is derived from the amino terminal region of plasminogen. The light chain originates from the carboxyl-terminus of plasminogen. In vivo, the MW of the heavy chain can vary from 63KDa to 12KDa depending on the extent of proteolysis to the plasminogen from which it is derived. *(http://www.sigmaaldrich.com/content/dam/sigmaaldrich/lifescience/biochemicals/migrationbiochemicals/Plasmin_Image.gif)*.

Plasmin is the product of the activation of plasminogen, a key enzyme of the fibrinolytic system, the physiological mechanism responsible for dissolving fibrin clots, plasmin exerts its proteolytic function on not only fibrin clots, but also a variety of substrates, including some components of the extracellular matrix. Its broad-range proteolytic activity implies that interaction with fibrinolysis and recruitment of plasmin by blood and tissue parasites is an important mechanism that mediates the invasion and establishment of pathogen in the hosts. However, recent studies have linked an excess of plasmin generated by this interaction with serious pathological events at the vascular level, including the proliferation and migration of arterial wall cells, inflammation, and degradation of the extracellular matrix.

A large number of blood and tissue parasites interact with the fibrinolytic system of their hosts by the expression and secretion of plasminogen receptors, which have been linked to parasite survival mechanisms.

The indiscriminate proteolytic activity of plasmin has been recently related to cardiovascular disease and important pathological mechanisms, such as cell proliferation and migration and inflammation in blood vessels *(González-Miguel J. et al., 2016)*.

3.1 Plasminogen activators

3.1.1 Tissue Plasminogen Activator (tPA)

Tissue Plasminogen Activator (tPA) is a serine protease of approximately 70 kD. tPA is synthesized as a protein of 562 aminoacids, which includes a signal peptide and a propeptide of 22 and 10 amino acids, respectively. Three additional aminoacids are removed leaving a mature protein that is composed of a single chain polypeptide of 527 amino acids. The protein comprises five domains, which are homologous to those found in other proteins (Fig. 15). Residues 39–81 form the finger (or fibronectin type-I module) domain found in related proteases FXII (Factor XII) and HGFa (Hepatocyte Grow Factor activator), residues 82–120 compose an epidermal growth factor-like domain (EGF-like-domain), residues 127–208 (K1) and 215–296 (K2) are kringle domains homologous to regions in plasminogen, and residues 311–561 (P) contain the protease domain.

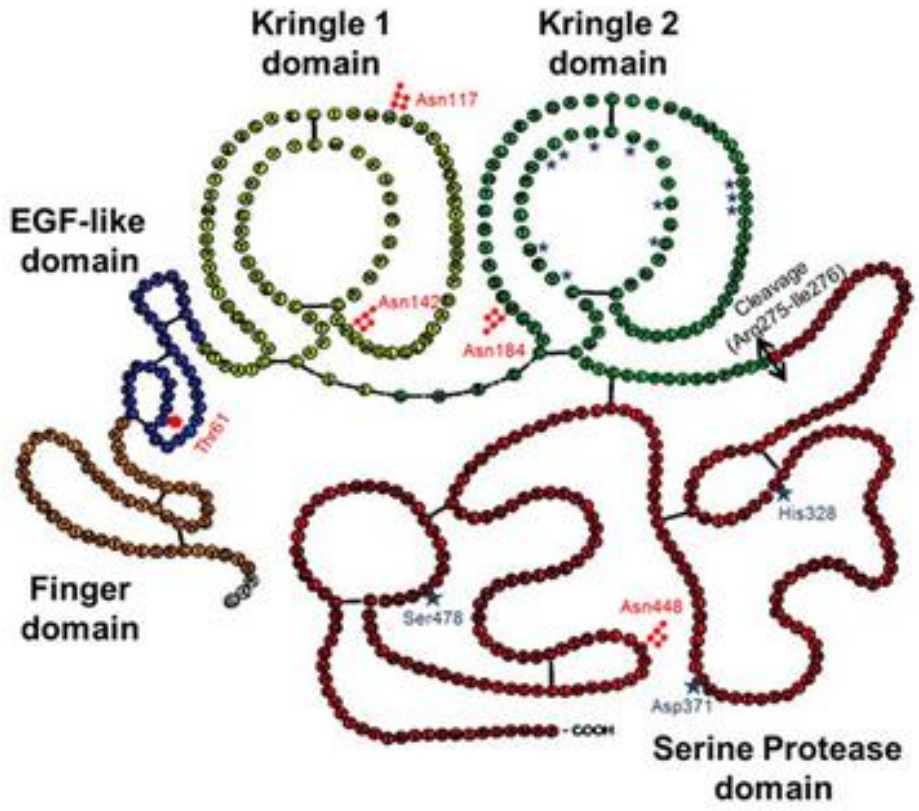


Figure 15. Schematic representation of structure of tPA. (*Chevilley A. et al., 2015*)

tPA facilitates the dissolution of blood clots by inducing the degradation of fibrin, the insoluble network that is a major component of a blood clot. It does this indirectly through the activation of plasminogen. tPA is present in only very low amounts in blood, but it is stored in small vesicles in the endothelial cells that line up the blood vessels and its release can be stimulated *(Gebbink M.F., 2011)*.The conversion of the zymogen, plasminogen to plasmin involves the proteolytic cleavage of Arg^{561}-Val^{562} by the tissue-type plasminogen activator (tPA). This results in the generation of two-chain Glu-plasmin which consists of a heavy chain of 561 residues (A chain) disulfide linked by two disulfide bonds to the light chain of 230 residues (B chain). The light chain contains the serine protease domain including the catalytic triad of His^{603}, Asp^{646}, and Ser^{741}. The proteolytic cleavage of plasminogen by the plasminogen activator creates a new amino-terminal Val^{562} residue. The free amino group of the Val^{562} residue stabilizes the structure of plasmin by interacting via a salt bridge with Asp^{740}. This interaction leads to the stabilization of the oxyanion hole and substrate binding pocket of the active site of plasmin, resulting in the formation of fully active plasmin *(Kwon M. et al., 2005)*. Plasmin in turn similarly converts single chain

tPA into a two-chain form. However, in contrast to plasminogen, single-chain tPA is active and the activity of single-chain and two-chain tPA is comparable *(Gebbink M.F., 2011)*.

3.1.2 Urokinase Plasminogen Activator (uPA)

The other human Plasminogen activator is urokinase Plasminogen Activator (uPA). uPA is a 53-kDa serine protease initially synthesized as a catalytically inactive single chain polypeptide, pro-uPA whose proteolytic cleavage - mediated by different proteases including plasmin, cathepsin B, cathepsin L, and human kallikrein type 2 - gives the mature form that is a two-chain protein linked by a single disulfide bond. uPA can be structurally divided into three domains, an amino-terminal domain, which contains the binding site for its receptor uPAR, a carboxy terminal sequence containing the catalytic site, and a kringle domain of a yet unknown function *(Gouri A. et al., 2016)*.

3.1.3 Staphylokinase

Staphylokinase (SAK) is an extra-cellular protein that is synthesized during the late exponential growth phase and positively regulated by the accessory gene regulator (agr) *(Arvidson S. et al., 2001)*. Sak is also a plasminogen activator protein that is secreted by many *Staphylococcus aureus* strains. It contains 136 amino acids and has a molecular weight of 15.5 kDa. Its 3D structure features a central α-helix lying across a five-stranded β-sheet (Fig.16) (*Nguyen L.T.& Vogel H.J., 2016*).

Several molecular forms of staphylokinase have been purified with slightly different molecular weights (Mr; 16,500 to 18,000) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] and iso-electric points. Lower MW derivatives of mature staphylokinase were obtained lacking the 6 (Sak-A6) or the 10 (Sak-AlO) NH_2-terminal amino acids.

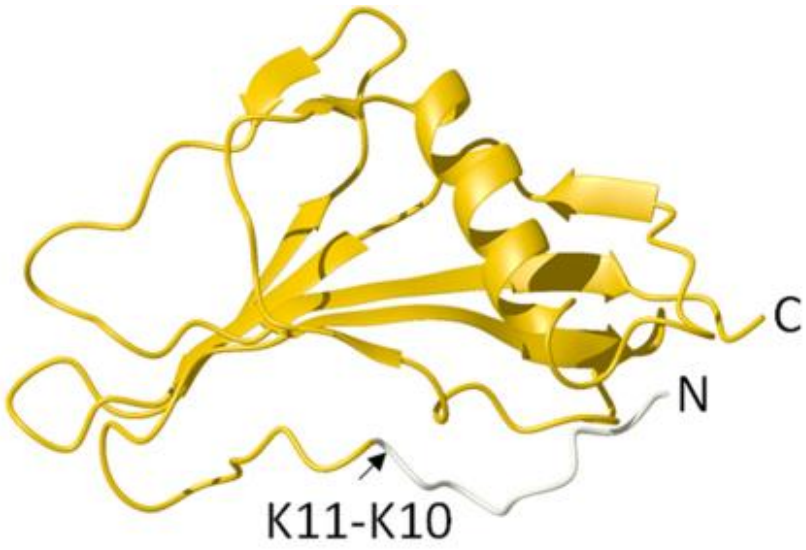


Figure 16. Structure of Staphylokinase, (*Nguyen L.T. et al., 2016*)

Like streptokinase (another activator produced by *Streptococcus*), staphylokinase is not an enzyme but it forms a 1:1 stoichiometric complex with plasminogen that activates other plasminogen molecules in plasmin (Pli). Streptokinase and plasminogen produce a complex that exposes the active site in the plasminogen molecule without proteolytic cleavage. According to this model, plasminogen and staphylokinase produce an inactive complex (PLG-Sak) in which active plasmin-Staphylokinase (Pli-Sak) is generated in a rate-limiting step that is accelerated by plasminogen activators (eg, Pli-Sak itself) and delayed by plasmin inhibitors (eg, a2-antiplasmin). In mixtures with excess plasminogen over staphylokinase, the generated Pli-Sak complex converts the excess plasminogen to plasmin. In contrast to the indirect mechanism of plasminogen activation with streptokinase and staphylokinase, the physiologic plasminogen activators, t-PA and urokinase, are enzymes that directly activate plasminogen by cleavage of a single Arg561-Va1562 peptide bond. Studies on the interaction of staphylokinase with different molecular forms of plasminogen have shown that the lysine-binding sites in kringles l through 4 of plasminogen are not required for formation of the complex with staphylokinase, nor for the enzymatic activity of the complex. (*Collen D. & Lijnen H.R. 1994*).

SAK-mediated PLG conversion at the staphylococcal surface has been implicated with a role in degradation of ECM proteins surrounding the bacterium and thus is an important pathogenetic mechanism of this Gram-positive bacterium (*Rooijakkers S.H. et al., 2005*).

4 Fibrinogen

Fibrinogen (FBG), a soluble plasma protein, is synthesized by hepatocytes and is composed of three pairs of non-identical chains, Aα, Bβ and γ, reciprocally linked by a number of disulfide bonds to form an elongated trinodular rod (Fig.17). Interchain disulfide bonds involving the N-terminal regions of the three polypeptides cooperate to form the central nodular E domain, while the C- terminal of Bβ- and γ- chain represent the D domain. D and E domains are connected by the coiled-coil regions of the three chains, while the Aα C- terminal appears as a flexible appendix of the oblong molecule. FBG is converted by thrombin into fibrin through the hydrolysis of four Arg-Gly peptide bonds in the central globular region. The resulting product spontaneously forms a long, insoluble homopolymeric structure, the fibrin clot. FBG is the most abundant ligand for the integrin GPIIb/IIIa on the surface of platelets. The binding of FBG to this integrin on activated platelets results in platelet aggregation and the formation of platelet- fibrin thrombi (*Kreis T.& Vale R., 1999*)

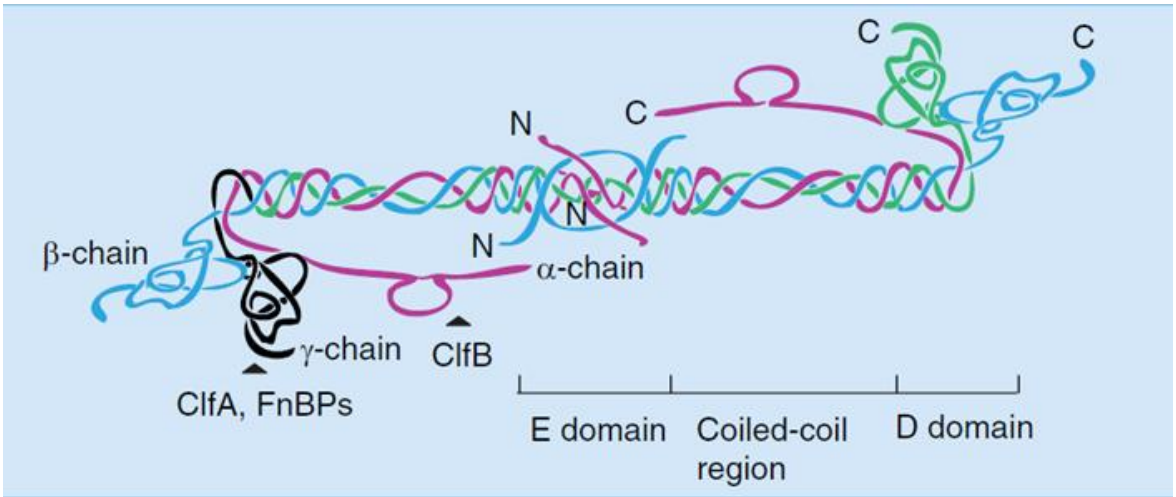


Figure 17. Schematic model of the structural organization of fibrinogen. Pairs of Aα-, Bβ- and γ-chains are linked by interchain disulphide bonds. The E domain, corresponding to the N-termini of the three pairs of chains, is connected to the C-terminal D domains by the coiled-coil regions. The C-termini of the Aα-chains extend away from the D domain region (*Speziale et al., 2009*).

Fibrinogen not only plays a pivotal role in hemostasis but also serves key roles in antimicrobial host defence. As a rapidly assembled provisional matrix protein, fibrin(ogen) can function as an early line of host protection by limiting bacterial growth, suppressing dissemination of microbes to distant sites, and mediating host bacterial killing. Fibrinogen-mediated host antimicrobial activity occurs predominantly through two general

mechanisms, namely, fibrin matrices functioning as a protective barrier and fibrin(ogen) directly or indirectly driving host protective immune function. The potential of fibrin to limit bacterial infection and disease has been countered by numerous bacterial species evolving and maintaining virulence factors that engage hemostatic system components within vertebrate hosts (*Ko Y.P. & FlicK M.J., 2016*).

AIM OF THE WORK

Staphylococcus aureus is an important opportunistic human pathogen that persistently colonizes about 20% of the human population and can cause a variety of diseases including skin infections, pneumonia, endocarditis, and sepsis. Nowadays, the emergence of methicillin (MRSA) as well as vancomycin-resistant (VRSA) strains is of great concern. In spite of the advance in antibiotic development, treating these infections remains a huge challenge reviving research for a vaccine to prevent infections. Potentially effective targets for vaccine design in Gram-positive pathogenic bacteria, including *S*. *aureus*, are the surface proteins that play important roles during infection. Among the multitude of interactions with host proteins, *S*. *aureus* binds the human plasma protein plasminogen (PLG) and expresses a PLG activator, staphylokinase, that converts PLG to the serine protease plasmin. Plasmin controls several processes such as fibrinolysis, wound healing and tissue remodelling. Moreover plasmin can cleave native C3 leading to the formation of the C3b, which is subsequently degraded and inactivated to iC3b. Thus, binding of PLG to the surface of *S*. *aureus* and its activation to plasmin is an important mechanism for the staphylococcal pathogenesis. It turns bacteria into proteolytic organisms capable of degrading extracellular matrix and other crucial components involved in the host defense. Several staphylococcal proteins have been reported to interact with PLG, however the molecular aspects of these interactions have not been investigated in detail. In this contest I wanted to examine whether other staphylococcal surface proteins, named Cell Wall Anchored (CWA) could support the interactions with PLG. In particular I found that FnBPB staphylococcal proteins, previously characterized as binders of Fibrinogen and Fibronectin, can bind also PLG.

I wanted to show that sortase-anchored cell wall associated proteins are responsible for capturing the bulk of plasminogen. I tried to identify the FnBPB binding site to PLG and to characterize the mechanism behind this interaction. Finally, I wanted to test if the formation of the receptor complex staphylococcal - PLG leads to enzyme activation of protein (PLG), which might result in the degradation of substrates such as fibrinogen.

MATERIAL AND METHODS

1 Bacterial strains and culture conditions

Staphylococcus aureus strains USA 300 LAC, USA 300 LAC Δ*srtA* , USA 300 LAC Δ*sak*, USA 300 LAC Δ*fnbB*/*fnbA*, Newman, SH1000 and 8325-4 were grown at 37°C in TSB culture medium (Tryptic Soy Broth, BD Bacto^{TM}) with constant shaking (150 rpm) for different times depending on the required growth phase. Addition of 10 μg/ml of erythromycin was necessary only for USA 300 LAC Δ*sak*. The bacteria were kindly donated by the research groups of Prof. Timoty J.Foster and Prof. Joan Geoghegan of the Microbiology Department, Moyne Institute of Preventive Medicine, Trinity College, Dublin 2, Ireland.

Escherichia coli strain TOPP3 (Stratagene, LA Jolla, CA) used for cloning and for expression of recombinant fragment of FnBPB N2N3, was grown in Luria Broth (LB, BD, Sparks, MD) at 37°C, under steady agitation, ampicillin at 100 μg/ml was added at the medium.

Lactococcus lactis strain carrying the expression vector pNZ8037 alone or harboring the *fnbB* gene (pNZ8037::*fnbB*) were grown at 30°C in M17 culture medium (Difco, Detroit, MI, USA), plus 10% lactose, 0,5% glucose and chloramphenicol (10 μg/ml), overnight, without shaking. Cultures were diluted 1:100 in the above medium and allowed to reach exponential phase A_{600nm}/0.4. Nisin (6,4 ng/ml) was added and cultures were allowed to grow overnight at 30°C without shaking.

For my pourposes the staphylococcal cells were harvested from cultures by centrifugation (ALC Refrigerated Centrifuge PK 120 R) 10 minutes at 4000 rpm, washed in PBS (Phosphate Buffered Saline, 10 mM Na_2HPO_4, 130 mM NaCl, pH 7.4) and resuspended at the suitable density by spectrophotometer measurements at A_{600nm} (Jasco, V-630).

2 Proteins

The Plasminogen (PLG) was purified from human plasma using the method of lysine-Sepharose affinity chromatography by Sanderson-Smith *(Sanderson-Smith. M., et.al 2006)*.

The Human fibrinogen (FBG) was bought from Calbiochem, which is a Merck company's branch (Darmstad, Germany). FBG was further purified on gelatin-Sepharose column to remove contaminating fibronectin (FN).

Human FN was purified from plasma by a combination of gelatin and arginine-Sepharose affinity chromatography. To exclude the presence of trace amounts of contaminants, affinity-purified FN was spotted onto nitrocellulose membranes at different concentrations and overlaid with anti-FBG and anti PLG antibodies.

The Albumin (BSA), plasminogen tissues activators tPA and the substrate chromogen S-2251 were bought from Sigma (St Louis, MO).

Kringle 1-3 and kringle 1-4 were purchased from Sigma (St.Louis, MO, USA) and MyBioSource (San Diego, Ca, USA), respectively.

Kringle 5 or mini-PLG (residues Val_{442} Asn_{790}) was obtained by digestion of Plasminogen with porcin pancreatic elastase (Sigma) as described by Cao Y., 1996.

The recombinant E. coli strains expressing recombinant FnBPBN2N3, FnBPBN2, FnBPBN3, FnBPAN2N3, SdrDN2N3, ClfAN2N3, ClfBN2N3, IsdBN2N3, SasINEAT1, SasINEAT2, Srap and Sbi were kindly donated by Prof. Timoty J.Foster and Prof. Joan Geoghegan. The *E.coli* clone expressing recombinant SAK protein was generously provided by Dr. S.H.M. Rooijakkers, University Medical Center Utrecht. The proteins were then purified in our labs according to the paragraph Expression and Purification of Recombinant Proteins (see below).

The purity of the proteins was assessed by 7.5-12.5% SDS-PAGE and Comassie brilliant blue staining.

Human plasma, normal human plasma was prepared from freshly drawn blood obtained healthy volunteers with informed consent and permission of the ethical board of University of Pavia (permit #19092013). After centrifugation, the plasma fraction was frozen in aliquots and stored at -20°C.

3 Antibodies

All the polyclonal antibodies used were available in the Speziale's Lab collection. Brefly, polyclonal antiserum against purified PLG was raised in a rabbit by routine immunisation procedures using purified human PLG as antigen. Polyclonal antiserum against human FBG or

recombinant SAK were raised in mice using human purified FBG or recombinant SAK as antigens. The purification of rabbit or mouse antibodies from sera was performed by affinity chromatography using a column of HiTrap™ (0.7x2.5 cm) (GE Healthcare, Buckinghamshire, United Kingdom) packed with 1 mL of Sepharose, which is bound to protein G, that is able to bind with high specificity the constants portions (Fc) of antibodies. The anti-human Fn rabbit polyclonal IgG was purchased from Pierce (Rockford, IL, USA). Rabbit anti-mouse or goat anti-rabbit HorseRadish Peroxidase (HRP)-conjugated secondary antibodies were purchased from Dako Cytomation (Glostrup, Denmark).

4 Plasmid and DNA manipulation

Plasmid DNA (Table 4) was isolated using the Wizard®Plus SV Miniprep Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions, and finally transformed into *E. coli* TOPP3 cells using standard procedures (*Sambrook J. et al., 1989*). Transformants were screened by restriction analysis and verified by DNA sequencing (Eurofins Genomics, Milan, Italy). Chromosomal DNA was extracted using the Bacterial Genomic DNA Purification Kit (Edge Biosystems, Gaithersberg, MD, USA).

Plasmid	Features	Marker	Source/reference
pQE30	*E. coli* vector for the expression of hexa-His-tagged recombinant proteins	Amp^R	Qiagen
pQE30::rFnBPB$_{163–480}$	pQE30 derivative encoding the N2N3 subdomain of FnBPB from *S. aureus* 8325-4	Amp^R	Burke et al., 2011
pQE30::rFnBPB$_{163–480}$ Site 1	pQE30 derivative encoding the N2N3 incorporating alanine substitutions in site 1 (residues K330, K334, K362)	Amp^R	This study
pQE30::rFnBPB$_{163–480}$ Site 2	pQE30 derivative encoding the N2N3 incorporating alanine substitutions in site 2 (residues K342, K374, K423)	Amp^R	This study

pQE30::rFnBPB$_{163–480}$ Site 3	pQE30 derivative encoding the N2N3 incorporating alanine substitutions site 1 and 2 substitutions combined	Amp^R	This study
pQE30::rFnBPB$_{163–463}$	pQE30 derivative encoding residues 163–463 of FnBPB from *S. aureus* 8325-4	Amp^R	Burke et al., 2011
pQE30::rFnBPB$_{163–480}$ N312A/F314A	pQE30 derivative encoding the N2N3 subdomain of FnBPB from *S. aureus* 8325-4 with mutations encoding the changes N312A and F314A	Amp^R	Burke et al., 2011
pQE30::rFnBPB$_{163–308}$	pQE30 derivative encoding the N2 subdomain of FnBPB from *S. aureus* 8325-4	Amp^R	Burke et al., 2011
pQE30::rFnBPB$_{309–480}$	pQE30 derivative encoding the N3 subdomain of FnBPB from *S. aureus* 8325-4	Amp^R	Burke et al., 2011

Abbreviation, Em^r erythromycin resistance; Tc^r tetracicline resistence; Cm^r chloramphenicol resistance

Table 4. Plasmids

4.1 Cloning of the lysine substitute DNA fragments

DNA encoding residues 163-480 of FnBPB incorporating alanine substitutions in site 1 (residues K330, K334, K362), site 2 (K342, K374, K423) or site 1 and 2 substitutions combined, referred to as site 3, was synthesized as g-Block® double stranded DNA fragments by Integrated DNA Technologies (Coralville, IA). To determine the lysin residues exposed on the surface of the protein and potentially involved in PLG-binding FnBPB structural prediction analysis (UniProt platform) was used. Primers rFnBPB$_{163-480}$F and rFnBPB$_{163-480}$R (Integrated DNA Technologies, Leuven, Belgium) (Table 5) were used to amplify the sequence for cloning into pQE30. Restriction digests and ligations were carried out using enzymes from New England Biolabs (Ipswich, MA, USA) according to the manufacturers' protocols. DNA purification was carried out using the Wizard® SV Gel and PCR Clean-up System (Promega). Transformants were screened by restriction analysis and verified by DNA sequencing (Eurofins Genomics, Milan, Italy).

Primer	Sequence (5'–3')[a]	5' restriction site
rFnBPB$_{163–480}$ F	CGGGGATCCGCATCGGAACAAAACAATAC	BamHI
rFnBPB$_{163–480}$ R	AAT*CCCGGG*TTACTTTAGTTTATCTTTGCC	SmaI

Table 5. FnBPB N2N3 primer sequences. The primers were created in order to comprise two restriction sequences, one for BamHI and one for SmaI.

[a] **Restriction sites used for cloning are shown in italics.**

4.2 Amplification of the lysine substitute DNA fragments

The three modified gene fragments, *site1*, *site2*, *site3* were amplified using the Phusion® High-Fidelity DNA Polymerase (BioLabs), an enzyme that is characterized by the high fidelity of the amplification. The steps of polymerase chain reaction were carried out using the amplification profile described in Table 6; the annealing temperature was inferred by the melting temperature of the primers. The PCR product was quantified using the Nanodrop.

STEPS	Temp	Time	
Initial denaturation	98°C	30"	
Denaturation	98°C	10"	35 cycles
Annealing	55°C	15"	
Elongation	72°C	30"	
Extension	72°C	10'	

Table 6. Phusion® High-Fidelity DNA Polymerase amplification profile. The annealing temperature was inferred using the following formula: Ta=Tm - 5°C

4.3 Purification of pQE30 plasmid

The pQE30 plasmid was purified from *E. coli* TOPP3 strain using the MiniPrep protocol (EuroClone) (Fig. 18) and quantified using the Nanodrop.

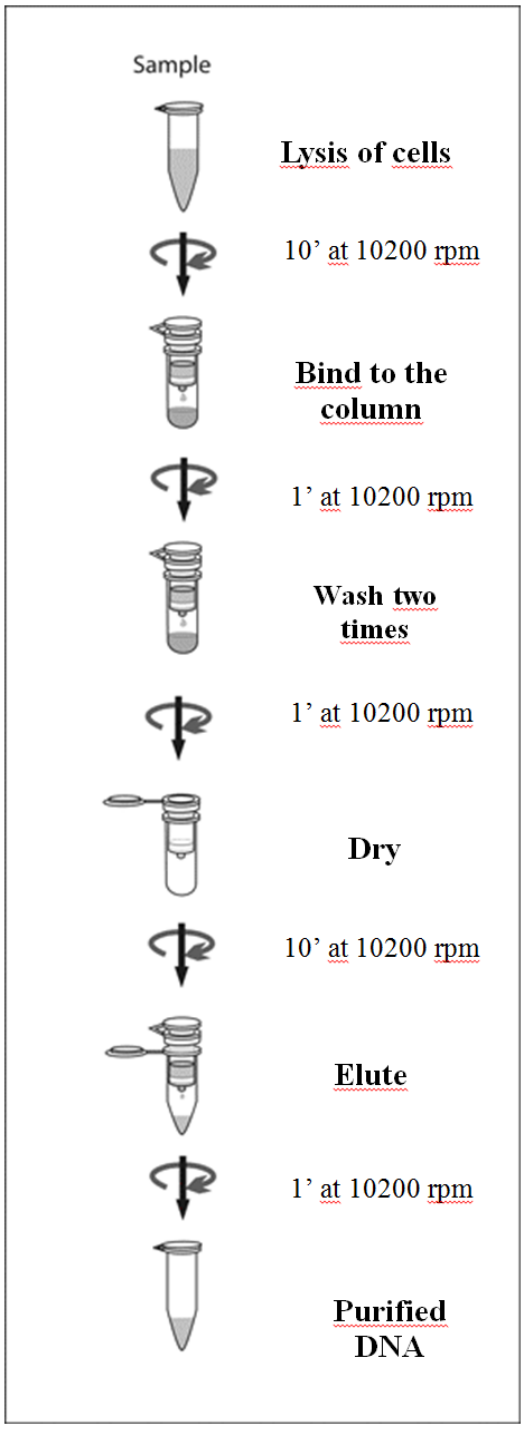


Figure 18. Plasmid extraction protocol. Cells are first lysed thanks to three solutions: i) RNAse added solution in order to get rid of non-DNA nucleic acids; ii) Solution II which lyses the bacterial cell walls; iii) solution III that counteracts the action of solution II, neutralizing the environment. The mixture is centrifuged in order to separate cells debris from the plasmid DNA that is present in the supernatant, which is then loaded on the column. The product is centrifuged and the flow-through is discarded while the DNA that binds the membrane in the column is washed twice with an ethanol-added solution. In order to dry the column getting rid of an excess of ethanol, the sample is again centrifuged at 10200 rpm for 10 minutes. The plasmid DNA is finally eluted using 35 µl of Elution buffer.

4.4 Digestion of plasmid and insert

pQE30 contains restriction sites inside its sequence and the primers for the N2N3 fragment were designed to have the restriction sequences for *Bam*HI (BioLabs) and *Sma*I (BioLabs): the former

cuts DNA forming sticky ends, the latter forming blunt ends (Fig. 19). At first, the reaction was set up with *Sma*I only: the reaction mix was kept at 30°C for 15 minutes and the activity of the resctriction enzyme was stopped by an incubation at 65°C for 20 minutes. Then BamHI was added and the reaction was continued at 37°C for 15 minutes. The digestion profiles of site1, site2, site3 and the plasmid are shown in Tables 7, 8, 9, 10.

Ingredients	Mix 1x (*µl*)
FNBPB N2N3 site1 (94 ng/µl)	10,6 (1 µg)
10x cutsmart buffer	5
SmaI (10 U/ml)	1
BamHI (10 U/ml)	1
H_2O	32,4
Final volume	50

Table 7. Digestion profile of site1. 94 ng/µl is the concentration of the DNA obtained after the PCR amplification

Ingredients	Mix 1x (*µl*)
FNBPB N2N3 site2 (71,7 ng/µl)	14 (1 µg)
10x cutsmart buffer	5
SmaI (10 U/ml)	1
BamHI (10 U/ml)	1
H_2O	29
Final volume	50

Table 8. Digestion profile of site2. 71,7 ng/µl is the concentration of the DNA obtained after the PCR amplification

Ingredients	Mix 1x (*µl*)
FNBPB N2N3 site3 (106,4 ng/µl)	9,4 (1 µg)
10x cutsmart buffer	5
SmaI (10 U/ml)	1
BamHI (10 U/ml)	1
H_2O	33,6
Final volume	50

Table 9. Digestion profile of site3. 106,4 ng/µl is the concentration of the DNA obtained after the PCR amplification

Ingredients	Mix 1x (*µl*)
pQE30 (185 ng/ µl)	6 (1 µg)
10x cutsmart buffer	5
SmaI (10 U/ml)	1
BamHI (10 U/ml)	1
H_2O	37
Final volume	50

Table 10. Digestion profile used for the plasmid. 185 ng/µl is the concentration obtained after the purification by means of the MiniPrep kit

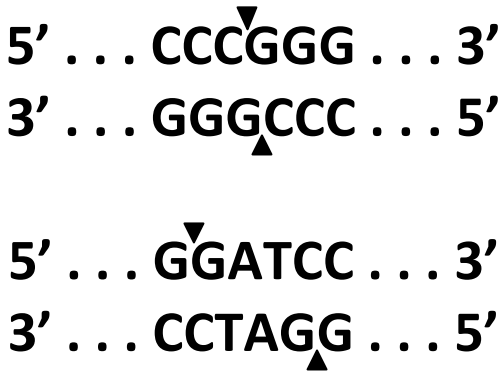


Figure 19. Restriction sequences of the enzyme used in the digestion step. Above: SmaI sequence. Below: BamHI sequence

Site1, site2, site3 digestion products were purified using the Eurogold Cycle Pure Kit (EuroClone). The plasmid digestion products instead were purified by means of a gel extraction: a 1% agarose gel without ethidium bromide was prepared and the product was loaded using a staining buffer provided by the digestion kit. Afterwards the agarose gel was stained with methilene blue for 10’ and decolorated using water until the product was visible in the gel. The band was excided from the gel and treated using a EuroGold Gel Extraction kit (EuroClone) (Fig. 20). Both the digested plasmid and fragment were quantified using the NanoDrop.

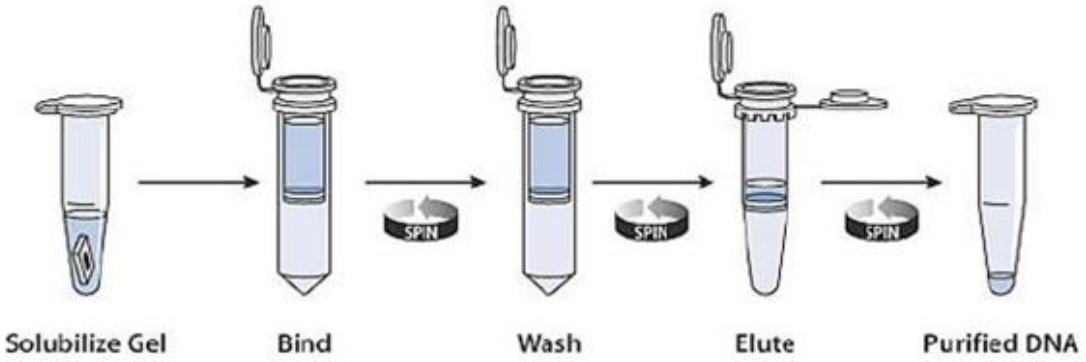


Figure 20. Extraction of DNA from agarose gel

4.5 Ligation

The restriction enzymes used allowed the formation of complementary DNA ends both on the plasmid and on the modified N2N3 fragments so that it was possible to ligate the two components together using the T4 ligase kit (Promega). In order to understand which were the best DNA quantities to use in the reaction, the following formula has been used:

$$\frac{\text{ng of pQE30 x kb size of insert}}{\text{kb size of pQE30}} \text{ x molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

The molar ratio adopted was 3:1. The insert is 1 kb in size, and the plasmid 3,4 kb. The ligation reaction was carried out as shown in Tables 11, 12, 13. The reaction mixes were left at room temperature for 15 minutes and then each of them was added to 100 µl of competent *E. coli* TOPP3 cells to perform the DNA transformation.

Ingredients	Mix 1x (*µl*)
Insert site1 (25,9 ng/µl)	1,7
Vector (28 ng/µl)	1,8
2x Rapid Ligation Buffer	5
T4 ligase	1
H_2O	0,5
Final volume	10

Table 11. Transformation reaction. 25,9 ng/µl is the concentration of the site 1 DNA obtained after fragment digestion, while 28 ng/µl is the one obtained for the plasmid after digestion and purification.

Ingredients	Mix 1x (*µl*)
Insert site3 (50 ng/ µl))	1
Vector (28 ng/µl))	1,8
2x Rapid Ligation Buffer	5
T4 ligase	1
H_2O	1,2
Final volume	10

Table 12. Transformation reaction. 50 ng/µl is the concentration of the site 2 DNA obtained after fragment digestion, while 28 ng/µl is the one obtained for the plasmid after digestion and purification.

Ingredients	Mix 1x (*µl*)
Insert site2 (26,8 ng/µl)	1,6
Vector (28 ng/µl)	1,8
2x Rapid Ligation Buffer	5
T4 ligase	1
H_2O	0,6
Final volume	10

Table 13. Transformation reaction. 26,8 ng/µl is the concentration of the site 3 DNA obtained after fragment digestion, while 28 ng/µl is the one obtained for the plasmid after digestion and purification.

4.6 Preparation of competent *E. coli* cells for heat shock transformation

E. coli from TOPP3 strain was made competent by a chemical and physical treatment which alters the structure of the cell wall allowing the cells to absorb exogenous DNA from the environment through a heat shock. Non-competent *E. coli* TOPP3 cells were grown overnight at 37°C with constant shaking. 1.5 ml of the overnight culture was diluted in 50 ml of fresh LB medium and incubated at 180 rpm at 37°C until the culture reached the exponential phase (OD_{600nm}= 0.4 – 0.5). The bacterial cells were then transferred in a 50 ml sterile falcon, left it in ice for 1 minute and centrifuged at 4000 rpm for 10 minutes at 4°C. Maintaining the sterile conditions, the supernatant was thrown whilst the pellet was kept in ice and resuspended with 10 ml of buffer for competent cells (0.3 g PIPES, 0.88 g $CaCl_2$, 15 ml Glycerol and dH_2O till 100 ml, pH 7). The suspension was kept on ice for 30 minutes and centrifuged at 4000 rpm for 10 minutes at 4°C. The centrifugation step was repeated again and the pellet was resuspended in 3 ml of buffer for competent cells. Finally 100 µl aliquots of the suspension were stored at -80°C in sterile eppendorf tubes.

4.7 Heat shock transformation of *E. coli* TOPP3 competent cells

The ligation products (10 µl each) were added to 100 µl of TOPP3 competent cells (see below) thawed in ice (the DNA solution should not exceed 10% of the total volume). The cells were incubated in ice with DNA for 30 minutes, and after that they were heat-shocked for 90 seconds at 42°C then immediately placed in ice for 2 minutes. This step is crucial as it allows the passive entry of the exogenous DNA into the bacterial cells. At this point, 900 µl of liquid LB medium were added and the culture was left to grow at 37°C for 2 hours in static conditions. Then it was centrifuge for 5 min at 4000 rpm; after that 800µl of the supernatant were discarded and the pellet was resuspended in the remaining 200µl of supernatant. At this point the culture was plated on ampicillin-supplemented LB agar plates (Fig. 21).

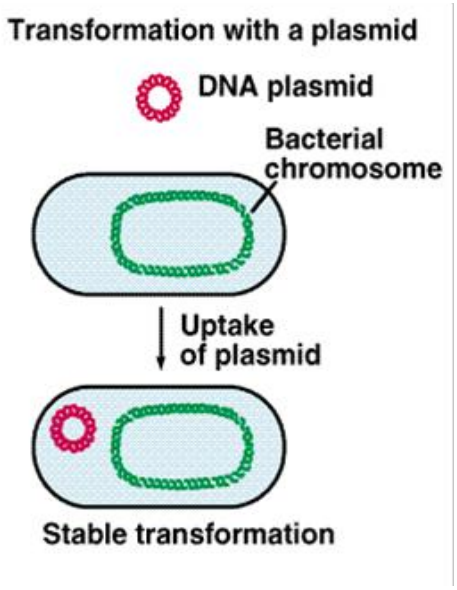


Figure 21. Transformation of bacterial cells with a plasmid. Only competent cells are able to uptake exogenous DNA. The plasmid must have a replication origin in order to make copies of itself and spread the plasmid as cells divide.

4.8 Colony PCR

Colony PCR is a technique used to identify the transformed cells that actually carry the gene of interest inside the plasmid. On the LB-agar ampicillin-added plate only the colonies that were successfully transformed can grow, as they carry the *ampR* gene on the plasmid: nevertheless, it is possible that the plasmid rejoined its ends without the insertion of the gene of interest. The colony PCR was performed on ten colonies that derived from three different plates. At first, ten PCR eppendorf tubes were filled with 10 µl of Nuclease-Free water (QIAGEN): a colony taken from the agar plate was stirred in each eppendorf tube, so that 2 microliters of it could be inoculated in 1 ml of LB medium containing ampicillin in order not to lose a potentially transformed colony. What remained in the PCR eppendorf tubes had been incubated at 98°C for 10' and subsequently put on ice, in order to break the bacterial cell walls thanks to the Heat-Shock methodology. The PCR was performed using the DreamTaq DNA Polymerase (Fermentas), as high fidelity replication was not needed. The result was detected by electrophoresis on a 1% agarose gel (see below). This technique was applied to all the three transformation product plated. The purified plasmids were further tested by sequencing.

4.9 Agarose Gel Electrophoresis

Electrophoresis on an agarose gel is a technique, which allows the separation of either circular molecules or linear fragments of DNA of different molecular weights in an electric field. In this study all agarose gels had a concentration of 1% agarose (GellyPhore, EuroClone), as this percentage is ideal to resolve fragments of 250-1000 bp and the DNA fragment of interest was 1000 base pairs long. To assess the dimensions of the fragments of DNA, in one of the gel's lanes a reference standard, selected according to the expected sizes of the DNA fragments, is loaded and the dimensions of the sample are inferred by comparison. The marker used in this study was GeneRuler 50 bp DNA ladder (Fermentas).

5 Expression and purification of recombinant proteins

Recombinant proteins were expressed from pQE30 (Qiagen,Chatsworth, CA, USA) in *E. coli* TOPP3(Stratagene). Overnight starter cultures were diluted 1:40 in LB containing ampicillin (100μg/ml) and incubated with shaking until reached the exponential phase (OD_{600nm} = 0.4) Recombinant protein expression was induced by addition of 1mM (final concentration) isopropyl IsoPropyl 1-Thio-β-D-Galactopyranoside (IPTG) (INALCO) to the culture. After 4 hours bacterial cells were harvested by centrifugation (4000 rpm for 20 minutes) and frozen at -80°C. The day after, the cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) containing Phenyl-Methane-Sulfonyl-Fluoride (PMSF) 1 mM as protease inhibitor and lysed by freezing with liquid nitrogen and subsequent defrosting with warm water. The lysing procedure was repeated twice. The cell debris was removed by centrifugation (20400 rpm, at 10°C 20 minutes) and the surnatant applied to a 1 ml His Trap HP Ni2+ chelating column (GE Healthcare) equilibrated with "binding buffer" (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.4). Fusion protein was eluted with five column volumes of "elution buffer" (500 mM imidazole in 50 mM NaH_2PO_4, 300 mM NaCl, pH 7.4). Protein purity was assessed to be 98% by SDS-PAGE, Coomassie brilliant blue staining, and densitometry analysis. A BicinChoninic acid protein Assay (BCA Pierce, Rockford, IL, USA) was used to measure concentrations of purified proteins.

6 BCA protein assay

The concentrations of recombinant proteins were determined with BCA protein assay kit (Pierce). The BCA Protein Assay combines the well-known reduction of cupric ion (Cu^{2+}) to cuprous ion (Cu^{1+}) by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid (BCA).

7 Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To evaluate the protein purity, a SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed. The protein samples, prior to being loaded on the gel, were treated by adding an equal volume of SDS-PAGE loading buffer (0.1% (w/v) bromophenol blue, 20% (v/v) glycerol, 2% (w/v) SDS in 50 mM Tris-HCl pH 6.8) containing (reducing conditions) or not (non reducing conditions) 20 mM Di-Thio-Threitol (DTT) and boiled for 5'. The gel was placed in a vertical migration electrophoresis tank (BioRad) with the running buffer (20 mM Tris-HCl pH 8.3, 0.2 M glycin, 0.1% SDS).

The protein was separeted by 10-15% (w/v) polyacrylamide, the gel was stained with Coomassie brilliant blue (BioRad, Hercules, CA, USA) staining solution (Coomassie blue 0.2% in 30% isopropanol, 20% acetic acid) and left to shake for 30-60 minutes before being destained in SDS-PAGE destaining solution (10% acetic acid 45% methanol) for 1-2 hours, so that the protein bands become visible. Alternatively the gel was transferred on a Nitrocellulose Blotting Membrane (0.45 µm NC) (GE Healthcare) for western blotting.

8 Western Blot Assays

For Western immunoblotting, proteins were subjected to SDS-PAGE, electroblotted onto a nitrocellulose membrane (0.45 µm NC) (GE Healthcare) The nitrocellulose membrane was wet with distilled water, soaked for 15 minutes in blot buffer (0.4 M Glycin, 40 mM Tris-HCl, 20% methanol, pH 8.3) and finally placed on the acrylamide gel before assembling them between two sheets of Whatman® blotting paper and sponge on either side. The transfer sandwich was then placed in a vertical blot tank immersed in transfer buffer with the gel at the negative pole and the

membrane at the positive. The membrane was blocked overnight at 4°C with 5% (w/v) skim milk (Sigma) in PBST (0,1% Tween 20 in PBS).

Blotted proteins were probed with rabbit polyclonal antibody against human PLG (1:5000) or with polyclonal mouse anti-FBG (1:2000) or mouse IgG against SAK (5μg/ml) for 1 h at 22°C. Following washes with PBST, the membrane was incubated for 1 h with either HRP-conjugated goat anti-rabbit IgG (1:10000) or rabbit anti-mouse IgG (1:10000). Finally, blots were developed using the ECL Advance Western blotting detection kit (GE Healthcare) and an ImageQuant TM LAS 4000 mini-biomolecular imager (GE Healthcare) was used to capture images of the bands. When the purified recombinant A domains of several CWA proteins of *Staphylococcus aureus* ($FnBPA_{194-511}$, $FnBPB_{163-480}$, $ClfA_{221-559}$, $ClfB_{201-542}$, Bbp_{40-599}, Pls_{49-694}) were subjected to SDS-PAGE and Far Western blot, membranes were incubated with 1μg/ml PLG in PBS for 1h at room temperature , washed and the complexes detected with anti-PLG antibody as reported above.

8.1 Capture of PLG from human plasma by *S. aureus*

To test the ability of *S. aureus* strain USA 300 LAC *wt*, USA 300 LAC *srtA*, USA 300 LAC *sak* mutant, and USA 300 LAC *fnbA*/*fnbB* double mutant, to sequester PLG that is present in the human serum in a concentration of 2.4 μM (Collen D., 1986), an assay of plasmatic PLG absorption by bacterial cells was performed. The overnight culture was diluted (1:40) in TSB medium and grown at 37°C with constant shaking (150 rpm) in order to obtain bacteria in exponential phase. When cultures reached the exponential phase (A_{600nm} about 0.4), the cells were diluited approximately at concentration of $2x10^8$ cells/mL, 500μl were harvested by spinning at 10000 rpm for 10 minutes at room temperature and the supernatant was discarded. Bacterial cells were resuspended in different human serum dilutions (10%, 5%, 2.5%, 1.25% in PBS) and incubated 1 hour at room temperature in end-over-end. The unbound PLG and the plasma were then removed washing the cells twice with PBS (spinning at 10000 rpm for 10 minutes). Proteins that were bound to the cell surface (pellets from the previus step) were dissociated by addition of extraction buffer (125 mM Tris/HCl, pH 7.0 containing 2% SDS) at 10μl/mg of pellet, and separated by SDS-PAGE (10% polyacrylamide gel) under non reducing conditions. The separated proteins were then transblotted onto a nitrocellulose membrane, incubated with a primary Rabbit anti-human PLG antibody (1:5000) followed by a secondary HRP-conjugated mouse anti Rabbit IgG (1:10000), as described above. The band intensities were quantified relative to the PLG sample (5 μg, 100% intensity) with the QuantityOne software (Bio Rad, Milan, Italy).

8.2 PLG binding to FnBPB N2N3 and its derivates

In order to screen which staphylococcal proteins were able to bind human PLG, which fragments of region A of rFnBPB, N2 or N3 (respectly $rFnBPB_{163-308}$ and $rFnBPB_{309-480}$) could interact with PLG or with FBG and if truncated variants ($rFnBPB_{163-463}$) or two aminoacids mutant (rFnBPB 163-480N312A/F314A) were still able to bind either PLG or FBG, the region A of a number of staphylococcal recombinant protein available in our lab, the single subdomains N2 and N3 of FnBPB and the truncated or mutated variants of FnBPB N2N3 subdomain, were subjected to SDS-PAGE (15%, or 12.5%, acrylamide respectively) and then transferred on a nitrocellulose membrane as described. The membrane was incubated overnight with a solution containing 5% dried milk in PBST (0.1% Tween 20 in PBS), in constant agitation, at 4°C to block the protein binding sites which were not occupied during the transfer. The membrane was incubated with human PLG (1μg/ml) or FBG (5 μg/ml) in PBST for 1 hour at room temperature and after three washes with PBST incubated for 1 hours with a primary antibody directed against PLG (polyclonal rabbit antibody) diluted 1:5000 in PBST or FBG (polyclonal mouse antibody) diluted 1:2000 in PBST. After other three washes, the secondary antibody, Goat-α- Rabbit IgG or Rabbit-α-Mouse IgG coupled to horse radish peroxidase (HRP) (DAKO) was diluted 1:10000 in PBST and added to the membrane.

8.3 Preparation of cell surface extracts and TCA-precipitated culture supernatants.

To detect SAK on the surface of *S. aureus* USA300 LAC *wt* or Δ*sak*, 1ml of bacteria ($1x10^8$/mL) grown on exponential phase was centrifuge at 4000 rpm for 10 minutes. The pellet was treated with buffer of extraction (2% SDS), heated at 95°C for 3 min and subjected to 15% SDS-PAGE and Western blotting. SAK was detected by incubation with mouse anti-SAK IgG (5 μg/mL) followed by a rabbit HRP-conjugated secondary antibody (1:10000). The supernatant was precipitated by treatment with 20% trichloroacetic acid (TCA) (v/v) at 4°C for 90 min. The pellet was collected by centrifugation at 10000 x *g* for 10 min and washed with cold acetone (v/v) for two times. The pellet was allowed to air dry overnight and dissolved in the sample buffer, boiled for 3 min and finally subjected to 15% SDS-PAGE and Western blotting. SAK was detected as reported above.

8.4 Release of cell wall anchored-proteins from *S. aureus* and detection of fibronectin-binding activity

To verify if the binding to FN of USA 300 LAC *fnbA*/*fnbB* double mutant was modified, cell wall-anchored proteins released from *S*. *aureus* were subjected to SDS-PAGE and Western blotting. For this purpose, bacteria were grown to an OD_{600} of 0.4 to 0.6, harvested by centrifugation at 7000 x *g* at 4°C for 15 min, washed 3 times with PBS, and resuspended to an OD_{600} of 40 in lysis buffer (50 mM Tris-HCl, 20 mM $MgCl_2$ [pH 7.5]) supplemented with 30% raffinose. Cell wall proteins were solubilized from *S*. *aureus* by incubation with lysostaphin (200 µg/ml) at 37°C for 20 min in the presence of protease inhibitors (Complete Mini; Roche Molecular Biochemicals, Indianapolis, IN, USA). Protoplasts were recovered by centrifugation at 6,000 x *g* for 20 min, and the supernatants were taken as the wall fraction. The material was adsorbed on IgG-Sepharose column to remove protein A and subjected to SDS-PAGE and Western blotting incubating the membrane with human FN (5 µg/ml) and a rabbit anti-FN antibody (2 µg/ml).

9 Functional activity of PLG captured by *S. aureus*

9.1 PLG capured by *S. aureus* USA 300 LAC wt or Δ sak

To check whether the PLG adsorbed on the surface of *S*. *aureus* can be activated to plasmin by SAK or tPA and therefore may degrade the FBG, an overnight culture of *S*. *aureus* USA 300 LAC wt was diluted 1:40 in Tryptic Soy Broth (TSB) and the bacterial cells were grown at 37°C, under constant shaking (150 rpm), until reaching of the exponential growth phase (A_{600nm} about 0.4). The staphylococcal cells were harvested by centrifugation (10 minutes at 4000 rpm), resuspended in PBS, washed with the same buffer and diluted at concentration of $5x10^8$ cells/ml. 100 uL of the bacterial suspension ($5x10^7$cells) were immobilized on microtiter wells and incubated overnight at 37°C without shaking. After washing three times with PBST, non-specific binding sites were blocked by incubating with 2% BSA in PBS (200µl /well) for 1 hour. 100 uL of human PLG (100µg /ml in PBS) were then added and the wells were incubated for 90 min at 37°C. Free PLG wells were used as control. After washing with PBST, the wells were incubated for 2h or 4h at room temperature with 30 µl of FBG (335µg /ml) in HEPES buffer (100 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1 mg / ml PEG 8000 pH 7.4) containing or not tPA (27nm in a molar ratio of 1:40 relative to the PLG) or recombinant SAK (15µg / ml) as PLG activators. From some wells 30 µl

samples were immediately collected and represent the t 0 of the reaction (undigested FBG). At the end of the incubation times, each collected sample (t0, t2 and t4) was then diluted v: v with "loading buffer" under reducing conditions, boiled at 95°C for 3 minutes, separated on to 12.5% polyacrylamide gel (see above). FBG degradation was detected after staining gel with Commassie Brilliant Blue R250 as previously indicated.

To verify then if *S. aureus* cell-bound PLG can be activated to plasmin by endogenous SAK a similar experiment was performed with *S. aureus* USA 300 LAC wt and *sak* mutant cells. For this purpose no activator was added in the incubation mixture containing FBG and the incubation time was prolonged for 4h.

9.2 PLG captured by heterologous FnBPB N2N3 on the surface of Lactococcus lactis

For this purposes cells of *Lactococcus lactis* expressing FnBPB from a gene cloned into a plasmid vector pNZ8037 (*L.lactis* pNZ8037:: *fnbB*) and cells of the same strain carrying the empty vector (pNZ8037) were grown as above described (see section: Bacterial strains and growth conditions).

Bacteria were harvested by centrifugation from the cultural medium (10 minutes, 4000 rpm), resuspended in PBS, washed two times and diluted at concentration of $5x10^8$ cells/ml in PBS. 100 µL of the bacterial suspension ($5x10^7$ cells) were then immobilized on microtiter wells and incubated overnight at 37 ° C without shaking. The next steps of the experiment were performed as above described for staphylococcal cells.

10 ELISA assays

To screen *S. aureus* strains PLG-binding and to characterize this interaction and the bacterial proteins involved, several ELISA (Enzyme Linked Immuno Sorbent Assay) assays were performed.

10.1 Attachment of *S. aureus* strains to surface-coated PLG

Clinical isolates and laboratory strains of *S. aureus* namely SH 1000, Newman, 8325-4 and USA 300 LAC were tested for attachment to PLG coating ELISA wells. For this purpose cells of these

strains were grown in Tryptic Soy Broth (TSB) medium overnight at 37°C with shaking (150 rpm). At the same time increasing concentrations (from 0 to 12,5 μg/well) of human PLG were coated on microtiter wells (Nunc, Denmark) in 100 μl of 50 mM Na_2CO_3 pH 9.5 and incubated overnight at 4°C. The overnight bacterial cultures, representing the stationary phase, were diluted (1:40) in fresh TSB medium and grown 37°C with shaking in order to obtain culture at exponential phases (OD_{600nm} about 0.4). The staphylococcal cells of both phases were harvested by centrifugation (10 minutes at 4000 rpm) and resuspended in PBS at a concentration of $2x10^8$ cells/ml. In the meantime the microtiter wells were washed three times with 0.1% (v/v) Tween 20 in PBS (PBST) and incubated with bovine serum albumin (BSA, 2% v/v) in PBS (200 μl/well) for 1 h at room temperature to block additional protein-binding sites. Bacterial cells grown both to stationary and exponential phases (100 μL of $2x10^8$ cells/ml in PBS) were then added to the microtiter plate and incubated at 37°C for 2 hours in static conditions. After washing three times with PBST, a mouse-α-rabbit HRP-conjugated antibody (Dako, Glostrup, Denmark) diluted 1:1000 in BSA 1 % was added to the microplate and incubated 1 hour at room temperature. Indeed the IgG binding SpA on *S. aureus* staphylococcal surface allowed detecting straight bacterial cells only with HRP conjugated secondary antibody. After washing with PBST, the antibody binding was detected by incubation of the wells with *ortho*-phenylenediamine dihydrochloride (OPD Dako Cytomation). The absorbance at 490 nm was read with a plate reader (Microplate Reader, model 680, BioRad, Richmond, CA).

10.2 Characterization of PLG binding to FnBPB

10.2.1 Saturation kinetics of the binding of human PLG to surface coated FnBPBN2N3

To characterize PLG binding to FnBPB N2N3, microtiter wells were coated with 1μg/well of recombinant FnBPB N2N3 subdomain in 100 μL 50 mM Na_2CO_3 pH 9.5 and incubated at 4°C overnight. The wells were washed three times with PBST and incubated with BSA 2% for 1 hour at room temperature to block aspecific binding sites. Increasing concentrations (from 0 to 5 μM) of human PLG were then added in 100 μL PBS and incubated 1 hour at room temperature. After an incubation of 1 hour with policlonal rabbit anti-PLG IgG (1:2000 in BSA 1%) followed by an incubation with HRP-conjugated goat-α rabbit antibody (1:1000 in BSA 1%), PLG- FnBPB N2N3 interaction was detected by adding OPD and reading OD at 490 nm in a plate reader.

10.2.2 Reactivity of truncated variants or trench mutants of recombinant FnBPB N2N3 to PLG and to FBG

Microtiter wells were coated with 1 μg/well (2,5μM) of either a truncated version of the FnBPB N2-N3 domain, lacking the 17 residues at the C-terminus of subdomain N3 (rFnBPB163-463), or a N2-N3 domain carrying two amino acid substitutions in residues N312 and F314 (rFnBPB 163-480N312A/F314A), in 100 μl 50 mM Na_2CO_3 pH 9.5 and incubated at 4°C overnight. The wells were washed three times with PBST and incubated with BSA 2%, 1 hour at room temperature to block aspecific binding sites. Increasing concentration of human PLG (added by twelve 1:2 dilutions from 4.5 μM to 0 μM) or of human FBG (added by twelve 1:2 dilutions from 18 μM to 0 μM) in 100 μl of PBS were then added and incubated 1 hour at room temperature. After washings, the plates were incubated 1 hour with polyclonal rabbit anti-PLG (1:2000) or with polyclonal mouse anti-FBG (1:2000) in BSA 1% and then for another hour with HRP-conjugated Goat anti rabbit or Rabbit anti mouse IgG diluted 1:1000 in BSA 1%. The interaction of PLG and FBG with truncated variants or trench mutants of the recombinant FnBPB was detected by adding OPD and reading the absorbance at 490 nm in a plate reader.

10.2.3 Reactivity of recombinant subdomains of FnBPB N2 and N3 to PLG and to FBG

Microtiter wells were coated with 1 μg/well (5 μM) of recombinant fragments of FnBPB N2 and N3 in 100 μl 50 mM Na_2CO_3 pH 9.5 and incubated at 4°C overnight. The wells were washed three times with PBST and incubated with BSA 2%, 1 hour at room temperature to block aspecific binding sites. After washings increasing concentration of human PLG (added by twelve 1:2 dilutions from 4.5 μM to 0 μM) or of human FBG (added by twelve 1:2 dilutions from 18 μM to 0 μM) in 100 μl of PBS were added and incubated 1 hour at room temperature. After washings, the plates were incubated 1 hour with polyclonal rabbit anti-PLG (1:2000) or with polyclonal mouse anti-Fbg (1:2000) in BSA 1% and then for another hour with HRP-conjugated Goat anti rabbit or Rabbit anti mouse IgG diluted 1:1000 in BSA 1%. The interaction of PLG and FBG with recombinant FnBPB subdomains was detected by adding OPD and reading the absorbance at 490 nm in a plate reader.

10.2.4 Effect of L-Lysin and ε–amino caproic acid (εACA) on the attachment of *S. aureus* USA 300 LAC to immobilized PLG

To determine whether lysine residues on the surface of staphylococcal cells could be involved in the interaction with PLG, an overnight culture of *S. aureus* cells from USA 300 LAC strain was diluted 1:40 in fresh TSB medium and grown 37°C to OD_{600nm} = 0.4. Staphylococcal cells at exponential phase were harvested by centrifugation (10 minutes at 4000 rpm) and resuspended in PBS at a OD_{600nm}= 1. 50 µL (approximately 5x 10^7 cells/well) of staphylococcal suspension were then added to microtiter wells previously coated, over night, with human PLG (2 µg/well), blocked with 2% BSA in PBS (200 µL/well) and incubated 30 minutes at 37°C in static conditions with 50 µL of increasing concentration of L-Lys or εACA in PBS (0, 3.125 mM , 6.25 mM, 12.5 mM, 25 mM, 50 mM, 100 mM). Therefore final concentrations of L-Lys and of εACA obtained after dilution 1:2 with bacterial suspension were: 0, 1.56 mM, 3.125 mM, 6.25 mM, 12.5 mM, 25 mM, 50 mM and, at the same time, final OD_{600nm} of bacterial suspension in the wells was 0.5. The plates were incubated two hours at 37°C, washed with PBST and the attached bacteria were detected with a mouse-α-rabbit HRP-conjugated antibody diluted 1:1000 in BSA 1% as reported above. The attached bacteria were detected by adding OPD and reading the absorbance at 490 nm in a plate reader.

10.2.5 Reactivity of lysine substituted mutants of FnBPB N2N3 to PLG and to FBG

Microtiter wells were coated with 1 µg/well (10 µg/ml) of recombinant lysine substituted mutants of FnBPB N2N3 (site 1, site 2, site 3) in 100 µl of 50 mM Na_2CO_3 pH 9.5 and incubated at 4°C overnight. After washings and blocking with BSA 2%, saturating concentrations of human PLG (0.5 µg/well) or of human FBG (2.5 µg/well) in 100 µl of PBS were added and incubated 1 hour at room temperature. After washings, the plates were incubated 1 hour with polyclonal rabbit anti-PLG (1:2000) or with polyclonal mouse anti-FBG (1:2000) in BSA 1% and then for another hour with HRP-conjugated Goat anti rabbit or Rabbit anti mouse IgG diluted 1:1000 in BSA 1%. The interaction of PLG and FBG with the lysine substituted mutants of FnBPB N2N3 was detected by adding OPD and reading the absorbance at 490 nm in a plate reader.

10.2.6 Binding of FnBPB N2N3 to PLG in the presence of FBG

In order to determine if PLG and FBG bind FnBPB at the same or distinct site, 0.5 µg of rFnBPBN2N3 was immobilized on to the surface of microtitre wells in 100 µl 50 mM Na_2CO_3 pH 9.5 and incubated at 4°C overnight. The wells were washed three times with PBST and incubated with BSA 2% 1 hour at room temperature. After washings a set of wells were incubated with 1µM of PLG (saturating concentration) along with increasing concentrations of FBG (from 0 µM to 18 µM). The remains wells were incubated with 4 µM of FBG (saturating concentration) along with increasing concentrations of PLG (from 0 µM to 4.5 µM) and incubated 1 hour at room temperature. After washings, bound PLG was detected with polyclonal rabbit anti-PLG (1:2000) instead bound FBG with polyclonal mouse anti-FBG (1:2000) in BSA 1% and then for another hour with HRP-conjugated Goat anti rabbit or Rabbit anti mouse IgG diluted 1:1000 in BSA 1% respectively. PLG and FBG bound to recombinant FnBPB were detected by adding OPD and reading the absorbance at 490 nm in a plate reader.

10.2.7 Identification of PLG kringle(s) involved in FnBPB N2N3 binding

To identify PLG kringle(s) involved in $FnBPB_{163-480}$(N2N3) binding, microtitre plates were coated with 0.5 µg of $FnBPB_{163-480}$/well in 100 µl 50 mM Na_2CO_3 pH 9.5 and incubated at 4°C overnight. After washings and blocking with BSA 2%, the plates were incubated with PLG, PLG fragments K1-K3, K1-K4 or mini-PLG (1µM) 1 hour at room temperature. Proteins bound to $FnBPB_{163-480}$ were detected by polyclonal rabbit anti-PLG (1:2000) followed by secondary HRP-conjugated Goat anti rabbit (1:1000).

10.3 Effect of bacteria-bound-PLG on the cleavage of chromogenic substrate S-2251

10.3.1 PLG captured by *S. aureus* USA 300 LAC *wt* and Δ*sak* mutant

To evaluate the ability of *S. aureus* USA 300 LAC *wt* and *Δsak* mutant to bind and activate PLG, an assay based on cleavage of the synthetic plasmin chromogenic substrate D-Val-Leu-Lys

Nitroanilide dihydrochloride (S-2251 Sigma) was performed. S-2251 is an oligopeptide (D-Val-Leu-Lys-4-nitroanilide dihydrochloride), the carboxyl group of its lysine, is bound to a molecule of para-nitroaniline (pNA) by a carbamide bond. Plasmin cleaves the peptide bond releasing pNA (colored yellow) proportionally to protease activity. The amount of pNA released is then detectable by measuring the absorbance at 415 nm with a common spectrophotometer. For this purpose cells of *S. aureus* USA 300 LAC *wt* and *Δsak* were grown in TSB medium at 37°C (150 rpm), the overnight culture was diluted 1:40 in fresh TSB medium and grown 37°C to OD_{600nm} = 0.4. Staphylococcal cells at exponential phase were harvested by centrifugation (10 minutes at 4000 rpm) and resuspended in PBS at a concentration of $5x10^8$ cells/ml. 100 µL of bacterial suspensions ($5x10^7$cells) were immobilized on a microtiter plate and incubated overnight at 37°C in static conditions. The microtiter wells were washed three time with PBST to discard not adhered bacteria, blocked with BSA 2% and incubated with 100 µL of human PLG 100 µg/mL in PBS (10 µg/well), at 37°C for 1 hour, with mild shaking (50 rpm). In some wells, used as a control was not added PLG. After washing with PBST, chromogenic substrate S-2251(H-D-Val-Leu-Lys p-Nitronilide di-hydrocloride SIGMA) was added at concentration of 0.6 µM in 100µl a chloride-containing buffer (100 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1 mg/ml PEG 8000, pH 7.4) in absence or in presence of tissue Plasminogen Activator (tPA) diluted to 27 nM (in 1:40 molar ratio with respect to human PLG) or rSAK 15µg/ml. In some control wells only tPA or SAK was added in absence of chromogenic substrate S-2251. The PLG-activation reaction was followed spectrophotometrically by measuring the absorbance at 415 nm at precise time point (0, 20', 40', 1 h, 2 h, 4 h, 6 h, 8 h, 20 h, 24 h) with a plate reader (Microplate Reader, model 680, BioRad).

10.3.2 PLG capured by heterologous FnBPB N2N3 on the surface of Lactococcus lactis.

To test if cells of *Lactococcus lactis* expressing FnBPB are able to capture plasminogen and activate it to plasmin an ELISA assay based on cleavage of the synthetic plasmin chromogenic substrate D-Val-Leu-Lys Nitroanilide dihydrochloride (S- 2251 Sigma) was performed. For this purposes the strain of *Lactococcus lactis* expressing FnBPB from a gene cloned into a plasmid vector pNZ8037 (*L.lactis* pNZ8037:: *fnbB*) and the same strain carrying the empty vector (pNZ8037) were grown according to the method described. Bacteria were collected by centrifugation from the culture medium (10 minutes, 4000 rpm), resuspended in PBS, washed with the same buffer and finally diluted at concentration of $5x10^8$ cells/ml in PBS. 100 µL of the bacterial suspension ($5x10^7$ cells) were then immobilized on microtiter wells and incubated overnight at 37°C under static conditions. The successive phases of the experiment were performed as above described for staphylococcal cells.

11 Surface plasmon resonance analysis of PLG binding to $FnBPB_{163-480}$

The optical phenomenon of Surface Plasmon Resonance (SPR) used by BIAcore systems enables the detection and quantification of protein-protein interactions in real time. BIAcore systems are widely used for characterizing the interactions of proteins with other proteins, peptides, nucleic acids, lipids, and small molecules. When BIAcore is used to measure protein interactions, one of the interactants is immobilized onto a sensor chip surface and the other interactant is passed over that surface in solution via an integrated microfluidic flow system. The immobilized interaction is referred to as the ligand, and the injected interactant in solution is referred to as the analyte. Binding responses are measured in resonance units (RU) and are proportional to the molecular mass on the sensor chip surface and, consequently, to the number of molecules on the surface *(Guiducci C., Advanced Bioengineering Methods Laboratory SPR.)*

Generally, a glass surface is coated with an inert metal (gold). On the metal, a self-assembled monolayer (SAM) is deposited, on the SAM a matrix of non-cross-linked carboxymethylated dextran is bound which provides a hydrophilic layer. To allow the detection the ligand is immobilized onto the sensor surface, the analyte is then injected in aqueous solution (ideally with the same components and composition as the running buffer) through the flow cell, under continuous flow. As the analyte binds to the ligand the accumulation of protein on the sensor surface causes an increase in refractive index. This refractive index change is measured in real time and the result plotted as response units (RU). Importantly, a response (background response) will also be generated if there is a difference in the refractive indices of the running and sample buffers. This background response must be subtracted from the sensorgram to obtain the actual binding response. The SPR optical unit consists of a source for a light beam that passes through a prism and strikes the surface of a flow cell at an angle, such that the beam is totally reflected. The SPR angle is sensitive to the composition of the layer at the surface of the gold. A baseline SPR angle is first determined by washing buffer over the surface with a fixed amount of ligand attached. To this flow of buffer, some analyte is then added. The binding of analyte to immobilized ligand causes an increase in the refractive index at the surface, thereby changing the SPR angle because it is directly proportional to the amount of bound analyte. (Fig. 22)

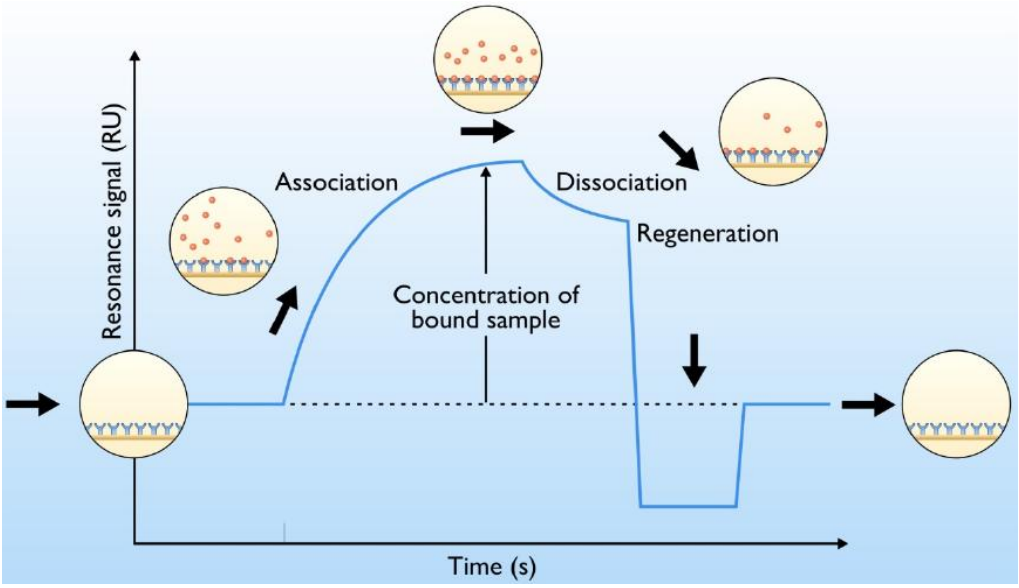


Figure 22. Surface Plasmon Resonance (SPR)

To estimate the affinity of the interaction between PLG and $FnBPB_{163-480}$ surface plasmon resonance (SPR) was conducted using a BIAcore X-100 instrument (GE Healthcare, Piscataway, NJ, USA). $FnBPB_{163-480}$ was covalently immobilized on dextran matrix CM5 sensor chip surface by using a $FnBPB_{163-480}$ solution (30 µg/ml in 50 mM sodium acetate buffer, pH 5) in a 1:1 dilution with N-hydroxysuccinimide and N-ethylN9-(3- dimethylaminopropyl) carbodiimide hydrochloride. The excess of active groups on the dextran matrix was blocked using 1 M ethanolamine (pH 8.5). On another flow cell, the dextran matrix was treated as described above but without any ligand to provide an uncoated reference flow cell. The running buffer used was PBS containing 0.005% (v/v) Tween 20. Two-fold linear dilution series (0.078–5 µM) of PLG in running buffer were passed over the ligand at the flow rate of 10 µl/min and all the sensorgrams were recorded at 22˚C. Assay channel data was subtracted from reference flow cell data. The response units (RU) at steady state were plotted as a function of PLG concentration, and fitted to the Langmuir equation to yield the KD of the PLG-FnBPB163-480 interaction. We are grateful to Professor V. De Filippis (University of Padua, Italy) for assistance and advice with BIAcore measurements.

12 Statistical methods

Continuous data were expressed as means and standard deviations. Two-group comparisons were performed by Student's *t* test. One-way analysis of variance, followed by Bonferroni's post hoc tests, was exploited for comparison of three or more groups. Analyses were performed using Prism 4.0 (GraphPad). Two-tailed *p* values of 0.05 were considered statistically significant.

RESULTS

1 *Staphylococcus aureus* interacts with PLG

In our research we focused on the interaction between *S. aureus* and PLG. Preliminary data showed that clinical isolates and laboratory strains of *S. aureus* (SH1000,Newman, 83254, USA 300 LAC) strongly attached to human PLG coating Elisa wells. Staphylococcal adherence was dependent on amount of PLG immobilized; strains grown to stationary or exponential phases attached to PLG with similar kinetics (Fig. 23).

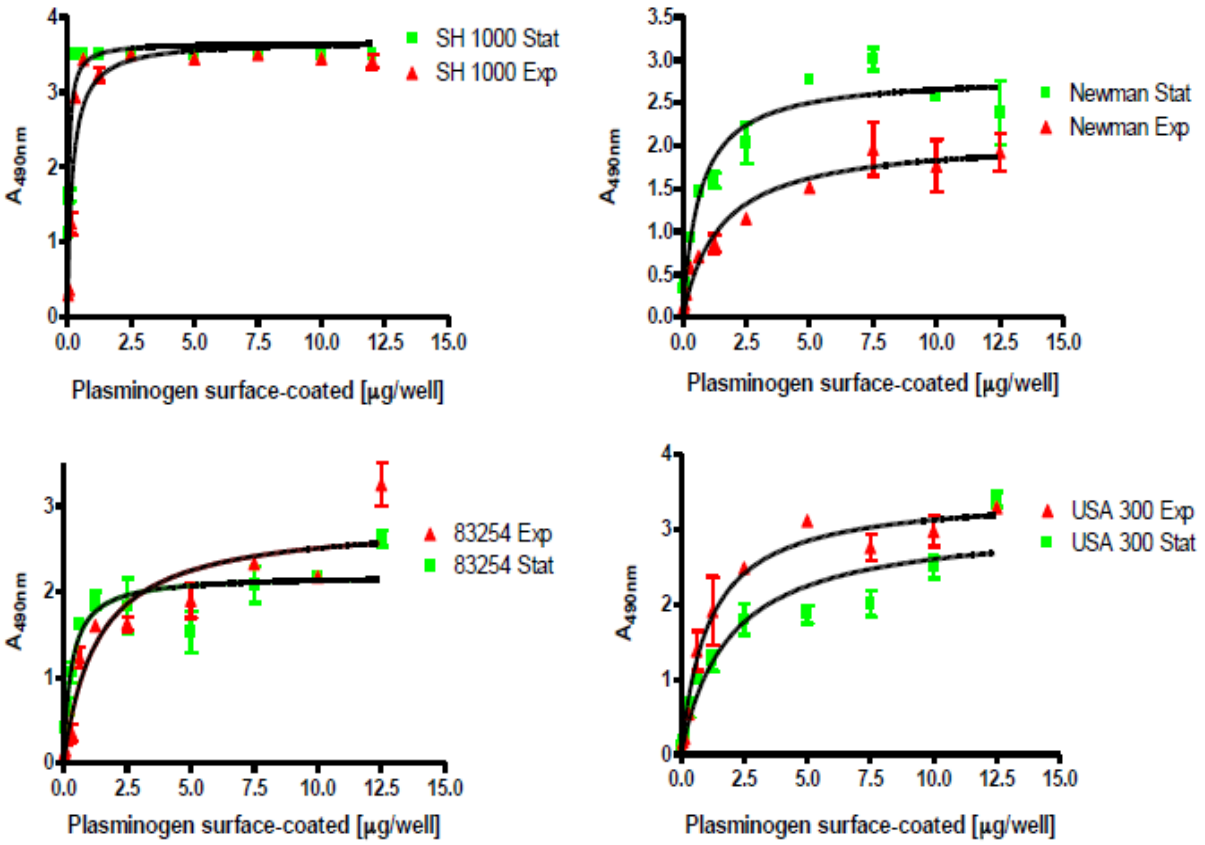


Figure 23. ELISA assays measuring the attachment of *S. aureus* strains to immobilized PLG. The concentrations of PLG on microtiter wells is given on the X-axis. Bacterial adherence is indicated on the Y-axis. Data points represent the mean of triplicate vials. Each graph is representative of three independent experiments.

2 *S. aureus* cell wall-anchored proteins bind PLG in human plasma

To go in more detail with this analysis, the *S. aureus* USA 300 LAC wt and *ΔsrtA* were grown to exponential phase and tested for their ability to sequester PLG from human serum. Following incubation of staphylococci with different concentrations of human plasma, bacteria bound proteins were detached and analyzed by SDS-PAGE under non-reducing conditions, subjected to immunoblotting and detected with an anti-PLG antibody. In these conditions, whatever was the concentration of plasma tested, a clear band with 90 kDa corresponding to PLG was detected, suggesting that PLG can be recognized *in vivo* by *S. aureus* cells (Fig. 24A).

S. aureus are known to bind to human PLG but the bacterial surface components responsible have not been identified. It is well documented in the literature that the proteins covalently anchored to the bacterial cell wall (CWA) are able to bind different host proteins, thus contributing to the process of colonization and bacterial infection. In order to determine if cell wall-anchored proteins on the surface of *S. aureus* contribute to PLG binding, the wild-type strain USA 300 LAC and sortase A deficient mutant (*ΔsrtA*) were compared. Bacteria were incubated with different concentrations of human plasma and proteins that were bound non-covalently to the cell surface were dissociated, separeted by SDS-PAGE under non reducing conditions and analysed by Western immunoblotting, probing with anti-PLG IgG. Bacterial cells captured a 90 KDa immuno-reactive protein in a dose-dependent manner (Fig. 24A).

Densitometric analysis of the blots showed that the wild-type strain captured at least 10-fold more PLG than sortase A mutant (Fig. 24B). To exclude the possibility that the markedly reduced capture of PLG from human plasma by sortase A mutant was due to higher proteolytic activity of mutant, LAC and its srtA mutant were grown to exponential phase and were incubated with purified PLG and the integrity of human protein in the supernatants was then examined by SDS-PAGE. As expected, the amounts of unbound PLG in the supernatant from srtA mutant was even higher than that from the wt strain (data not shown).

Therefore it can be preliminarily conclude that one or more CWA proteins represent receptors for human PLG and that other surface-located proteins contribute minimally.

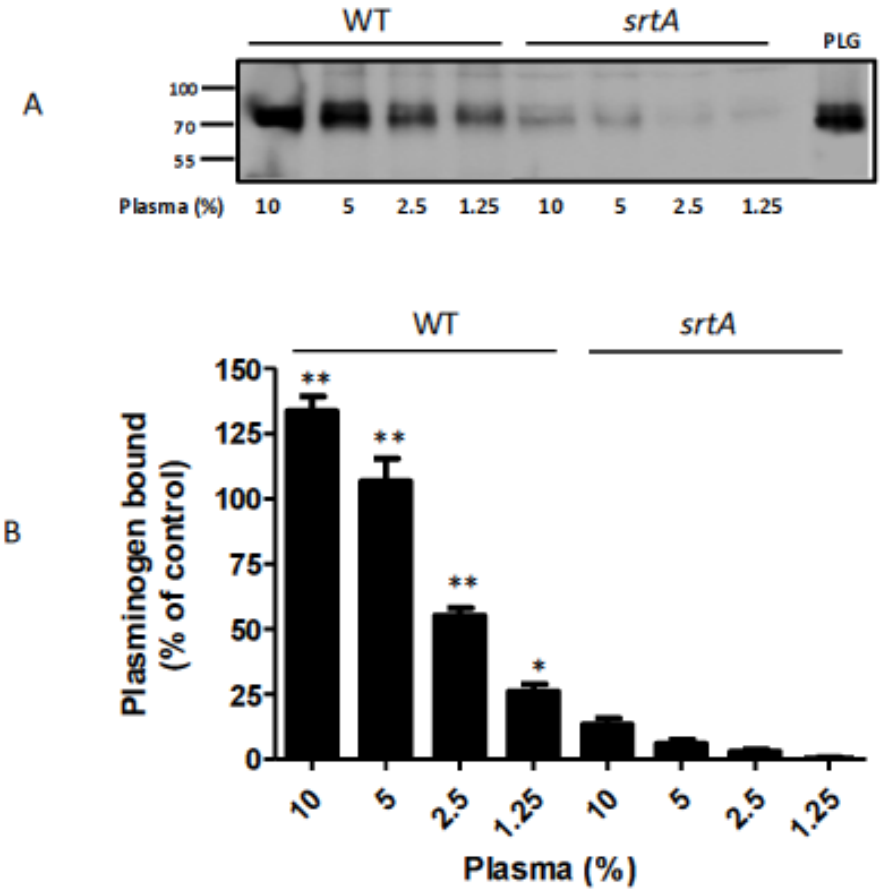


Figure 24: Capture of PLG from human plasma by *S*. *aureus*. **A)***S*. *aureus* strain LAC wt and LAC Δ*srtA* were mixed with different concentrations of human plasma for 60 min. Proteins that were boundto the cell surface were released by extraction buffer and separated by SDS-PAGE under nonreducingconditions and analysed by Western immunoblotting. The membranes were probed withrabbit anti-human PLG followed by HRP-conjugated mouse anti-rabbit IgG and developed with theECL Western blotting detection kit. **B)** Densitometric analysis of PLG bound to *S*. *aureus* LAC and the sortase A mutant as reported in panel A. The band intensity was quantified relative to a sample ofpure human PLG. The reported data are the mean values +/- SD from three independent experiments.

3 PLG bound to the surface of *S. aureus* can be activated.

The ability of PLG bound to the surface of *S*. *aureus* LAC to be activated by exogenous or endogenous PLG activators was tested. *S*. *aureus* LAC is lysogenized by a bacteriophage that carries the gene for SAK, a known activator of the zymogen PLG. Human tissue PLG activator was also tested. Bacterial cells (USA 300 LAC and Δ*srtA)* were immobilized on the surface of microtitre wells, incubated with PLG and then for 24h with exogenous purified recombinant SAK (rSAK) or tPA and the chromogenic plasmin substrate S-2251. The substrate was consumed following incubation with the wild-type but minimal substrate consumption was seen with Δ*srtA* (Fig. 25A and 25B). Controls where PLG was omitted did not have the ability to cleave the

substrate. Interestingly, incubation of wild-type LAC with PLG and S-2251 without activators showed a significantly higher cleavage of the substrate as compared to the controls. Conversely until 10 hours, the level of substrate consumption was significantly lower than that observed with samples with exogenous SAK or tPA added. These data show that PLG bound to the cell surface by CWA proteins can be activated efficiently by exogenously added rSAK or tPA (Fig. 25A and 25B, respectively) and furthermore the immobilized cells can release an endogenous PLG activator, presumably SAK.

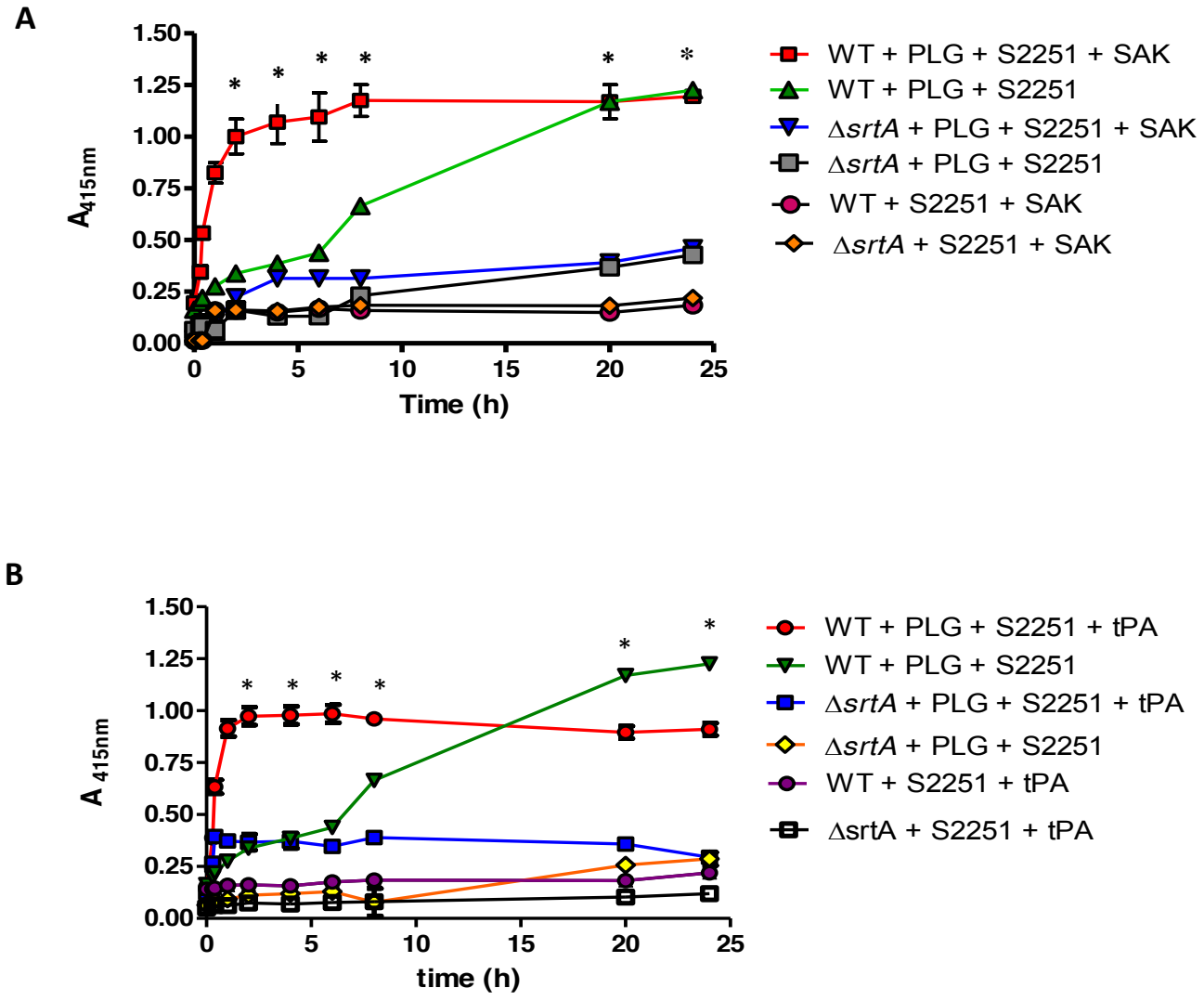


Figure 25. PLG captured by *S. aureus* LAC is functionally active. *S. aureus* LAC and the *srtA* mutant were immobilized on the surface of microtitre plates and then incubated with human PLG for 1 h at 37ºC. After washing, cells were incubated with recombinant SAK (A) or tPA (B) and S-2251 for 24 h, and the activity of plasmin was determined measuring the absorbance at 415 nm. Controls lacking PLG or activator were included. Data points are the means of 3 independent experiments each performed in triplicate +/- the SD. Statistically significant differences are indicated (Student's *t* test; *, $p < 0.05$).

4 *S. aureus*-bound PLG activated can degrade FBG

Plasmin is a central component of the fibrinolytic system and can actively cleave fibrin and FBG. In order to determine if PLG captured by CWA proteins on the surface of *S. aureus* can be activated by SAK and cleave FBG, immobilized *S. aureus* LAC cells were incubated with PLG. After washing rSAK and human FBG were added. The integrity of FBG was assessed by SDS-PAGE and staining with Coomassie Blue. After 2 and 4h incubation FBG was progressively cleaved (Fig. 26A). Samples where rSAK was omitted also cleaved FBG which was presumably due to secretion of endogenous SAK (Fig. 26A, tracks D and F). When cells were incubated with tPA rather than rSAK, FBG was digested more efficiently (25B). In fact, all three FBG chains were hydrolyzed compared to the mild hydrolysis observed when PLG was activated by SAK. This is probably due to the different mechanisms of PLG activation promoted by tPA and SAK, respectively (Fig.26 B and 26A).

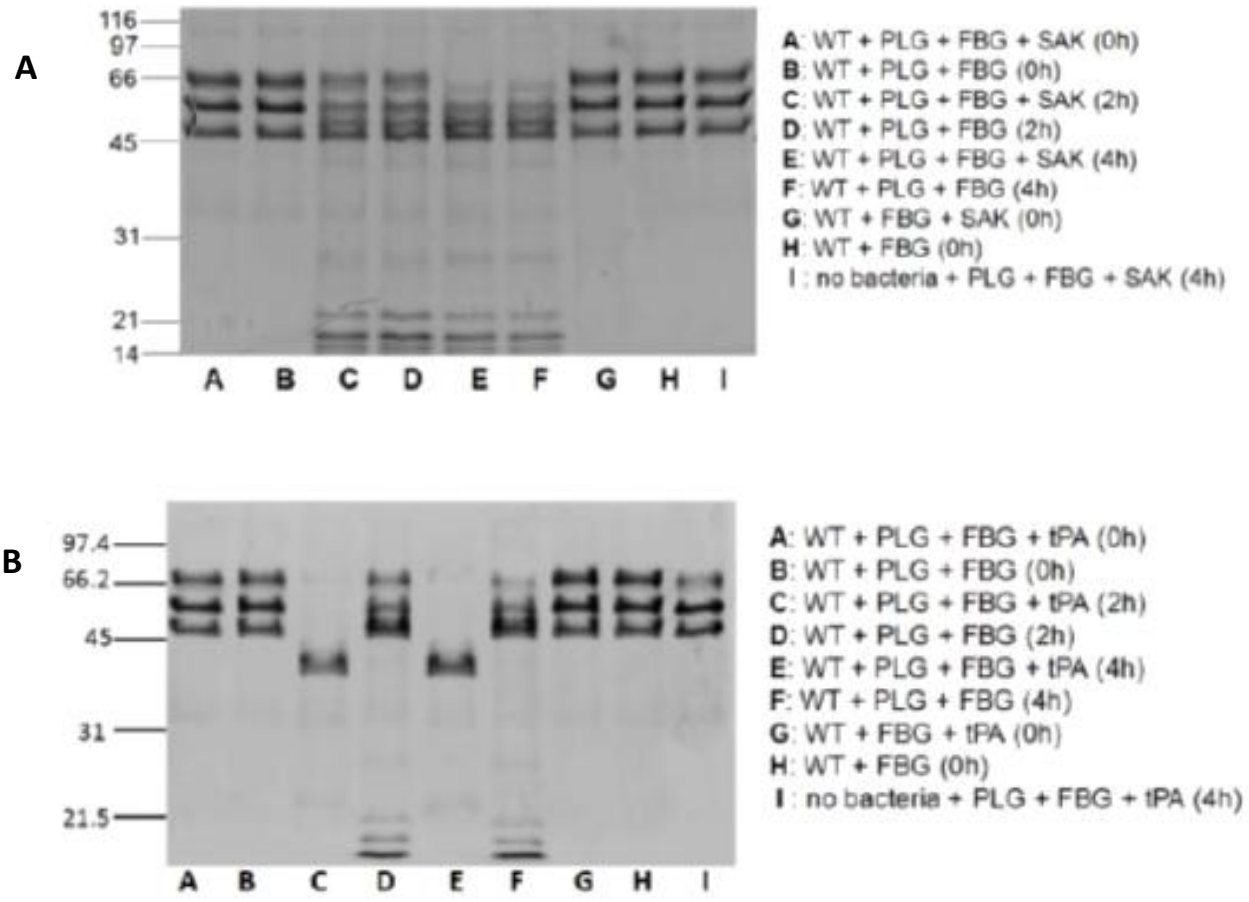


Figure 26. PLG captured by *S. aureus* LAC is functionally active. *S. aureus* LAC wild-type cells were also immobilized on microtitre plates and incubated with human PLG for 1 h at 37ºC. After washing, PLG was activated by addition of rSAK (A) or tPA(B) and the plasmin substrate FBG. Samples were incubated for 2h or 4h and FBG in the fluid phase was subjected to SDS-PAGE and stained with Coomassie Blue. The figure is the representative of three independent experiments.

5 Captured PLG can be activated by endogenously expressed SAK

In order to determine if cleavage of plasmin substrates S2251 and whole FBG in the experiments described in Fig. 25 and 26, where exogenous rSAK or tPA was not present, was due to SAK expressed by the bacterium, a null mutation in the chromosomal *sak* gene of LAC was constructed (LAC *Δsak)*. Culture supernatants and cell surface extracts were examined by SDS-PAGE and Western immunoblotting using anti-SAK IgG. SAK was present both in the supernatant and was associated with the cell surface of LAC and no SAK was detected infractions from LAC *sak* (Fig.27A). To determine if endogenously expressed SAK was responsible for activating captured PLG, wild-type and mutant cells were compared. Cleavage of the substrate S-2251 was evident with wild-type cells whereas no cleavage occurred with the *sak* mutant (Fig. 27B). Similarly, wild-type cells could capture and activate PLG and cleave FBG (Fig. 27C) whereas the *sak* mutant was defective. In order to demonstrate directly that LAC Δ*sak* could still capture PLG, bacteria were incubated with different concentrations of plasma and the amount of PLG captured was quantified by Western immunoblotting and densitometry (Fig. 27D). No difference in PLG bound to the wild-type or *sak* mutant cells was observed. Thus it can be concluded that SAK secreted by *S. aureus* LAC in the supernatant or bound to the cell surface is capable of activating captured PLG.

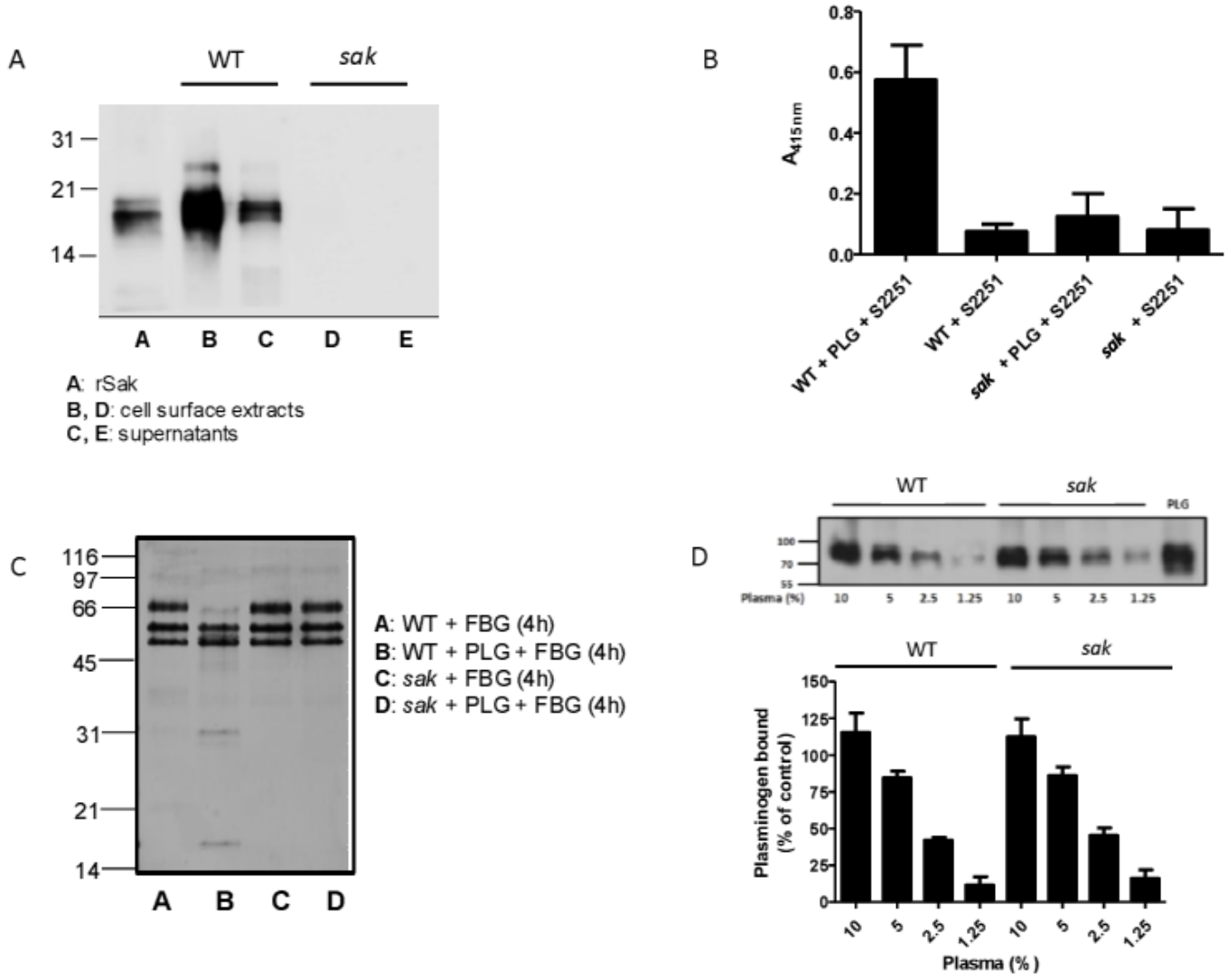
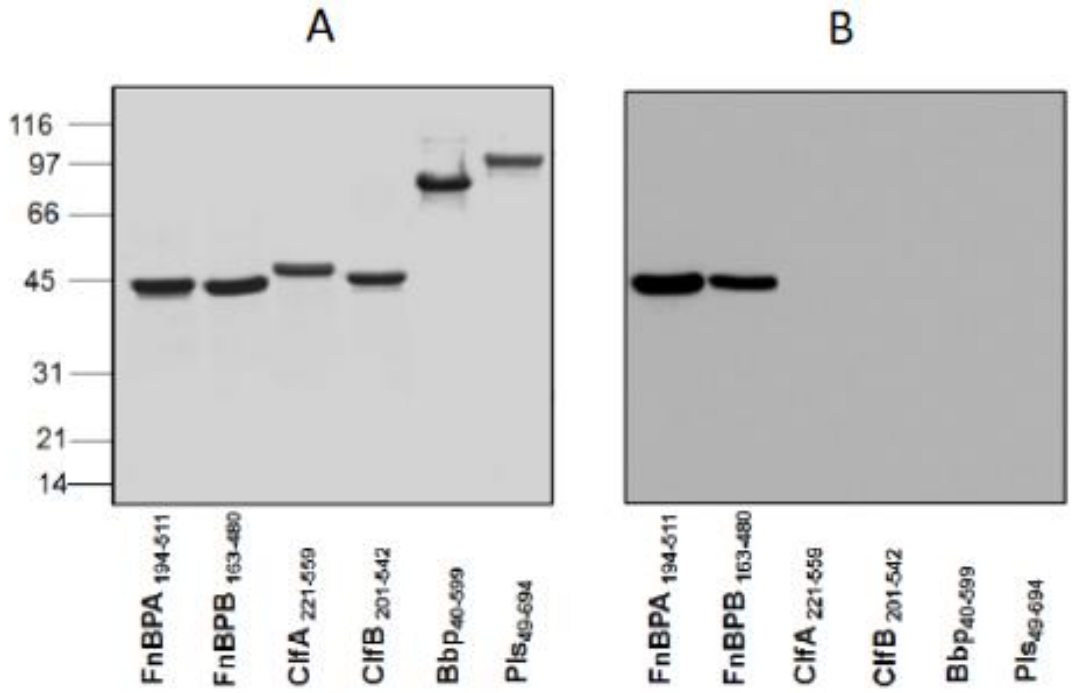


FIGURE 27. ***S. aureus*** **cells-bound PLG can be activated by endogenous SAK.** A) TCA precipitated culture supernatants and proteins extracted from the cell surface were subjected to SDS-PAGE and Western immunoblotting probing with mouse anti-rSAK IgG and HRP-conjugated rabbit anti-mouse IgG. B) *S. aureus* LAC and the *sak* mutant were immobilized on the surface of microtitre plates and then incubated with human PLG for 1 h at 37C. After washing, cells were incubated withS-2251 for 8h and the activity of plasmin was determined measuring the absorbance at 415nm.Controls lacking PLG were included. Data points are the means of 3 independent experiments each performed in triplicate +/- the SD. C) *S. aureus* LAC wild-type and *sak* mutant cells were immobilized on microtitre plates and incubated with human PLG for 1 h at 37C. After washing the plasmin substrate FBG was added. Samples were incubated for 2h or 4h and FBG in the fluid phase was subjected to SDS-PAGE and stained with Coomassie Blue. The figure is the representative of three independent experiments. D) *S. aureus* strain LAC and *sak* mutant cells were mixed with different concentrations of human plasma for 60 min. Upper panel: proteins that were bound to the cell surface were released with extraction buffer and separated by SDS-PAGE under non-reducing conditions and analysed by Western immunoblotting. The membranes were probed with rabbit anti human PLG followed by HRP-conjugated mouse anti-rabbit IgG. Lower panel: densitometric analysisof released PLG. The band intensities were quantified relative to a sample of pure human PLG. The reported data are the mean values from three independent experiments.

6 Fibronectin binding proteins A and B bind PLG

The inability of the *srtA* mutant of *S*. *aureus* LAC to capture PLG indicates that one or more sortase-anchored cell wall-associated protein(s) is involved. In order to begin the process of identification which CWA proteins can bind PLG, purified recombinant ligand binding domains (A region) of several CWA proteins were tested by Western blotting. Proteins were subjected to SDS-PAGE (Fig.28A) and Western blots (Fig.28B) were probed with purified human PLG and anti-PLG IgG. Subdomains N2 and N3 of the N-terminal A regions of both FnBPA and FnBPB bound PLG whereas the A domains of ClfA and ClfB, the bone sialoprotein binding protein (Bbp) and the plasmin-sensitive protein (Pls) did not (Fig. 28B). In order to investigate if FnBPA and FnBPB are the sole PLG binding proteins of *S*. *aureus* LAC, a mutant that lacks both proteins was tested for PLG capture. No statistically significant difference between the wild type and the *fnbA fnbB* mutant was seen(Fig. 28C) which indicates that one or more CWA protein(s) in addition to FnBPA and FnBPB can bind PLG.



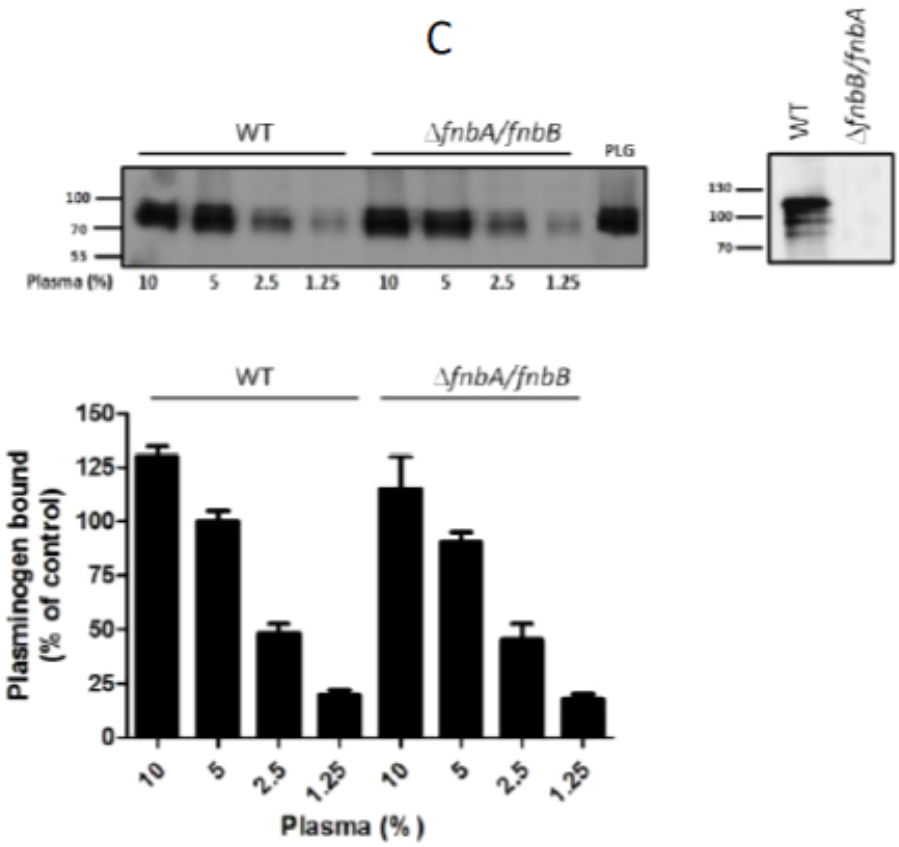
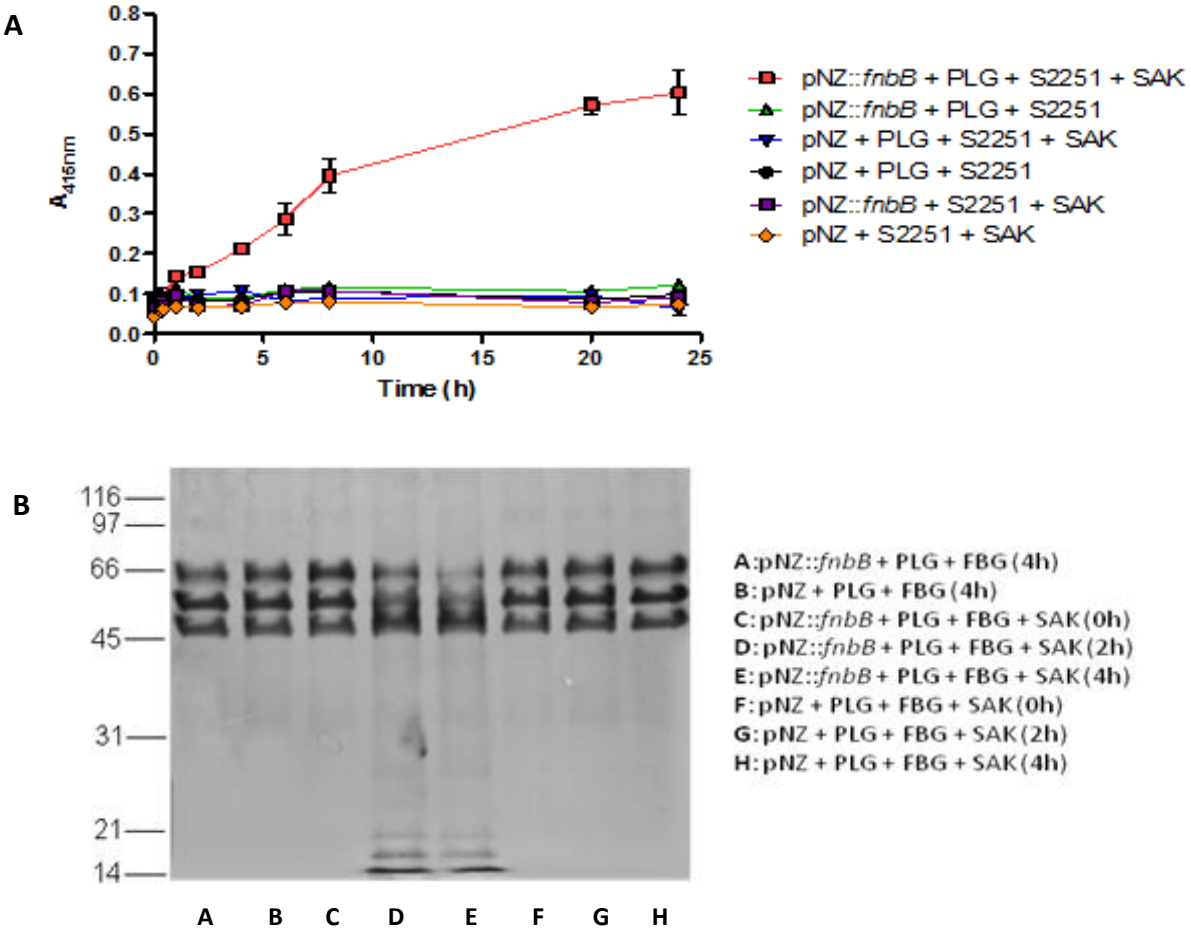


Figure 28. Recombinant cell wall-anchored proteins binding to PLG. Purified recombinant A domains of several CWA proteins of *S*. *aureus* were subjected to SDS-PAGE and either A) stained with Coomassie Blue or B) transferred to a nitrocellulose membrane and probed with human PLG followed by rabbit anti-PLG serum and then HRP-conjugated goat anti-rabbit IgG. C) *S*. *aureus* strains LAC and its *fnbA*/*fnbB* double mutant cells (upper panel) were mixed with different concentrations of human plasma for 60 min. Proteins that were bound to the cell surface were released by extraction buffer and separated by SDS-PAGE under non-reducing conditions and analyzed by Western immunoblotting. The membranes were probed with rabbit anti-human PLG followed by HRP-conjugated mouse anti-rabbit IgG and developed with the ECL Western blotting detection kit. Densitometric analysis of PLG bound to the strains (lower panels of C). The band intensity was quantified relative to a sample of pure human PLG. The reported data are the mean values+/- SD from three independent experiments. In the insets the material released by treatment of bacteria with lysostaphin was subjected to Western blot and probed with FN.

7 *Lactococcus lactis* expressing FnBPB can capture PLG

In order to study FnBPB inisolation from other *S*. *aureus* CWA proteins, a strain of *Lactococcus lactis* expressing FnBPB from a gene cloned into the plasmid vector pNZ8037 was used. *L*. *lactis* pNZ8037::*fnbB* and the same strain carrying the empty vector were immobilized inmicrotitre wells, incubated with PLG and following washing steps the chromogenic plasmin substrate S-2251 and rSAK (Fig. 29A) or tPA (Fig. 29C) were added. Cleavage of the substrate was observed whereas the cells bearing the empty vector had no activity. It should be noted that this system was somewhat

less active than *S. aureus* LAC cells. *L. lactis* (pNZ8037::*fnbB)* cells were also incubated with FBG and rSAK (Fig. 29B) or tPA (Fig. 29D). SDS-PAGE analysis showed that FBG was cleaved by the FnBPB expressing cells and not by those carrying the empty vector. These experiments show that *L.lactis* cells normally lack the ability to capture PLG and that FnBPB expressed on the bacterial cell surface can capture PLG in a form that can be activated by exogenous PLG activators.



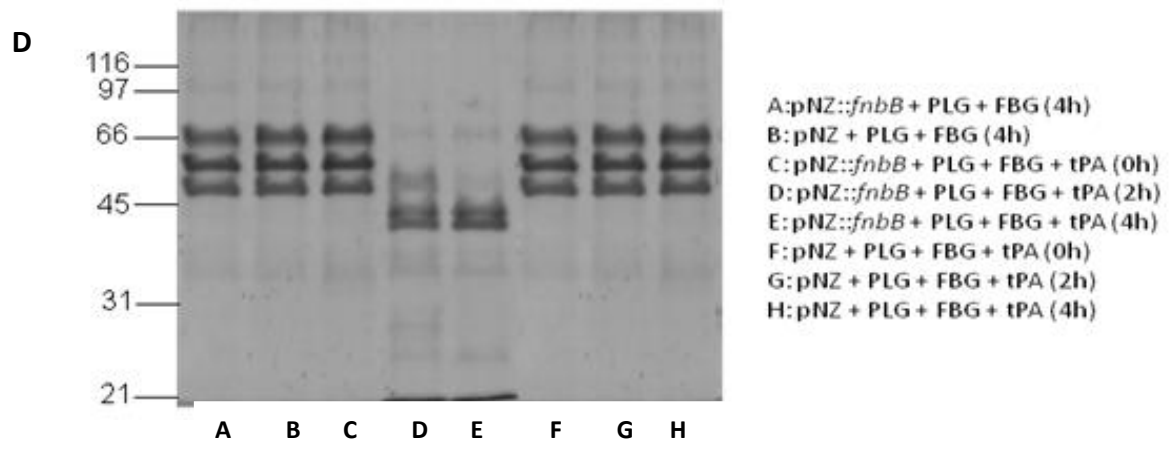


Figure 29. Activation of PLG bound to *L. lactis* expressing FnBPB. *L. lactis* expressing FnBPB (pNZ8037fnbB) and cells carrying the empty vector were immobilized in microtitre plates and incubated with PLG for 1 h at 37°C. After washing cells were incubated with rSAK (A) or tPA (C) and the chromogenic plasmin substrate S-2251 for 24h. The activity of plasmin was determined measuring the absorbance at 415 nm. The data points are the means (+/- SD) of three independent experiments each performed in triplicate.
Immobilized *L. lactis* expressing FnBPB and cells carrying the empty vector were also incubated with PLG for 1h at 37°C. After washing cells were incubated with FBG and rSAK (B) or tPA (D) for 2h and 4h. Controls lacking rSAK or tPA were included (tracks A and B). The fluid was analyzed by SDS-PAGE followed by staining with Coomassie Blue.

8 PLG and FBG bind FnBPB at distinct sites

The A domain of FnBPB binds to FBG by the "dock, lock, and latch" mechanism. In order todetermine if PLG and FBG bind FnBPB at the same or distinct sites, rFnBPB163-480 (rFnBPBN2N3) was immobilized onto the surface of microtitre wells and tested for binding to saturating concentrations of PLG in the presence of increasing amounts of FBG by an ELISA-type assay. Bound PLG was detected with specific antibodies and the amount of PLG bound did not change as the concentration of FBG added increased (Fig. 30A). A similar result was obtained when saturating concentrations of FBG were tested in the presence of increasing amounts of PLG (Fig. 30B). In both panels dose-dependent binding of increasing amounts of either FBG or PLG to FnBPB is also reported. Together these data indicate that PLG and FBG bind to distinct sites on the A domain of FnBPB without steric hindrance. The failure of FBG to interfere with rFnBPB binding to PLG suggested that the ligands bind to different sites within the A domain of the protein.

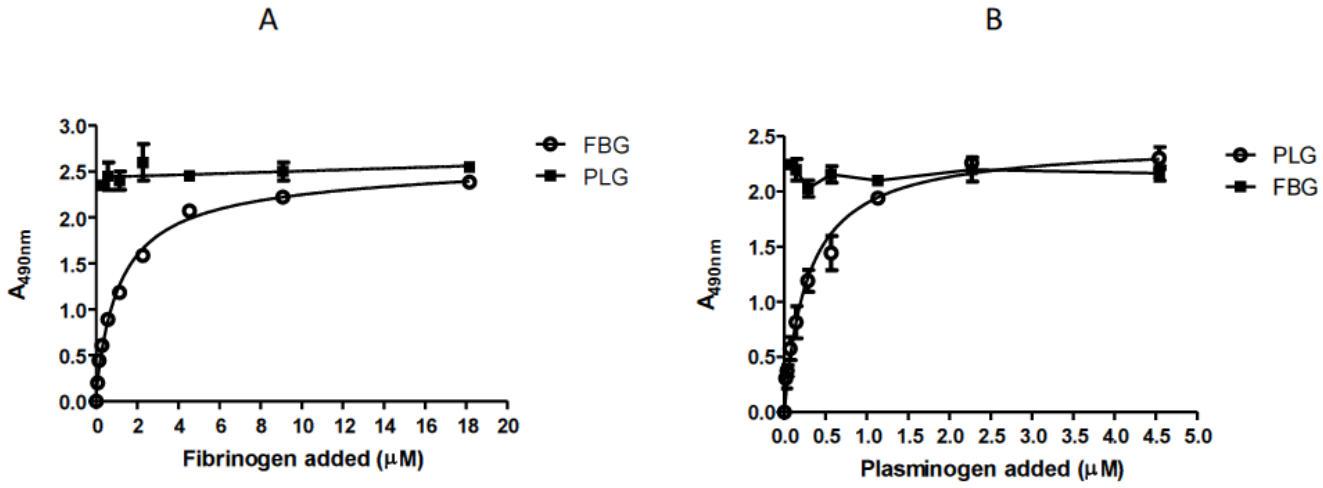
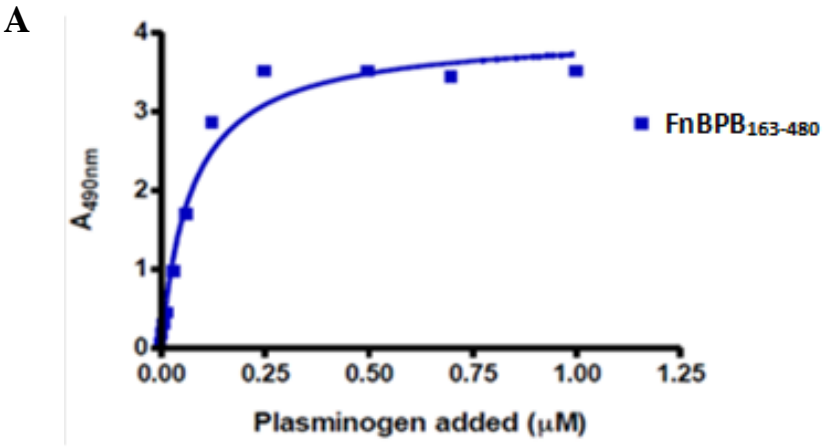


Figure 30. Binding of FnBPB to PLG in the presence of FBG. A) rFnBPB was immobilized on the surface of microtitre wells. Saturating concentrations of PLG were added to along with increasing concentrations of FBG. Bound PLG was detected with rabbit anti-PLG followed by HRP-conjugated goat anti-rabbit IgG (full squares). In the same panel binding of increasing amounts of FBG to the wells is also reported (open dots). Bound FBG was detected with mouse anti-FBG serum followed by HRP-conjugated rabbit anti-mouse IgG. The data points are the means +/- SD of three independent experiments each performed in triplicate. B) ELISA type assay with rFnBPB immobilized on the surface of microtitre wells. Saturating concentrations of FBG were added to along with increasing concentrations of PLG. Bound FBG was detected with mouse anti-FBG followed by HRP-conjugated rabbit anti-mouse IgG (full squares). The panel reports also binding of increasing amounts of PLG to the wells (open dots). Bound proteins were detected with specific antibodies as in A. The data points are the means (+/- SD) of three independent experiments each performed in triplicate.

9 FnBPB N2N3 domain binds PLG with high affinity

Recombinant $FnBPB_{163-480}$ (N2N3 domain) was tested for PLG binding also by ELISA-based solid phase assay. As shown in figure 31A, N2N3 subdomain interacts with human PLG in a saturable and dose-dependent manner. Purified $rFnBPB_{163-480}$ was immobilized onto the surface of a dextran chip and PLG was passed over the chip in concentrations ranging from 0.78 –5 μM (Fig. 31B). The K_D for the interaction was 0.532 ±0.028 μM, an affinity that is nearly 4 fold higher than the interaction between FnBPB and FBG (*Burke F.M. et al., 2010*).



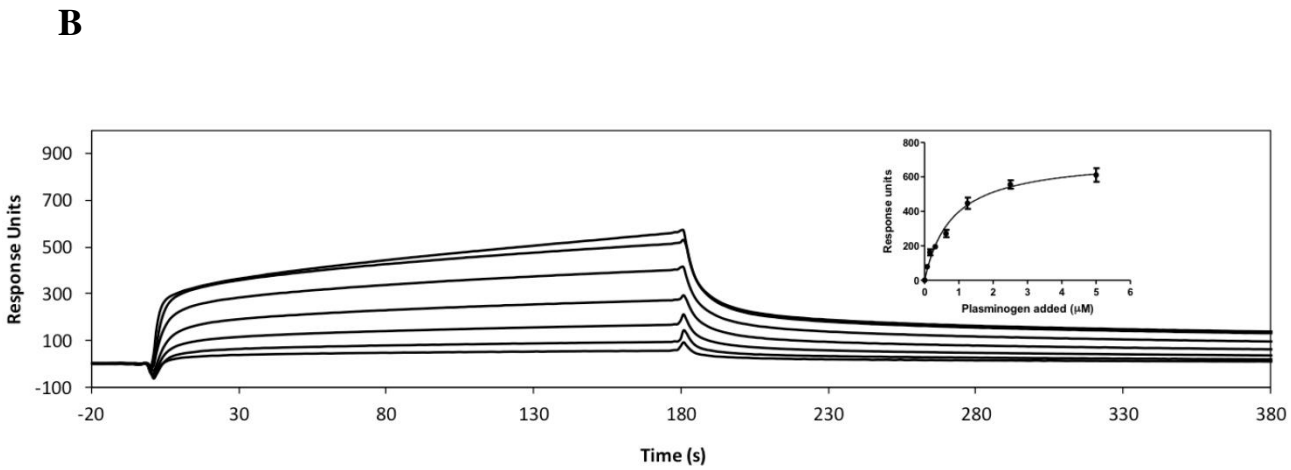
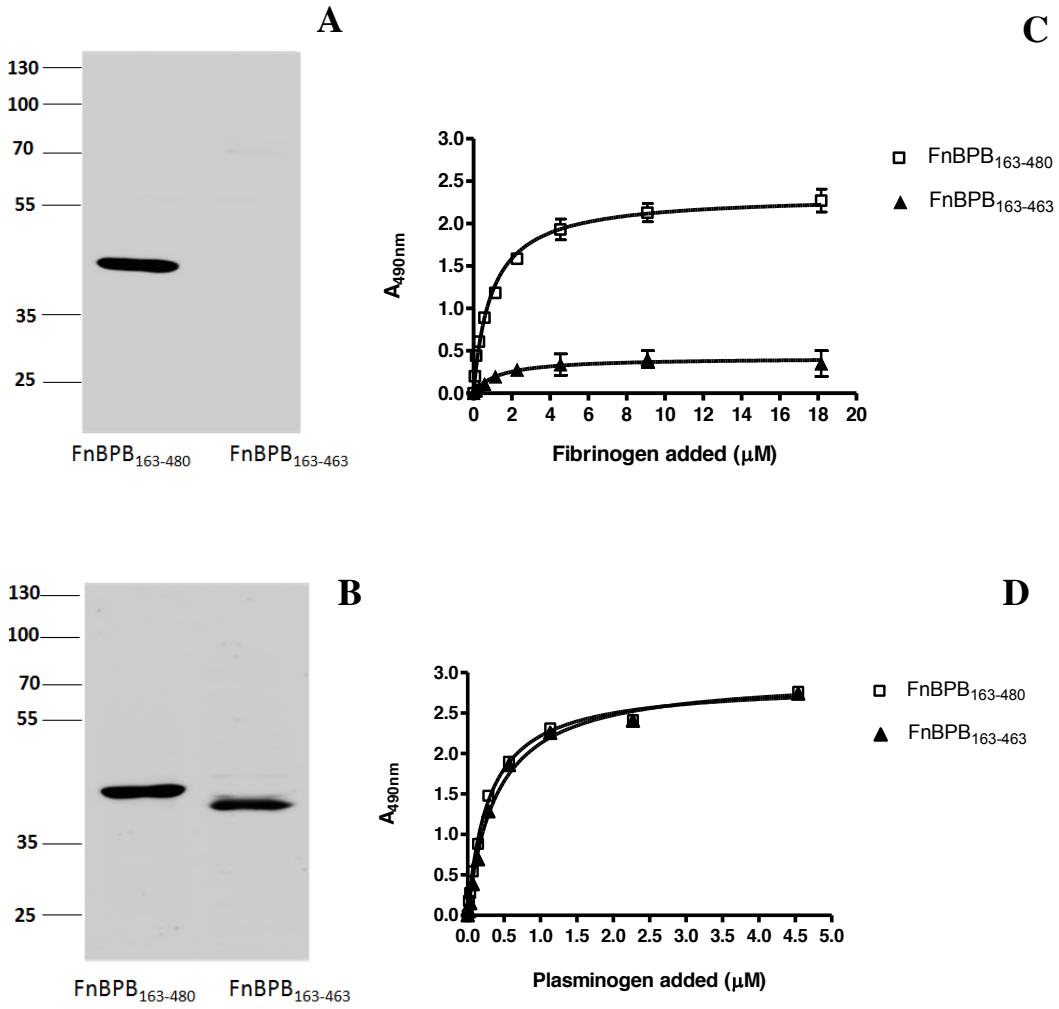


Figure 31. PLG binds FnBPB with high affinity A) ELISA assay of the binding of FnBPB N2N3 subdomain to PLG. The concentrations of PLG added on microtiter wells is given on the X-axis. FnBPB N2N3 binding is indicated on the Y-axis. Values represent the means of triplicate samples ±SD.
B) Surface plasmon resonance analysis of the interaction of $rFnBPB_{163-480}$ with PLG. Representative sensorgrams display binding of PLG to and dissociation from rFnBPB163-480. The affinity was calculated from curve fitting to a plot of the response units RU values against concentrations of PLG (inset). The figure shows one representative of three experiments.

10 Characterization of PLG binding to N2N3 of FnBPB

From literature it is known that the N-terminal A domain of FnBPs binds to fibrinogen by the "dock, lock, and latch" mechanism (*Loughman A. et al., 2008*, *Burke F.M. et al., 2011*) in a similar fashion to the archetypal MSCRAMM ClfA (*Deivanayagam C.C. et al., 2002*). A hydrophobic trench located between subdomains N2 and N3 accepts the C-terminal peptide of the fibrinogen α-chain. A flexible region at the C-terminus of N3 (the locking and latching peptide) undergoes a conformational change that locks the ligand in the trench and forms an additional β-strand in a β-

sheet in subdomain N2. To investigate if the same mechanism is implicated into PLG binding to FnBPB, variants of the recombinant A domain were tested for ligand binding in solid phase ELISA-type assays and through Western blot. To this purpose we used either a truncated version of the FnBPBN2-N3 domain ($FnBPB_{163-480}$), lacking the 17 residues at the C-terminus of subdomain N3 that are involved in the locking and latching steps of the dock, lock, and latch mechanism ($rFnBPB_{163-463}$), and a N2-N3 domain carrying two amino acid substitutions in residues N312 and F314 of the FnBPB fibrinogen binding trench (rFnBPB 163-480N312A/F314A). The recombinant constructs were tested for binding to PLG and, as a control, FBG (Fig. 32). Neither the C-terminal truncated mutant nor the trench one could bind to FBG but each bound to PLG dose-dependently and saturably in a manner that was indistinguishable from $rFnBPB_{163-463}$. These data show that PLG does not bind to the FnBPB A domain using the dock, lock, and latch mechanism.



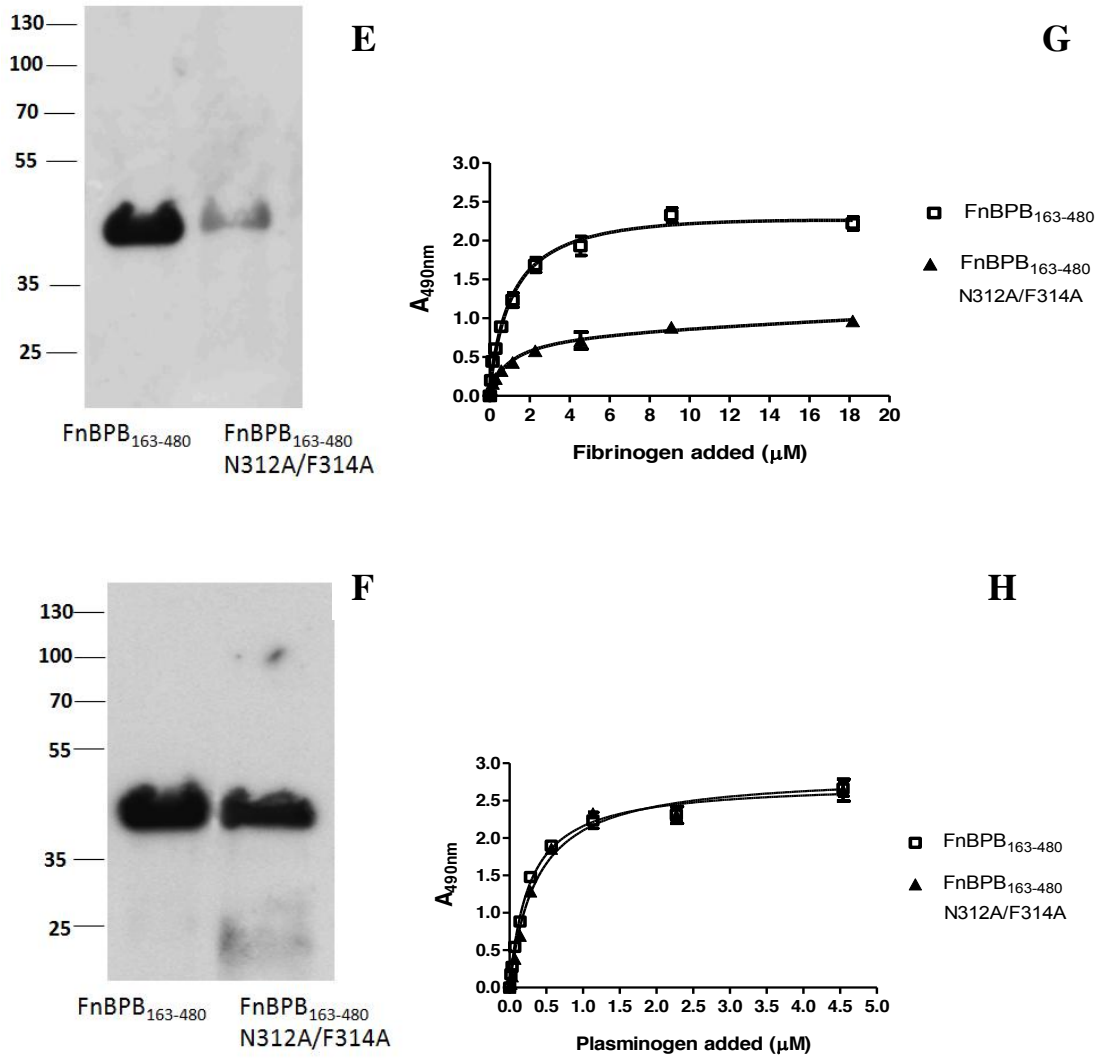


Figure 32. PLG binding to $FnBPB_{163-480}$ and its derivatives. Truncated variants (rFnBPB163-463) or two aminoacid substituted mutants (rFnBPB $_{163-480}$ N312A/F314A) of rFnBPB N2N3 subdomain were immobilized in microtiter plate wells and tested for binding to PLG (D, H) or FBG (C, G), or instead they were blotted onto a nitrocellulose filter and probed with PLG (B, F) or FBG (A, E). The recombinant N2N3 subdomain of FnBPB (rFnBPB163-480) was used as control. The data points are the means (+/- SD) of three independent experiments each performed in triplicate.

11 The N3 module of FnBPB is involved in PLG binding

It has been previously reported that FnBPB N2N3 interacts with immobilized fibrinogen, while single recombinant N2 ($FnBPB_{163-308}$) or N3 ($FnBPB_{309-480}$) subdomains do not (*Burke F.M. et al.*, 2006). To confirm these data in our conditions, FnBPB N2N3 and the single subdomains N2 and N3 were tested for fibrinogen binding both by Western blotting (Fig. 33A) and ELISA assay (Fig. 33B), as reported in Materials and Methods. We observed a saturable and dose-dependent interaction of N2N3 to FBG, while neither recombinant N2, nor N3 subdomains were able to bind.

Starting from these evidences, we tried to localize the PLG-binding site in the N2N3 subdomain of FnBPB. The recombinant N2N3, and the single N2 and N3 subdomains were tested for binding to PLG by using the same assays. As reported in figures 34A and 34B, the N2N3 as well as N3 proteins, maintained the ability to interact with PLG, while no binding was detected when PLG was incubated with N2 subdomain. Moreover, N3 subdomains bound dose dependently to PLG with an affinity constant (K_D) of 0,64 ± 0,015 μM (Fig. 34B).

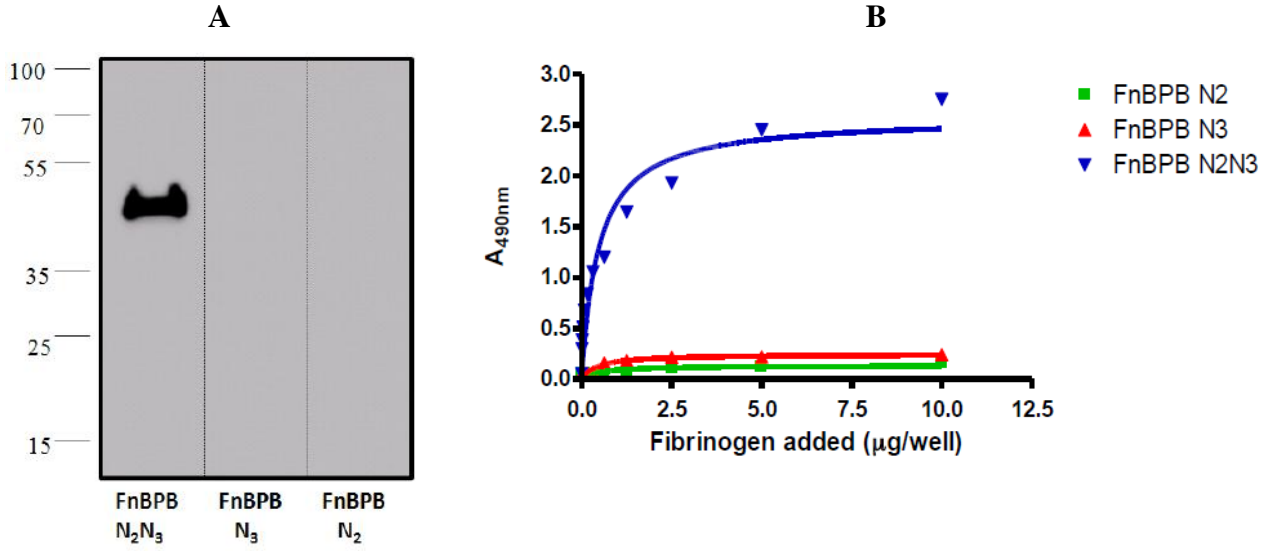


Figure 33. **Binding of N2 and N3 modules to fibrinogen**. A) Recombinant N2-N3 subdomain, N2 (FnBPB163- 308) and N3 (FnBPB309-480) modules were subjected to SDS/PAGE and tested for interaction with fibrinogen by Western Blot. B) ELISA assay measuring the dose-dependent binding of FBG to surface-coated N2N3, and the N2 and N3 modules of FnBPB.

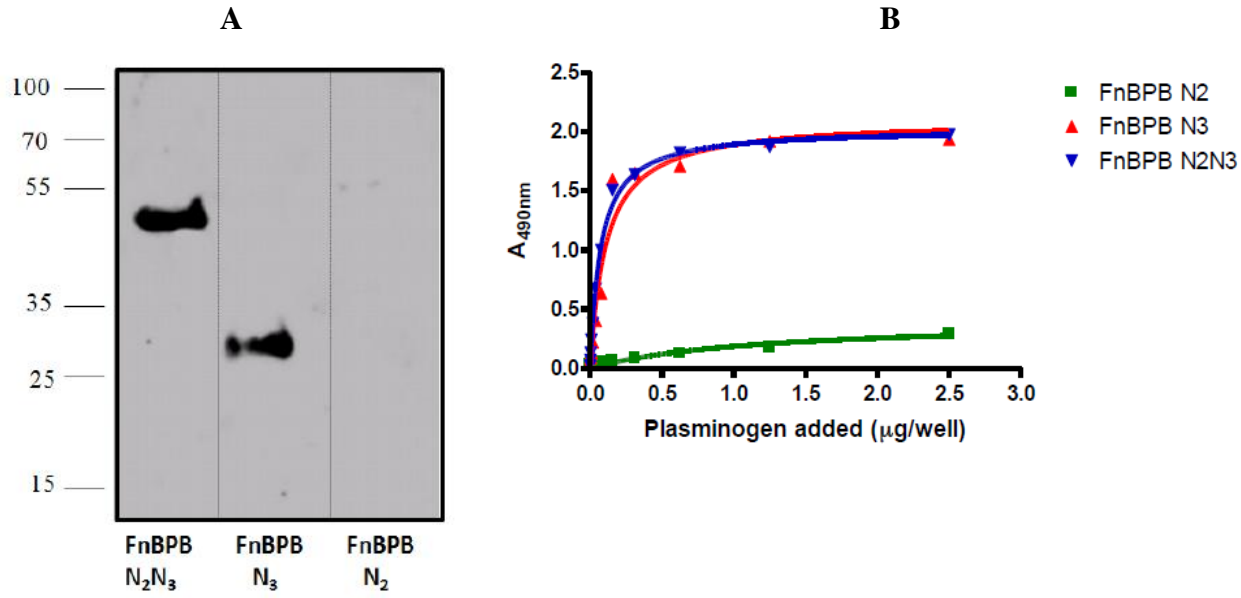


Figure 34. **Binding of N2 and N3 modules to PLG**. A) Recombinant N2N3 subdomain, N2 (FnBPB163-308) and N3 ($FnBPB_{309-480}$) modules were subjected to SDS/PAGE and tested for interaction with PLG by Western Blot. B) ELISA assay measuring the dose-dependent binding of PLG to surface-coated N2N3, and the N2 and N3 modules of FnBPB.

12 Role of lysine residues in PLG binding to FnBPB

The binding of PLG to its natural ligands, such as fibrin and FBG, or lysine-containing host and bacterial binding proteins, is attributed to the strong affinity for lysine rich regions present in those target proteins by the PLG kringle domain(s). To investigate if lysine residues are important in the binding between FnBPB N2N3 and PLG, a plasminogen binding assays were performed with the recombinant bacterial protein subdomain in the presence of increasing concentrations of lysine and the lysine analogue ε-amino caproic acid (EACA).Both molecules caused dose-dependent and complete inhibition of the interaction, with EACA being far more potent (Fig. 35). For further investigating the role of lysine residues in PLG binding, a structural model of the N3 subdomain of FnBPB was designed based on the X-ray crystal structure of the related protein FnBPA. Then amino-acid sequence alignments of isotypes I-VII of FnBPB were used to identify conserved lysine residues in subdomain N3. The conserved lysine residues were visualised using the molecular model and two conserved lysine-rich surface regions were identified. Alanine substitutions were isolated in site 1 (K330, K334, K362) and site 2 (K342, K374 and K423) and then combined to form a double site 1-site 2 mutant (site 3). The N3 variants were tested for their ability to bind to PLG and FBG. Both site 1 and site 2 mutants showed a significant reduction in binding to PLG, a difference that was even greater when the site 1 and site 2 substitutions were combined (site 3). In contrast, FBG binding was not significantly reduced (Fig. 36).

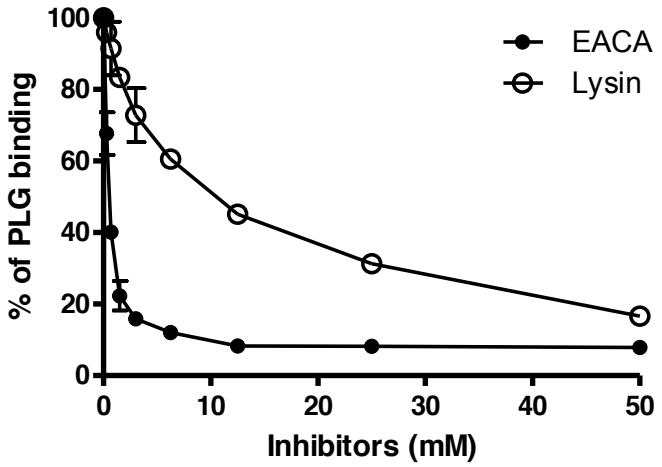


Figure 35. Recombinant FnBPB N2N3 was immobilized onto microtiter plates and incubated with PLG in the presence of increasing concentrations of lysine and the lysine analog ε-amino caproic acid. Binding of PLG by FnBPB in the absence of inhibitor was set to 100%. The concentrations of inhibitors added on microtiter wells is given on the X-axis. The percentage of PLG binding to immobilized FnBPB N2N3 is indicated on the Y-axis. Values represent the means of three separate experiments performed in triplicate, and error bars show SD.

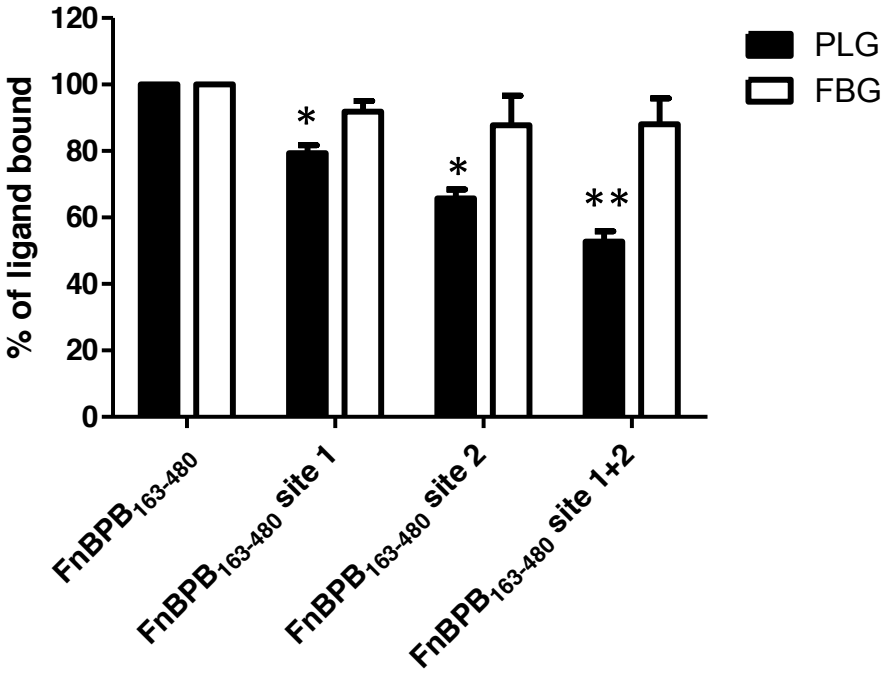


Figure 36. Microtiter plates coated with FnBPB N2N3 and its isoforms mutated at site 1, site 2 or both sites (site 3) were incubated with PLG or FBG. Binding of each ligand to the variants was expressed as percentage of the controls where binding of PLG or FBG to FnBPB N2N3 was set to 100%. The data points are the means +/- SD of three independent experiments each performed in triplicate. Statistically significant differences are indicated (Student's *t* test; *, $P < 0.05$; **, $P < 0.01$).

13 Kringle 4 of PLG binds FnBPB N2N3

To determine which part of the PLG protein binds FnBPB, truncates comprising kringles 1-3 and 1-4, and mini-PLG, which is composed of kringle 5 along with C-terminal residues,were tested for binding to the bacterial protein. Kringles 1-3 and kringle 5 failed to bind FnBPB whereas kringles 1-4 bound similarly to full length PLG. Thus, kringle 4 seems to be the sole binding domain within PLG forFnBPB (Fig.37).

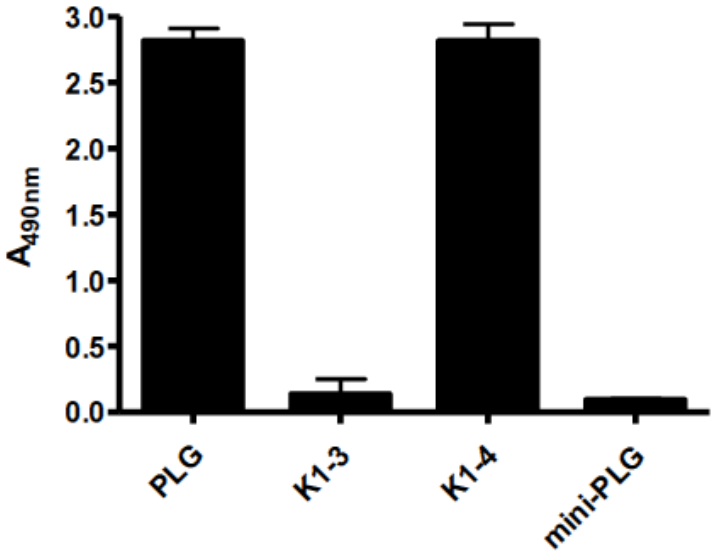


Figure 37. Microtitre plates coated with $FnBPB_{163-480}$ were incubated with equimolar concentrations of PLG or PLG fragments K1-K3, K1-K4 and mini-PLG. PLG and the fragments bound to FnBPB were detected by incubation of the plates with a rabbit PLG antibody followed by goat HRP-conjugated secondary IgG. Three independent experiments were conducted in triplicate and graphs represent means +/- SD.

These datas are published in "Molecular Interactions of Human Plasminogen with Fibronectin-binding Protein B (FnBPB), a Fibrinogen/Fibronectin-binding Protein from Staphylococcus aureus". **Pietrocola G., Nobile G., Gianotti V., Zapotoczna M., Foster T.J., Geoghegan Joan A., Speziale P**., (2016). *J Biol Chem*. **291**(35):18148-62

DISCUSSION

Staphylococcus aureus is the causative agent of potentially harmful diseases, like sepsis, endocarditis and necrotizing pneumonia and it is also responsible for less severe clinical manifestations such as epithelial and mucosal-associated infections. Nowadays, the emergence of methicillin (MRSA) as well as vancomycin-resistant (VRSA) strains is of great concern (*Salazar N. et.al., 2014)*.

S. aureus, like other invasive pathogens such as *Streptococcus pneumoniae (Agarwal, V. et.al., 2013; Bergmann S., et.al 2013)*, *Streptococcus pyogenes* (*Berge, A. & Sjöbring, U. 1993*, Sanderson-Smith, M.L *et.al 2007*) *Streptococcus agalactiae* (*Six, A et.al 2015*, *Wiles K.G et.al 2010*, *Buscetta M. et.al 2014)* can capture PLG from human plasma.

From the literature it is known that several cell surface associated staphylococcal proteins interact with PLG for example the secreted and wall associated protein Sbi (*Koch T.K. et.al 2012*), the manganese transporter MntC *(Salazar N. et.al 2014*), and the moonlighting cytoplasmic protein enolase (*Antikainen, J et.al 2007*) but the molecular aspects of these interactions have not been investigated in detail. However this redundancy provides a number of advantages for *S. aureus*, For example, various PLG-binding proteins may be expressed at different time periods during the infection, another advantage of having multiple PLG binding proteins in a single microorganism could be explained by differences in affinity. While in the blood stream, at PLG concentrations of 200 μg/ml, low affinity binding proteins may be sufficient for recruitment of PLG to the staphylococcal surface, high affinity binding proteins may be required in compartments where the concentration of PLG is low.

Most of the staphylococcal surface proteins with PLG binding activity such as α enolase, Sbi and MntC so far identified are secreted proteins that can rebind to the bacterial surface. Thus we asked whether CWA proteins covalently anchored to the peptidoglycan surface, could potentially express a PLG-binding activity.

Preliminary investigations showed that both clinical isolates and laboratory strains of *S. aureus* interact strongly with human PLG, suggesting that binding of staphylococci to PLG is a general property of this species. Here I have shown for the first time that sortase A-anchored cell wall-associated proteins are the dominant PLG binding proteins on the *S. aureus* cell surface. A sortase A mutant of *S. aureus* USA300 LAC bound at least a 10-fold lower level of PLG than the wild-type. It is noteworthy that PLG captured from human plasma could be activated by exogenously added tissue PLG activator t-PA and SAK. To identify the repertoire of PLG binding CWA proteins

and to determine their relative importance in the capacity of the bacterium to bind PLG, I screened the region A of several recombinant CWA proteins by Western Blot. Two of them, FnBPA and FnBPB, were shown to bind PLG *in vitro,* the binding appeared specific, instead no interaction was observed with other CWA proteins such as ClfA/B, Bbp and Pls. Once verified that PLG doesn't bind the repetitive region of FnBPB (tandemly repeated fibronectin-binding motifs, *Burke F.M. et al., 2010*) the A domain of this staphylococcal protein (FnBPBN2N3, aa 163-480) was chosen for detailed analysis of PLG binding. Surface plasmon resonance analysis of this binding indicates a high affinity interaction in the nanomolar range. This domain can bind to FBG by the dock, lock, and latch mechanism (*Burke F.M et.al 2011*). I have shown that PLG and FBG bind FnBPB at distinct non-overlapping sites and that PLG binding does not involve the dock, lock, and latch mechanism. This is important since PLG and FBG are both components of blood plasma. FnBPB can simultaneously capture both PLG and FBG (a substrate for plasmin). The PLG binding site is confined to subdomain N3, it does not overlap the FBG binding site and variants of the recombinant A domain with substitutions at two distinct conserved lysine-rich sites in subdomain N3 bound PLG with reduced affinity. X ray crystal structure analysis of the FnBPB-PLG complex will provide molecular details of the interaction. The seven distinct isoforms of FnBPB each bound PLG with similar affinities in solid phase assays (data not shown). This attests to the idea that PLG binding is an important property which has been retained during diversification of the N3 subdomain surface residues. Moreover my data indicates that kringle 4 probably comprises the only binding domain within PLG for FnBPB and that this binds the two lysine-rich patches within subdomain N3 of FnBPB. Therefore FnBPB can be considerate as a new receptor of PLG. Structural analysis of streptokinase binding to PLG showed that the bacterial zymogen activator bound in such a way as to avoid the kringle domains. SAK and streptokinase belong to the same protein superfamily even though they share little amino acid sequence similarity *(Parry M.A.A et.al 2000*) so it seems likely that they interact with PLG at the same or a similar site leaving kringle 4 free (*Collen D. & Lijnen H.R. 1994)* to bind to FnBPB. In addition, the PLG-FnBPB interaction does not reduce the ability of PLG to be activated by the activators as SAK or tPA. Indeed they can activate PLG bound on the surface of *S. aureus* USA 300 LAC and use it as a proteolytic reactor with the ability to digest the artificial substrate S2251 and human FBG, suggesting that binding of PLG to the staphylococcal surface does not compromise its biological functionality.

Interestingly, surface coated cells of USA 300 LAC incubated with PLG even in the absence of PLG's activators maintained an important proteolytic activity both on the artificial substrate (S-2251) and on the human FBG, while immobilized LAC *sak* mutant cells, incubated with PLG, did not affect the integrity of substrates, suggesting that endogenous SAK could activate PLG. To

confirm the above finding the direct expression of SAK by *S. aureus* strain USA 300 LAC and LAC *sak* mutant was investigated both in cell surface and in TCA-precipitated culture supernatants. SAK was detected in both fractions only in USA 300 LAC, instead no SAK was presented in *Δsak,* allowing to conclude that *S. aureus* can secrete SAK in the culture environment (*Arvidson S. et al 2001)* and re-capturing it, acquires the ability to activate adsorbed PLG. The presence of active plasmin on the surface of *S. aureus* cells most likely contributes to the pathogenesis of different infections. For example, *S. aureus* promotes clotting of plasma and this ability constitutes a mechanism that support the aggregation, survival and persistence of the bacteria within the fibrin network (*Loof, T.G et al 2015*). Damage to subcutaneous tissue could contribute to the pathogenesis of skin soft tissue infections (SSTIs) caused by CA- MRSA (Wang X., *et al 2013*). Degradation of opsonins could contribute to the avoidance of neutrophil-mediated phagocytosis and killing (*Attali C et al 2008*; *Rooijakkers S.H et al 2005*). Thus, recruitment of plasmin(ogen) to staphylococcal surface may represent an important virulence mechanism leading to effective evasion of proteolytically active bacteria from thrombus within the vasculature of the host and to bacterial dissemination into deeper tissue sites. To investigate this further requires the employment of appropriate animal models, and to test the role of SAK in these processes requires the use of a transgenic mouse expressing human PLG.

In conclusion in my study I have shown that CWA proteins are the dominant bacterial receptors for capturing host PLG by *S. aureus* and I have investigated in detail the mechanism of PLG binding by one such CWA protein, FnBPB. Furthermore I have analyzed the intrinsic staphylococcal capacity to activate PLG in plasmin and provided evidence that it was the surface associated SAK.

BIBLIOGRAPHY

1. **Agarwal, V., Kuchipudi, A., Fulde, M., Riesbeck, K., Bergmann, S,. Blom, A.M**. (2013) *Streptococcus pneumoniae* endopeptidase O (PepO) is a multifunctional plasminogen- and fibronectin-binding protein, facilitating evasion of innate immunity and invasion of host cells. *J. Biol. Chem.* **288,** 6849-6863.
2 **Agerer F., Michel A., Ohlsen K., Hauck C.R.**, (2003). Integrin-mediated invasion of Staphylococcus aureus into human cells requires Src family protein-tyrosine kinases. *J Biol Chem*., **278**(43):42524-31.
3 **Aguilar J., Urday-Cornejo V., Donabedian S., Perri M., Tibbetts R., Zervos M.**, (2010). *Staphylococcus aureus* meningitis: case series and literature review. *Medicine*, **89**(2):117-25.
4 **Antikainen J., Kuparinen V., Lähteenmäki K., Korhonen T.K.** (2007) Enolases from Grampositive bacterial pathogens and commensal lactobacilli share functional similarity in virulence-associated traits. *FEMS Immunol. Med. Microbiol.***51,** 526-534.
5 **Antikainen J., Kuparinen V., Lähteenmäki K., Korhonen T.K.**, (2007). Enolases from Gram-positive bacterial pathogens and commensal lactobacilli share functional similarity in virulence-associated traits. *FEMS Immunol Med Microbiol*. ; **51**(3):526-34.
6 **Arvidson S. & Tegmark K.**, (2001). Regulation of virulence determinants in *Staphylococcus aureus*. *Int. J. Med. Microbiol*., **291**(2):159–170.
7 **Attali C., Durmort C., Vernet T., Di Guilmi A.M.**, (2008). The interaction of *Streptococcus pneumoniae* with plasmin mediates transmigration across endothelial and epithelial monolayers by intercellular junction cleavage. *Infect. Immun*., **76**: 5350-5356.
8 **Berge, A., Sjöbring, U.** (1993) PAM, a novel plasminogen-binding protein from *Streptococcus pyogenes*. *J. Biol. Chem*. **268,** 25417-2524.
9 **Bergmann S., Schoenen H., Hammerschmidt S**. (2013) The interaction between bacterialenolase and plasminogen promotes adherence of *Streptococcus pneumoniae* to epithelial and endothelial cells. *Int. J. Med. Microbiol*. **303,** 452-462.
10 **Bischoff M., Dunman P., Kormanec J., Macapagal D., Murphy E., Mounts W., Berger-Bächi B., Projan S.**, (2004). Microarray-based analysis of the *Staphylococcus aureus* σB regulon. *J. Bacteriol*., **186**:4085– 4099.
11 **Borgia M.B., Borgia A., Best R.B., Steward A., Nettels D., Wunderlich B., Schuler B., Clarke J.**, (2011). Singlemolecule fluorescence reveals sequence-specific misfolding in multidomain proteins. *Nature*, **474**:662–665
12 **Bowden M.G., Chen W., Singvall J., Xu Y., Peacock S.J., Valtulina V., Speziale P., Höök M.**, (2005). Identification and preliminary characterization of Cell-Wall-Anchored proteins of *Staphylococcus epidermidis*. *Microbiology*, **151**(Pt 5):1453-64.
13 **Burke F.M., Di Poto A., Speziale P., Foster T.J.**, (2011). The A domain of fibronectin-binding protein B of *Staphylococcus aureus* contains a novel fibronectin binding site. *FEBS Journal,* **278**:2359–2371.
14 **Burke F.M., McCormack N., Rindi S., Speziale P., Foster T.J.**, (2010). Fibronectin-binding protein B variation in *Staphylococcus aureus*. *BMC Microbiology,* **10**:160.
15 **Burman J.D., Leung E., Atkins K.L., O'Seaghdha M.N., Lango L., Bernadó P., Bagby S., Svergun D.I., Foster T.J., Isenman D.E., van den Elsen J.M.H.**, (2008). Interaction of human complement with Sbi, a Staphylococcal Immunoglobulin-binding protein: indications of a novel mechanism of complement evasion by *Staphylococcus aureus*. *J Biol Chem*., **283**(25):17579–17593.
16 **Buscetta, M., Firon, A., Pietrocola, G., Biondo, C., Mancuso, G., Midiri, A., Romeo, L., Galbo, R., Venza, M., Venza, I., Kaminski, P.A., Gominet, M., Teti, G., Speziale, P., Trieu-Cuot, P., Beninati, C**. (2016) PbsP, a cell wall-anchored protein that binds plasminogen to promote hematogenous dissemination of Group B Streptococcus. *Mol. Microbiol*. doi: 10.1111/mmi.13357.
17 **Cao Y., Ji,Don R.W., Davidson D., Schaller J., Marti D., So¨hndel S., McCance S.G., O'Reilly M.S., Llina M., and Folkman J.**, (1996). Kringle Domains of Human Angiostatin. Characterization of the anti-proliferative activity on endothelial cells.*; J Biol Chem*., 29461–29467.

18 **Cassat J.E., Skaar E.P.**, (2012). Metal ion acquisition in *Staphylococcus aureus*: overcoming nutritional immunity. *Semin Immunopathol*., **34**(2):215-35.

19 **Castellino F.J., Ploplis V.A.**, (2005). Structure and function of the plasminogen/plasmin system. *Thromb Haemost 93: 647-54*

20 **Cedergren L., Andersson R., Jansson B., Uhlen M., Nilsson B.**, (1993). Mutational analysis of the interaction between staphylococcal protein A and human IgG1. *Protein Eng*., **6**:441–448.

21 **Cesarman-Maus G. and Hajjar C.A.,** (2005). Molecular mechanism of fibrinolysis. British Journal of Haematology., 129, 307–321. Review

22 **Cheung A.L., Koomey J.M., Butler C.A., Pojan S.J., Fischetti V.A.**, (1992). Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. *Proc*. *Natl*. *Acad*. *Sci*. *USA*, **89**:6462-6466.

23 **Chevilley A., Lesept F., Lenoir S., Ali C., Jéróme P., Vivien D.,** (2015). Impacts of tissue-type plasminogen activator (tPA) on neuronal survival. *Front*. *Cell*. *Neurosci,* 10.3389/Fncel.2015.00415.

24 **Clauditz A., Resch A., Wieland K.P., Peschel A., Götz F.**, (2006). Staphyloxantin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxydative stress. *Infection and immunity*, **74**(8):4950- 3.

25 **Cleaveland M.G., Gorham J.D., Murphy T.L., Tuomanen E., Murphy K.M.**, (1996). Lipoteichoic acid preparations of Gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. *Infect*. *Immun*., **59**:4614-4620.

26 **Collen D. and Lijnen H.R.,** (1994). Staphylokinase, a Fibrin-Specific Plasminogen Activator With Therapeutic Potential?. *Blood*, 84(3):680-6.

27 **Conrady D.G., Wilson J.J., Herr A.B.**, (2013). Structural basis for Zn2+-dependent intercellular adhesion in staphylococcal biofilms. *Proc*. *Natl Acad*. *Sci*. *USA*, **110**:E202–E211.

28 **Corrah T.W., Enoch D.A., Aliyu S.H., Lever A.M.**, (2011). Bacteraemia and subsequent vertebral osteomyelitis: a retrospective review of 125 patients. *QJM*., **104**(3):201-7.

29 **Cucarella C., Solano C., Valle J., Amorena B., Lasa I., Penadès J.R.**, (2001). Bap, a *Staphylococcus aureus* surface proteins involved in biofilm formation. *J*. *Bacteriol*., **183**(9):2888.

30 **Daum R.S., Spellberg B.,** (2012). Progress toward a Staphylococcus aureus vaccine. *Clin Infect Dis*., **54**(4):560-7.

31 **De Kimpe S.J., Kengatharan M., Thiemermann C., Vane J.R.** (1995). The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure. *Proc Natl Acad Sci U S A*., **92**(22):10359-63..

32 **DeDent A., Bae T., Missiakas D.M., Schneewind O.,** (2008). Signal peptides direct surface proteins to two distinct envelope locations of *Staphylococcus aureus*. *EMBO J*. **27**(20):2656-68

33 **Deisenhofer J.**, (1981). Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-Å resolution. *Biochemistry*, **20**:2361–2370.

34 **Deivanayagam C.C., Wann E.R., Chen W., Carson M., Rajashankar K.R., Höök M., Narayana S.V.**, (2002). A novel variant of the immunoglobulin fold in surface adhesins of *Staphylococcus aureus*: crystal structure of the fibrinogen-binding MSCRAMM, clumping factor A. *EMBO J*., **21**:6660–6672.

35 **DeLeo F.R., Chambers H.F.**, (2009). Reemergence of antibiotic-resistant *Staphylococcus aureus* in the genomics era. *J*. *Clin*. *Invest*., **119**(9):2464-74.

36 **Donlan R.M., and Costerton J.W**., (2002). Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. Clin Microbiol Rev, 15(2):167-93.

37 **Dunkelberger J.R., Song W.C.**, (2010). Complement and its role in innate and adaptive immune responses. *Cell Res*., **20**(1):34-50.

38 **Ebner P., Prax M., Nega M., Koch I., Dube L., Yu W., Rinker J., Popella P., Flötenmeyer M., Götz F.**, (2015). Excretion of cytoplasmic proteins (ECP) in*Staphylococcus aureus*. *Mol*. *Microbiol*., **96** (4) : 775-789.

39 **Emma J.S., Corrigan R.M., van der Sluis T., Gründling A., Speziale P., Geoghegan J.A., Foster T.J.**, (2012). The immune evasion protein Sbi of *Staphylococcus aureus* occurs both extracellularly and anchored to the cell envelope by binding lipoteichoic acid**.** *Mol Microbiol*. **83**(4): 789–804

40 **Fluit A.C., Schmitz F.J., Verhoef J.**, (2001). Frequency of isolation of pathogens from bloodstream, nosocomial pneumonia, skin and soft tissue and urinary tract infections occurring in European patients. *Eur*. *J*. *Clin*. *Microbiol*. *Infect*. *Dis*., **20**:188-191. 78

41 **Foster T.J., Geoghegan J.A., Ganesh V.K., Höök M.**, (2014). Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. *Nat*. *Rev*. *Microbiol*., **12**(1):49-62.

42 **Fowler T., Johansson S., Wary K.K., Höök M.**, (2003). Src kinase has a central role in in vitro cellular internalization of *Staphylococcus aureus*. *Cell Microbiol*., **5**(6):417-26.

43 **Fowler T., Wann E.R., Joh D., Johansson S., Foster T.J., Höök M.**, (2000). Cellular invasion by *Staphylococcus aureus* involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. *Eur J Cell Biol*., **79**(10):672-9.

44 **Furuya H., Ikeda R.**, (2011). Interaction of triosephosphate isomerase from *Staphylococcus aureus* with plasminogen. *Microbiol Immunol*., **55**(12):855-62.

45 **Ganesh V.K., Barbu E.M., Deivanayagam C.C., Le B., Anderson A.S., Matsuka Y.V., Lin S.L., Foster T.J., Narayana S.V., Höök M.**, (2011). Structural and biochemical characterization of *Staphylococcus aureus* clumping factor B/ligand interactions. *J*. *Biol*. *Chem*., **286**:25963–25972.

46 **Gebbink M.F.**, (2011). Tissue-type plasminogen activator-mediated plasminogen activation and contact activation, implications in and beyond haemostasis. *J Thromb Haemost*, 9 Suppl 1:174-81.

47 **Geoghegan, J.A. Corrigan R.M., Gruszka D.T., Speziale P., O'Gara J.P., Potts J.R., Foster T.J.**, (2010). Role of surface protein SasG in biofilm formation by *Staphylococcus aureus*. *J*. *Bacteriol*., **192**, 5663–5673.

48 **González-Miguel J., Siles-Lucas M., Kartashev V., Morchón R., Simón F.,** (2016). Plasmin in Parasitic Chronic Infections: Friend or Foe? *Trends Parasitol*., **32**(4):325-35, *Review*.

49 **Gouri A., Dekaken A., El Bairi K., Aissaoui A., Laabed N., Chefrour M., Ciccolini J., Milano G., and Benharkat S.,** (2016). Plasminogen Activator System and Breast Cancer: Potential Role in Therapy Decision Making and Precision Medicine. *Biomark Insights*., 11: 105–111.

50 **Greene C., McDevitt D., Francois P., Vaudaux P. E., Lew D.P., Foster T.J.**, (1995). Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of fnb genes. *Mol*. *Microbiol*., **17**:1143–1152.

51 **Gross M., Cramton S.E., Götz F., Peschel A.**, (2001). Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. *Infect*. *Immun*., **69**(5):3423.

52 **Gruszka D.T., Wojdyla J.A., Bingham R.J., Turkenburg J.P., Manfield I.W., Steward A., Leech A.P., Geoghegan J.A., Foster T.J., Clarke J., Potts J.R.**, (2012). Staphylococcal biofilm-forming protein has a contiguous rod-like structure. *Proc*. *Natl Acad*. *Sci*. *USA*, **109**:E1011–E1018.

53 **Guiducci C.**, Surface Plasmon Resonance Based Systems. *Advanced Bioengineering Methods Laboratory SPR*.

54 **Hair P.S., Ward M.D., Semmes O.J., Foster T.J., Cunnion K.M.**, (2008). *Staphylococcus aureus* clumping factor A binds to complement regulator factor I and increases factor I cleavage of C3b. *J*. *Infect*. *Dis*., **198**:125– 133.

55 **Hamill R.J., Vann J.M., Proctor R.A.**, (1986). Phagocytosis of *Staphylococcus aureus* by cultured bovine aortic endothelial cells: model for postadherence events in endovascular infections. *Infect*. *Immun*., **54**:833-836.

56 **Hammer N.D, Skaar E.P.**, (2011). Molecular mechanisms of *Staphylococcus aureus* iron acquisition. *Annu*. *Rev*. *Microbiol*., **65**:129–147.

57 **Hartman B., Tomasz A**., (1981). Altered penicillin-binding proteins in methicillin- resistant strains of *Staphylococcus aureus*. *Antimicrob Agents Chemother*., 19:726-35.

58 **Heilbronner S., Holden M.T., van Tonder A., Geoghegan J.A., Foster T.J., Parkhill J., Bentley S.D.**, (2011). Genome sequence of *Staphylococcus lugdunensis* N920143 allows identification of putative colonization and virulence factors. *FEMS Microbiol*. *Lett*., **322**:60–67.

59 **Heumann D., Barras C., Severin A., M.P. Glauser, Tomasz A.**, (1994). Gram-positive cell walls stimulate synthesis of tumor necrosis fator alpha and interleukin-6 by human monocytes. *Infect*. *Immun*., **62**:2715- 2721.

60 **Hiramatsu K.**, (2001). Vancomycin-resistant *Staphylococcus aureus*: a new model of antibiotic resistance. *Lancet Infect*. *Dis*., **1**(3):147-55.

61 **Janeway C.A., Jr, Paul Travers, Walport M., and Shlomchik M.J**., *Immunobiology, the Immune System in Health and Disease*. (2001) 6th edition.

62 **Jevon M., Guo C., Ma B., Mordan N., Nair S.P., Harris M., Henderson B., Bentley G., Meghji S.**, (1999). Mechanisms of internalization of *Staphylococcus aureus* by cultured human osteoblasts. *Infect Immun*., **67**:2677-2681.

63 **Jönsson K., Signäs C., Müller H.P., Lindberg M.,** (1991). Two different genes encode fibronectin binding proteins in *Staphylococcus aureus*. The complete nucleotide sequence and characterization of the second gene. *Eur J Biochem*., **202**(3):1041-8.

64 **Karchmer A.W., Gibbons G.W.**, (1994). Infection of prosthetic heart valves and vascular grafts. Bisno A.L., Waldovogel F.A. Ed.; American Society for Microbiology, Washington: pp. 213-49.

65 **Keane F.M., Loughman A., Valtulina V., Brennan M., Speziale P., Foster T.J.,** (2007). Fibrinogen and elastin bind to the same region within the A domain of fibronectin binding protein A, an MSCRAMM of *Staphylococcus aureus*. Mol Microbiol, **63**(3):711-723.

66 **Keane F.M., Loughman A., Valtulina V., Brennan M., Speziale P., Foster T.J.**, (2007). Fibrinogen and elastin bind to the same region within the A domain of fibronectin binding protein A, an MSCRAMM of *Staphylococcus aureus*. *Mol*. *Microbiol*., **63**:711–723.

67 **Kengatharan K.M., De Kimpe S., Robson C., Foster S.J., Thiemermann C.**, (1998). Mechanism of Gram-positive shock: identification of peptidoglycan and lipoteichoic acid moieties essential in the induction of nitric oxyde synthase, shock and multiple organ failure. *J*. *Exp*. *Med*. **188**:305-315.

68 **Kintarak S., Whawell S.A., Speight P.M., Packer S., Nair S.P.**, (2004). Internalization of *Staphylococcus aureus* by human keratinocytes. *Infect*. *Immun*., **72**:5668-5675.

69 **Kluytmans J., van Belkum A., Verbrugh H.**, (1997). Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms and associated risks. *Clin*. *Microbiol*. *Rev*., **10**:505-520.

70 **Knox K.W., Wicken A.J.**, (1973) . Immunological properties of teichoic acids. *Bacteriol Rev*., **37**(2):215-57.

71 **Ko YP, Flick MJ.**, (2016) Fibrinogen is at the interface of host defense and pathogen virulence in *Staphylococcus aureus* infection. *Semin Thromb Hemost*., **42**(4):408-21.

72 **Koch T.K., Reuter M., Barthel D., Böhm S., van den Elsen J., Kraiczy P., Zipfel P.F., Skerka C.**, (2012). *Staphylococcus aureus* proteins Sbi and Efb recruit human plasmin to degrade complement C3 and C3b. *PLoS One*, **7**(10):e47638.

73 **Kreis T., Vale R.**, (1999). Guidebook to the Extracellular Matrix, Anchor, and Adhesion Proteins. (2nd edition) Oxford University Press, Oxford.

74 **Kuroda M., Ohta T., Uchiyama I., Baba T., Yuzawa H., Kobayashi I., Cui L., Oguchi A., Aoki K., Nagai Y., Lian J., Ito T., Kanamori M., Matsumaru H., Maruyama A., Murakami H., Hosoyama A., Mizutani-UiY., Takahashi N.K., Sawano T., Inoue R., Kaito C., Sekimizu K., Hirakawa H., Kuhara S., Goto S.,Yabuzaki J., Kanehisa M., Yamashita A., Oshima K., Furuya K., Yoshino C., Shiba T., Hattori M.,Ogasawara N., Hayashi H., Hiramatsu K.**, (2001). Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet*, **357**:1225–40.

75 **Kuypers J.M., Proctor R.A.**, (1989). Reduced adherence to traumatized rat heart valves by a low fibronectinbinding mutant of *Staphylococcus aureus*. *Infect*. *Immun*., **57**:2306–2312.

76 **Kwon M., MacLeod T.J., Zhang Y and Morton Waisman D.** (2005). S100A10, annexin A2, and annexin A2 heterotetramer as candidate plasminogen receptors. *Frontiers in Bioscience*, **10**, 300-325.

77 **Kwon M., MacLeod T.J., Zhang Y., Waisman D.M.,** (2005). S100A10, annexin A2, and annexin a2 heterotetramer as **candidate** plasminogen receptors. *Front Biosci*., **10**:300-25.

78 **Lähteenmäki K., Kuusela P., Korhonen T.K.,** (2001). Bacterial plasminogen activators and receptors. *FEMS Microbiol Elsevier*., **25**(5):531-52, *Review*.

79 **Laursen N.S., Gordon N., Hermans S., Lorenz N., Jackson N., Wines B., Spillner E., Christensen J.B., Jensen M., Fredslund F., Bjerre M., Sottrup-Jensen L., Fraser J.D., Andersen G.R.**, (2010). Structural basis for inhibition of complement C5 by the SSL7 protein from *Staphylococcus aureus*. *Proc Natl Acad Sci U S A*, **107**(8):3681-6.

80 **Law R.H., Caradoc-Davies T., Cowieson N., Horvath A.J., Quek A.J., Encarnacao J.A., Steer D., Cowan A., Zhang Q., Lu B.G., Pike R.N., Smith A.I., Coughlin P.B., Whisstock J.C.**, (2012). The X-ray crystal structure of full-length human plasminogen. *Cell Rep*., **1**(3):185-90.

81 **Liu C., Graber C.J., Karr M, Diep B.A., Basuino L., Schwartz B.S., Enright M.C., O'Hanlon S.J., Thomas J.C., Perdreau-Remington F., Gordon S., Gunthorpe H., Jacobs R., Jensen P., Leoung G., Rumack J.S., Chambers H.F.**, (2008) A population-based study of the incidence and molecular epidemiology of methicillin- resistant *Staphylococcus aureus* disease in San Francisco, 2004-2005. *Clin Infect Dis*., **46**(11):1637-46.

82 **Liu G.Y., Essex A., Buchanan J.T., Datta V., Hoffmann H.M., Bastian J.F., Fierer J., Nizet V.**, (2005). *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxydant activity. *J Exp Med*., **202**(2)209-15.

83 **Loof, T.G., Goldmann, O., Naudin, C., Mörgelin, M., Neumann, Y., Pils, M.C., Foster, S.J., Medina, E., Herwald, H.** (2015) *Staphylococcus aureus*-induced clotting of plasma is an immune evasion mechanism for persistence within the fibrin network. *Microbiology* **161,**621-627.

84 **Loughman A., Sweeney T., Keane F.M., Pietrocola G., Speziale P., Foster T.J.,** (2008). Sequence diversity in the A domain of Staphylococcus aureus fibronectin-binding protein A. *BMC Microbiol*, **8**:74.

85 **Mack D., Haeder M., Siemssen N., Laufs R.**, (1996). Association of biofilm production of coagulase-negative staphylococci with expression of a specific polysaccharide intercellular adhesin. *J Infect Dis*., **174**(4):881-4.

86 **Madigan M.T., Martinko J.**, (2006). *Brock Biology of Microorganisms* (11th edition). Prentice Hall, Upper Saddle River, NJ, USA.

87 **Majcherczyk P.A., Rubli E., Heumann D., Glauser M.P., Moreillon P.**, (2003). Teichoic acids are not required for *Streptococcus pneumoniae* and *Staphylococcus aureus* cell walls to trigger the release of tumor necrosis factor by peripheral blood monocytes. *Infect*. *Immun*., **71**:3707-3713.

88 **Maresso AW., Schneewind O.,** (2008). Sortase as a target of anti-infective therapy. *Pharmacol Rev*.*;* **60**(1):128-41.

89 **Marraffini LA., Dedent AC, Schneewind O.,** (2006). Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. *Microbiol Mol Biol Rev*. **70**(1):192-221.

90 **Mattsonn E., Verhage L., Rollof J., Fleer A., Verhoef J., van Dijk H.**, (1993). Peptidoglycan and teichoic acid from Staphylococcus epidermidis stimulate human monocytes to release tumor necrosis factor-alpha, interleukin-1 beta and interleukin-6. *FEMS Immunol*. *Med*. *Microbiol*., **7**:281-287.

91 **Mazmanian S.K., Skaar E.P., Gaspar A.H., Humayun M., Gornicki P., Jelenska J., Joachmiak A., Missiakas D.M., Schneewind O.**, (2003). Passage of heme–iron across the envelope of *Staphylococcus aureus*. *Science,* **299**:906–909.

92 **McAleese F.M., Walsh E.J., Sieprawska M., Potempa J., Foster T.J.**, (2001). Loss of clumping factor B fibrinogen binding activity by *Staphylococcus aureus* involves cessation of transcription, shedding and cleavage by metalloprotease. *J*. *Biol*. *Chem*., **276**:29969–29978.

93 **McDevitt D., Francois P., Vaudaux P., Foster T.J.,** (1995). Identification of the ligand-binding domain of the surface-located fibrinogen receptor (clumping factor) of *Staphylococcus aureus*. *Mol Microbiol*, , **16**(5):895-907.

94 **Moreillon P., Que A.Y.**, (2004). Infective endocarditis. *Lancet*, **363**:139-49.

95 **Morfeldt E., Janzon L., Arvidson S., Lofdhal S.**, (1988). Cloning of a chromosomal locus (*exp*) which regulates the expression of several exoprotein genes in *Staphylococcus aureus*. *Mol*. *Gen*. *Genet*., **211**:435-440.

96 **Mulcahy M.E., Geoghegan J.A., Monk I.R., O'Keeffe K.M., Walsh E.J., Foster T.J., McLoughlin R.M.**, (2012). Nasal colonisation by *Staphylococcus aureus* depends upon clumping factor B binding to the squamous epithelial cell envelope protein loricrin. *PLoS Pathog*., **8**: e1003092.

97 **Murray P.R., Rosenthal K.S., Pfaller M.A.**, (2009). Medical Microbiology (6th edition). Mosby Inc., Philadelphia, PA, USA.

98 **Nguyen L.T. and Vogel H.J.,** (2016). Staphylokinase has distinct modes of interaction with antimicrobial peptides, modulating its plasminogen-activation properties., *Sci Rep*, 10.1038/srep31817.

99 **Novick R.P., Ross H.F., Projan S.J., Kornblum J., Kreiswirth B., Moghazeh S.**, (1993). Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J*., **12**:3967-3975.

100 **O'Brien L.M., Walsh E.J., Massey R.C., Peacock S.J., Foster T.J.**, (2002). *Staphylococcus aureus* clumping factor B (ClfB) promotes adherence to human type I cytokeratin 10: implications for nasal colonization. *Cell*. *Microbiol*., **4**:759–770.

101 **O'Neill E., Pozzi C., Huston P., Humphreys H., Robinson D.A., Loughman A., Foster T.J., O'Gara J.P.**, (2008). A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. *J*. *Bacteriol*., **190**(11):3835.

102 **Ogawa S.K., Yurberg E.R., Hatcher V.B., Levitt M.A., Lowy F.D.**, (1985). Bacterial adherence to human endothelial cells *in vitro*. *Infect Immun*., **50**(1):218-24.

103 **Parry M.A.A., Zhang X.C., Bode, I.**, (2000) Molecular mechanisms of plasminogen activation: bacterial cofactors provide clues. *Trends Biochem*. *Sci*. **25**, 53-59.

104 **Patel A.H., Nowlan P., Weavers E.D., Foster T.J.**, (1987). Virulence of protein A-deficient and α-toxin deficient mutants of *Staphylococcus aureus* isolated by allele replacement. *Infect*. *Immun*., **55**:3103–3110.

105 **Patti J.M., Bremell T., Krajewska-Pietrasik D., Abdelnour A., Tarkowski A., Rydén C., Höök M.**, (1994). The *Staphylococcus aureus* collagen adhesin is a virulence determinant in experimental septic arthritis. *Infect*. *Immun*., **62**:152–161.

106 **Peetermans M., Vanassche T., Liesenborghs L., Lijnen R.H., Verhamme P.**, (2015). Bacterial pathogens activate plasminogen to breach tissue barriers and escape from innate immunity. *Crit*. *Rev*. *Microbiol*., **20**:1-17.

107 **Peng H.L., Novick R.P., Kreiswirth B., Kornblum J., Schlievert P.**, (1988). Cloning, characterization and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *J*. *Bacteriol*., **170**:4365-4372.

108 **Pietrocola G., Nobile G., Gianotti V., Zapotoczna M., Foster T.J., Geoghegan Joan A., Speziale P**., (2016). Molecular Interactions of Human Plasminogen with Fibronectin-binding Protein B (FnBPB), a Fibrinogen/Fibronectin-binding Protein from *Staphylococcus aureus*. *J Biol Chem* **. 291**(35):18148-62.

109 **Ponnuraj K., Bowden M.G., Davis S., Gurusiddappa S., Moore D., Choe D., Xu Y., Hook M., Narayana S.V.**, (2003). A "dock, lock, and latch" structural model for a staphylococcal adhesin binding to fibrinogen. *Cell*, **115**:217–228.

110 **Postma B., Poppelier M.J., van Galen J.C., Prossnitz E.R., van Strijp J.A., de Haas C.J., van Kessel K.P.,** (2004). Chemotaxis inhibitory protein of *Staphylococcus aureus* binds specifically to the C5a and formylated peptide receptor. *J Immunol*. **172**(11):6994-7001.

111 **Projan S.J., Novick R.P.**, (1997). The molecular basis of pathogenicity,. *In* K.B. Crossley and G.L. Archer (ed.), *The staphylococci in human disease*. *Churchill Livingstone*, New-York, N.Y.

112 **Qin Z., Ou Y., Yang L., Zhu Y., Tolker-Nielsen T., Molin S., Qu D.**, (2007). Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. *Microbiology*, **157**(7):2083.

113 **Que Y.A., Haefliger J.A., Piroth L., François P., Widmer E., Entenza J.M., Sinha B., Herrmann M., Francioli P., Vaudaux P., Moreillon P.**, (2005). Fibrinogen and fibronectin binding cooperate for valve infection and invasion in *Staphylococcus aureus* experimental endocarditis. *J*. *Exp*. *Med*., **201**:1627–1635

114 **Roche F.M., Downer R., Keane F., Speziale P., Park P.W., Foster T.J.,** (2004).The N-terminal A domain of fibronectin-binding proteins A and B promotes adhesion of *Staphylococcus aureus* to elastin. *J Biol Chem*., **279**(37):38433-38440.

115 **Roche FM., Massey R., Peacock S.J., Day N.P., Visai L., Speziale P., Lam A., Pallen M., Foster T.J.,** (2003). Characterization of novel LPXTG-containing proteins of *Staphylococcus aureus* identified from genome sequences. *Microbiology*.; **149**(Pt 3):643-5**4**

116 **Rohde H., Burdelski C., Bartscht K., Hussain M., Buck F., Horstkotte M.A., Knobloch J.K., Heilmann C., Herrmann M., Mack D.**, (2005). Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. *Mol*. *Microbiol*., **55**:1883–1895.

117 **Rooijakkers S.H., Milder F.J., Bardoel B.W., Ruyken M., van Strijp J.A., Gros P.**, (2007). Staphylococcal complement inhibitor: structure and active sites. *J Immunol*., **179**(5):2989-98.

118 **Rooijakkers S.H., van Wamel W.J., Ruyken M., van Kessel K.P., van Strijp J.A.**, (2005). Anti-opsonic properties of staphylokinase. *Microbes Infect*., **7**: 476-484.

119 **Ruhland G.J., Fiedler F.**, (1990). Occurrence and structure of lipoteichoic acids in the genus *Staphylococcus*. *Arch*. *Microbiol*., **154**:375-379.

120 **Ryan K.J., Ray C.G.**, (2004). *Sherris Medical Microbiology* (4th edition). The McGraw-Hill Companies, Inc.

121 **Salazar N, Castiblanco-Valencia M.M., da Silva L.B., de Castro Í., Monaris D., Masuda H.P., Barbosa A.S., Arêas A.P.**, (2014). *Staphylococcus aureus* manganese transport protein C (MntC) is an extracellular matrix- and plasminogen-binding protein. *PLoS One*, **9**(11):e112730

122 **Sambrook J., Fritsch E. F., Maniatis T.**, (1989). Molecular Cloning: A Laboratory Manual, 2nd edn. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor*, New York.

123 **Sanderson-Smith M, Batzloff M, Sriprakash KS, Dowton M, Ranson M, Walker MJ**. (2006). Divergence in the plasminogen-binding group a streptococcal M protein family: functional conservation of binding site and potential role for immune selection of variants. J Biol Chem, **281**(6):3217-26.

124 **Sanderson-Smith, M.L., Dowton, M., Ranson, M., Walker, M.J.** (2007) The plasminogenbinding group A streptococcal M protein-related protein Prp binds plasminogen via arginine and histidine residues. *J. Bacteriol*. **189,** 1435-1440.

125 **Sarma J.V. & Ward P.A.,** (2011). The Complement System. *Cell Tissue Res*., **243**(1):227-235.

126 **Schwarz-Linek U., Werner J.M., Pickford A.R., Gurusiddappa S, Kim J.H., Pilka E.S., Briggs J.A., Gough T.S., Höök M, Campbell I.D., Potts J.R.**, (2003). Pathogenic bacteria attach to human fibronectin through a tandem β-zipper. *Nature*, **423**:177–181

127 **Shirtliff M.E., Mader J.T., Camper A.K.**, (2002). Molecular interactions in biofilms. *Chem. Biol*., **9**:859-871.

128 **Shittu A.O., Udo E.E., Johnson L.**, (2007). Insights on Virulence and Antibiotic Resistance: A Review of the Accessory Genome of *Staphylococcus aureus*. *Wounds*, **19**(9):237-244.

129 **Signas C., Raucci G., Jonsson K., Lindgren P.E., Anantharamaiah G.M., Hook M., Lindberg M.,** (1989). Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus*: use of this peptide sequence in the synthesis of biologically active peptides. *Proc Natl Acad Sci USA*., **86**(2):699-703.

130 **Sinha B., Francois P., Que Y.A., Hussain M., Heilmann C., Moreillon P., Lew D., Krause K.H., Peters G., Herrmann M.**, (2000). Heterologously expressed *Staphylococcus aureus* fibronectin-binding proteins are sufficient for invasion of host cells. *Infect. Immun*., **68**:6871–6878.

131 **Sinha B., François P.P., Nüsse O., Foti T., Hartford O.M., Vaudaux P., Foster T.J., Lew D.P., Herrmann M., Krause K.H.**, (1999). Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin alpha5beta1. *Cell Microbiol*., **1**(2):101-17.

132 **Six, A., Bellais, S., Bouaboud, A., Fouet, A., Gabriel, C., Tazi, A., Dramsi, S., Trieu-Cuot, P., Poyart, C**. (2015) Srr2, a multifaceted adhesin expressed by ST-17 hypervirulent Group B Streptococcus involved in binding to both fibrinogen and plasminogen. *Mol. Microbiol*. **97,** 1209-1222. doi: 10.1111/mmi.13097

133 **Smith E.J., Corrigan R.M., van der Sluis T., Gründling A., Speziale P., Geoghegan J.A., Foster T.J.** (2012). The immune evasion protein Sbi of *Staphylococcus aureus* occurs both extracellularly and anchored to the cell envelope by binding lipoteichoic acid. *Mol. Microbiol*., **83**:789–804.

134 **Speziale P., Pietrocola G., Foster T.J., Geoghegan J.A**. (2014). Protein-based biofilm matrices in Staphylococci. Front Cell Infect Microbiol, 10.3389/fcimb.2014.00171.

135 **Speziale P., Pietrocola G., Rindi S., Provenzano M., Provenza G., Di Poto A., Visai L., Arciola C.R.**, (2009). Structural and functional role of *Staphylococcus aureus* surface components recognizing adhesive matrix molecules of the host. *Future Microbiol*., **4**(10):1337-1352.

136 **Stewart P.S., Costerton J.W.**, (2001). Antibiotic resistance of bacteria in biofilms. *Lancet*, **358**(9276):135.

137 **Timmermann C.P., Mattsonn E., Martinez-Martinez L., De Graaf L., Van Strijp J.A., Verbrugh H.A., Verhoef J., Fleer A.**, (1993). Induction of release of tumor necrosis factor from human monocytes by staphylococci and staphylococcal peptidoglycans. *Infect. Immun*., **61**:4167-4172.

138 **Todar K.**, (2008). *Staphylococcus aureus* and staphylococcal disease. *Todar's Online Textbook of Bacteriology*.

139 **Tompkins D.C., Blackwell L.J., Hatcher V.B., Elliott D.A., O'Hagan-Sotsky C., Lowy F.D.**, (1992). *Staphylococcus aureus* proteins that bind to human endothelial cells. *Infect. Immun*., **60**:965-969.

140 **Upadhyay A., Burman J.D., Clark E.A., Leung E., Isenman D.E., van den Elsen J.M., Bagby S.**, (2008). Structure-function analysis of the C3 binding region of *Staphylococcus aureus* immune subversion protein Sbi. *J Biol Chem*., **283**(32):22113-20.

141 **von Aulock S., Morath S., Hareng L., Knapp S., van Kessel K.P., van Strijp J.A., Hartung T.**, (2003). Lipoteichoic acid from *Staphylococcus aureus* is a potent stimulus for neutrophils recruitment. *Immunobiology*, **208**:413-422.

142 **Vuong C., Voyich J.M., Fischer E.R., Braughton K.R., Whitney A.R., DeLeo F.R., Otto M.**, (2004). Polysaccharide intercellular adhesion (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. *Cell. Microbiol*., **6**(3):269.

143 **Wakabayashi G., Gelfand J.A., Jung W.K., Connolly R.J., Burke J.F., Dinarello C.A.**, (1991). *Staphylococcus epidermidis* induces complement activation, tumor necrosis factor and interleukin-1, a shock-like state and tissue injury in rabbits without endotoxemia. Comparison to *Escherichia coli*. *J. Clin. Investig*., **87**:1925-1935.

144 **Waldvogel F.A.**, (1990) *Staphylococcus aureus* (including toxic shock syndrome), In: Mandell GL, Douglas RG, Bennett JE (eds.). Principles and Practice of Infectious Disease, 3rded. Churchill Livingston, London. pp 1489- 1510.

145 **Walport M.J.**, (2001). Complement: First of two parts. *N Engl J Med*., **344**(14):1058–66. Review.

146 **Wang X., Towers S., Panchanathan S., Chowell G.**, (2013). A population based study of seasonality of skin and soft tissue infections: implications for the spread of CA-MRSA. *PLoS One*, **8**: e60872.

147 **Wann E.R., Gurusiddappa S., Hook M.,** (2000) : The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. *J Biol Chem*, 275(18):13863-13871.

148 **Wijaya L., Hsu L.Y, Kurup A**., (2006) Community-associated Methicillin-resistant *Staphylococcus aureus*: Overview and Local Situation. Ann Acad Med Singapore, **35**(7):479-86

149 **Wiles, K.G., Panizzi, P., Kroh, H.K., Bock, P.E**. (2010) Skizzle is a novel plasminogen- and plasmin-binding protein from *Streptococcus agalactiae* that targets proteins of human fibrinolysis to promote plasmin generation. *J*. *Biol*. *Chem*. **285,** 21153-21164. doi:10.1074/jbc.M110.107730

150 **Wilkinson B.J., Holmes K.M.**, (1979). *Staphylococcus aureus* cell surface: capsule as a barrier to bacteriophage adsorption. *Infect Immun*., **23**: 549-552.

151 **Wu H.L., Shi G.Y., Wohl R.C., and Bender M.L.,** (1987). Structure and formation of microplasmin. *Proc Natl Acad Sci U S A*., 84(24): 8793–8795.

152 **Wu S., de Lencastre H., Tomasz A.**, (1996). Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. *J*. *Bacteriol*., **178**:6036-6042.

153 **Zhang L., Jacobsson K., Vasi J., Lindberg M., Frykberg L.**, (1998). A second IgG-binding protein in *Staphylococcus aureus*. *Microbiology*, **144** (4):985-91.

154 **Zipfel P.F., Skerka C.**, (2009). Complement regulators and inhibitory proteins. *Nat Rev Immunol*., **9**(10):729-40.

ABSTRACT

Staphylococcus aureus is the causative agent of potentially harmful diseases, like sepsis, endocarditis and necrotizing pneumonia and it is also responsible of less severe clinical manifestations such as epithelial and mucosal-associated infections. Nowadays, the emergence of methicillin (MRSA) as well as vancomycin-resistant (VRSA) strains is of great concern. In spite of the advance in antibiotic development, treating these infections remains a huge challenge. The surface of Gram-positive pathogenic bacterium *Staphylococcus aureus* is decorated with cell wall-anchored (CWA) proteins, Like several other invasive pathogens *S. aureus* can capture plasminogen from the human host where it can be converted to plasmin by host plasminogen activators or by endogenously expressed staphylokinase. Sortase-anchored cell wall associated proteins are responsible for capturing the bulk of bound plasminogen. A mutant defective in the sortase enzyme responsible for anchoring CWA proteins to the *S. aureus* cell surface captured 10-fold less PLG compared to the wild type indicating that one or more CWA proteins is involved. Two cell wall associated proteins, the fibrinogen and fibronectin binding proteins A and B, were found to bind plasminogen and one of them, FnBPB, was studied in detail. Plasminogen captured on the surface of *S. aureus* or *Lactococcus lactis* expressing FnBPB could be activated to the potent serine protease plasmin by staphylokinase and tissue plasminogen activator. Plasminogen bound to recombinant FnBPB with KD of 0.532 μM as determined by surface plasmon resonance. Plasminogen binding did not to occur by the same mechanism of Dock Lock and Latch through which FnBPB binds to fibrinogen. Indeed, FnBPB could bind both ligands simultaneously indicating that their binding sites do not overlap. The N3 subdomain of FnBPB contains the full plasminogen binding site and this comprises, at least in part, two conserved patches of surface-located lysine residues which were recognized by kringle 4 of the host protein.

Structural analysis of streptokinase binding to PLG showed that the bacterial zymogen activator bound in such a way as to avoid the kringle domains. SAK and streptokinase belong to the same protein superfamily even though they share little amino acid sequence similarity so it seems likely that they interact with PLG at the same or a similar site leaving kringle 4 free to bind to FnBPB.

PLG-FnBPB interaction does not reduce the PLG ability to be activated by the activators as SAK or tPA. Indeed they can activate PLG bound on the surface of *S. aureus* USA 300 LAC and use it as a proteolytic reactor with the ability to digest the artificial substrate S2251 and human FBG, suggesting that binding of PLG to staphylococcal surface does not compromise its biological functionality.

Moreover SAK both in cell surface and in TCA-precipitated culture supernatants of USA 300 LAC was detected, allowing to conclude that *S*. *aureus* can capture SAK secreted in the culture environment and acquire the ability to active adsorbed PLG in plasmin.

The presence of active plasmin on the surface of *S*. *aureus* cells most likely contributes to the pathogenesis of different infections. For example, *S*. *aureus* promotes clotting of plasma and this ability constitutes a mechanism that support the aggregation, survival and persistence of the within the fibrin network (*Loof, T.G et al 2015*). Damage to subcutaneous tissue could contribute to the pathogenesis of skin soft tissue infections (SSTIs) caused by CA- MRSA (Wang X., *et al 2013*). Degradation of opsonins could contribute to the avoidance of neutrophilmediated phagocytosis and killing (*Attali C et al 2008*; *Rooijakkers S.H et al 2005*). Thus, recruitment of plasmin(ogen) to staphylococcal surface may represent an important virulence mechanism leading to effective evasion of proteolytically active bacteria from thrombus capture within the vasculature of the host and to bacterial dissemination into deeper tissue sites. To investigate this further requires the employment of appropriate animal models, and to test the role of SAK in these processes requires the use of a transgenic mouse expressing human PLG.

“To accomplish great things, we must not only act, but also dream; not only plan, but also believe”.

Anatole France

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Molecular Interactions of Human Plasminogen with Fibronectin-binding Protein B (FnBPB), a Fibrinogen/Fibronectin-binding Protein from *Staphylococcus aureus*[*]

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***Staphylococcus aureus* is a commensal bacterium that has the ability to cause superficial and deep-seated infections. Like several other invasive pathogens, *S. aureus* can capture plasminogen from the human host where it can be converted to plasmin by host plasminogen activators or by endogenously expressed staphylokinase. This study demonstrates that sortase-anchored cell wall-associated proteins are responsible for capturing the bulk of bound plasminogen. Two cell wall-associated proteins, the fibrinogen- and fibronectin-binding proteins A and B, were found to bind plasminogen, and one of them, FnBPB, was studied in detail. Plasminogen captured on the surface of *S. aureus*- or *Lactococcus lactis*-expressing FnBPB could be activated to the potent serine protease plasmin by staphylokinase and tissue plasminogen activator. Plasminogen bound to recombinant FnBPB with a K_D of 0.532 μM as determined by surface plasmon resonance. Plasminogen binding did not to occur by the same mechanism through which FnBPB binds to fibrinogen. Indeed, FnBPB could bind both ligands simultaneously indicating that their binding sites do not overlap. The N3 subdomain of FnBPB contains the full plasminogen-binding site, and this includes, at least in part, two conserved patches of surface-located lysine residues that were recognized by kringle 4 of the host protein.**

Staphylococcus aureus colonizes the anterior nares of ~25% of the healthy human population (1, 2). This commensal Gram-positive bacterium has the ability to cause a plethora of infections ranging from superficial skin abscesses to serious and potentially life-threatening invasive diseases such as osteomyelitis, endocarditis, and septic arthritis. Strains that are resistant to multiple antibiotics are associated with infections in hospitals. These are referred to as hospital or healthcare-associated methicillin-resistant *S. aureus* (HA-MRSA),[4] which have a propensity to cause bacteremia often associated with biofilm formation on indwelling medical devices (3, 4). Recently a global epidemic of MRSA has occurred in the community (community-associated MRSA (CA-MRSA)) exemplified by USA300 strains such as LAC (5). CA-MRSA strains have fewer antibiotic resistance determinants than HA-MRSA; they express a lower level of resistance to β-lactams, and they can survive on human skin and cause serious skin and soft tissue infections often requiring hospitalization.

The surface of *S. aureus* is decorated with proteins that are covalently anchored to the cell wall by sortases. During the process of secretion and anchoring to the cell wall peptidoglycan, the pre-proteins undergo post-translational changes both at the N terminus to remove the secretory signal sequence and at the C terminus where sortase recognizes the LP*X*TG motif, and it covalently links the COOH of the Thr to the amino group of the fifth Gly in the nascent cross-bridge of the peptidoglycan precursor lipid II. Transglycosylation joins the cell wall-associated (CWA) protein-linked precursor to peptidoglycan (6).

Fig. 1 shows the structural organization of two important CWA proteins, the fibronectin-binding proteins A and B (FnBPA and FnBPB), which are members of the microbial surface component recognizing adhesive matrix molecules (MSCRAMM) family. FnBPs are multifunctional proteins. The C-terminal fibronectin binding repeats are unstructured and form a flexible stalk-like region that projects the A domain away from the cell surface. The N-terminal A domain binds to fibrinogen by the "dock, lock, and latch" mechanism (7, 8) in a similar fashion to the archetypal MSCRAMM clumping factor A (9). A hydrophobic trench located between subdomains N2 and N3 accepts the C-terminal peptide of the fibrinogen γ-chain. A flexible region at the C terminus of N3 (the locking and latching peptide) undergoes a conformational change that locks the ligand in the trench and forms an additional β-strand in a

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[4] The abbreviations used are: HA-MRSA, hospital/healthcare-associated methicillin-resistant *S. aureus*; CA-MRSA, community-associated MRSA; Clf, clumping factor; CWA, cell wall-associated; EACA, ϵ-aminocaproic acid; FBG, fibrinogen; FN, fibronectin; FnBPA, fibronectin-binding protein A; FnBPB, fibronectin-binding protein B; MSCRAMM, microbial surface component recognizing adhesive matrix molecules; PBST, PBS containing Tween 20; PLG, plasminogen; SAK, staphylokinase; rSAK, recombinant SAK; Sbi, immunoglobulin binding protein; t-PA, tissue plasminogen activator; rFnBPB, recombinant FnBPB.

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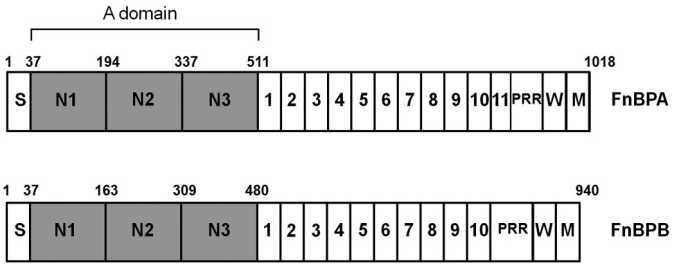

FIGURE 1. **Schematic diagram of FnBPA and FnBPB structure.** The primary translation products of FnBPA and FnBPB proteins contains a signal sequence (*S*) at the N terminus and a wall-spanning region (*M*) and sorting signal (*W*) at the C-terminal end. The N-terminal A region of FnBPA and FnBPB contains three separately folded subdomains, which are known as N1, N2, and N3. Structurally, N2 and N3 form IgG-like folds that combined bind ligands such FBG by the dock, lock, and latch mechanism. Located distal to the A domain is an unfolded region that contains multiple tandemly arranged motifs (11 in FnBPA and 10 in FnBPB) that bind to the N-terminal type I modules of fibronectin and a proline-rich region.

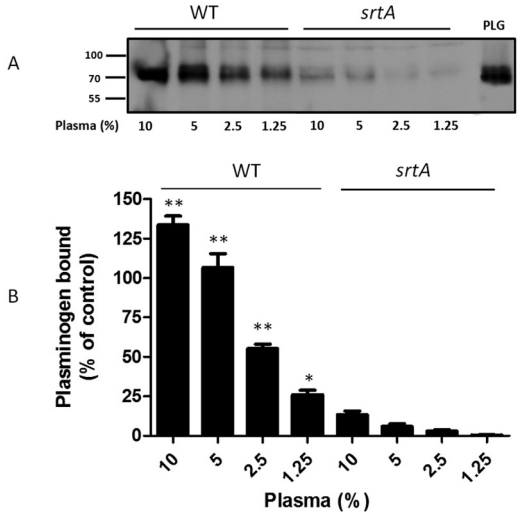

FIGURE 2. **Capture of PLG from human plasma by *S. aureus*.** *A, S. aureus* strain LAC and LAC *srtA* were mixed with different concentrations of human plasma for 60 min. Proteins that were bound to the cell surface were released by extraction buffer and separated by SDS-PAGE under non-reducing conditions and analyzed by Western immunoblotting. The membranes were probed with rabbit anti-human PLG followed by HRP-conjugated mouse anti-rabbit IgG and developed with the ECL Western blotting detection kit. *B,* densitometric analysis of PLG bound to *S. aureus* LAC and the sortase A mutant as reported in *A*. The band intensity was quantified relative to a sample of pure human PLG. The reported data are the mean values ± S.D. from three independent experiments. Statistically significant differences are indicated (Student's *t* test; *, $p < 0.05$; **, $p < 0.01$).

β-sheet in subdomain N2. The A domains of FnBPA and FnBPB both bind to elastin, most probably involving dock lock and latch. They can also engage in homophilic interactions to form dimers, and when two FnBP molecules on neighboring cells interact, this can lead to cell accumulation during biofilm formation (10). The HA-MRSA strain BH1CC and the CA-MRSA strain LAC form biofilm that is dependent on FnBPs (11, 12).

Several pathogenic bacteria that cause invasive infections (*Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae,* and *Borrelia burgdorferi*) can capture host plasminogen (PLG) and allow it to become activated to form the potent serine protease plasmin (13–19). Surface-bound plasmin enables bacteria to remove opsonins IgG and C3b, to degrade fibrin clots, and to promote bacterial spreading by cleaving tissue components (20–22).

Plasminogen is a 92-kDa zymogen that includes a PNA-apple N-terminal domain, five kringle domains (K1–5), and a serine proteinase catalytic domain (23). The kringle domains mediate interactions with fibrin clots and surface receptors from both eukaryotic and bacterial cells (24, 25). Proteolytic activation of PLG to plasmin occurs through cleavage of the Arg^{561}–Val^{562} peptide bond in the catalytic domain by the tissue plasminogen activator (t-PA) and urokinase plasminogen activator. The cleavage results in the formation of active plasmin enzyme that contains a serine protease active site in the C-terminal region (23, 26, 27).

S. aureus can capture PLG on its cell surface where the zymogen can be activated by host t-PA and urokinase, or by staphylokinase, a zymogen activator encoded by a gene located on lysogenic bacteriophages in *S. aureus* (28). Surprisingly little is known about the surface-located proteins of *S. aureus* that capture PLG. The second immunoglobulin-binding protein Sbi and the extracellular fibrinogen-binding protein Efb both occur in the culture supernatant and are associated non-covalently with the cell wall and both can bind PLG (29). The membrane-bound manganese transporter MntC and the moonlighting cytoplasmic proteins enolase and triose-phosphate isomerase can also bind PLG (30–32). However, the biological relevance of these PLG-binding proteins has never been elucidated.

Surprisingly, the ability of CWA proteins to bind PLG has never been examined. Here, for the first time we have tested the ability of CWA proteins to bind PLG and allow it to be activated to form plasmin. Initially, a sortase A mutant was compared with the wild type and was found to bind much less PLG. The FnBPs were found to contribute to the PLG-binding phenotype and thereafter a detailed analysis of one of these, FnBPB was undertaken.

Results

S. aureus Cell Wall-anchored Proteins Bind PLG in Human Plasma—S. aureus cells are known to bind to human PLG, but the bacterial surface components responsible have not been identified. To determine whether cell wall-anchored proteins on the surface of *S. aureus* contribute to PLG binding, the wild-type strain LAC and a sortase A-deficient mutant were compared. Bacteria were incubated with different concentrations of human plasma, and proteins that were bound non-covalently to the cell surface were dissociated by addition of extraction buffer, separated by SDS-PAGE under non-reducing conditions, and analyzed by Western immunoblot probing with anti-PLG IgG. Bacterial cells captured a 90-kDa immunoreactive protein in a dose-dependent manner (Fig. 2*A*). Densitometric analysis of the blots showed that the wild-type strain captured at least 10-fold more PLG than the sortase A mutant (Fig. 2*B*). To exclude the possibility that the markedly reduced capture of PLG from plasma by the sortase A mutant is due to higher proteolytic activity, LAC and its *srtA* mutant were grown to exponential phase and were incubated with purified PLG, and the integrity of the protein in the supernatants was then examined by SDS-PAGE. As expected, the amounts of unbound PLG in the supernatant from *srtA* mutant were even higher than that from the parental strain (data not shown). It can be concluded

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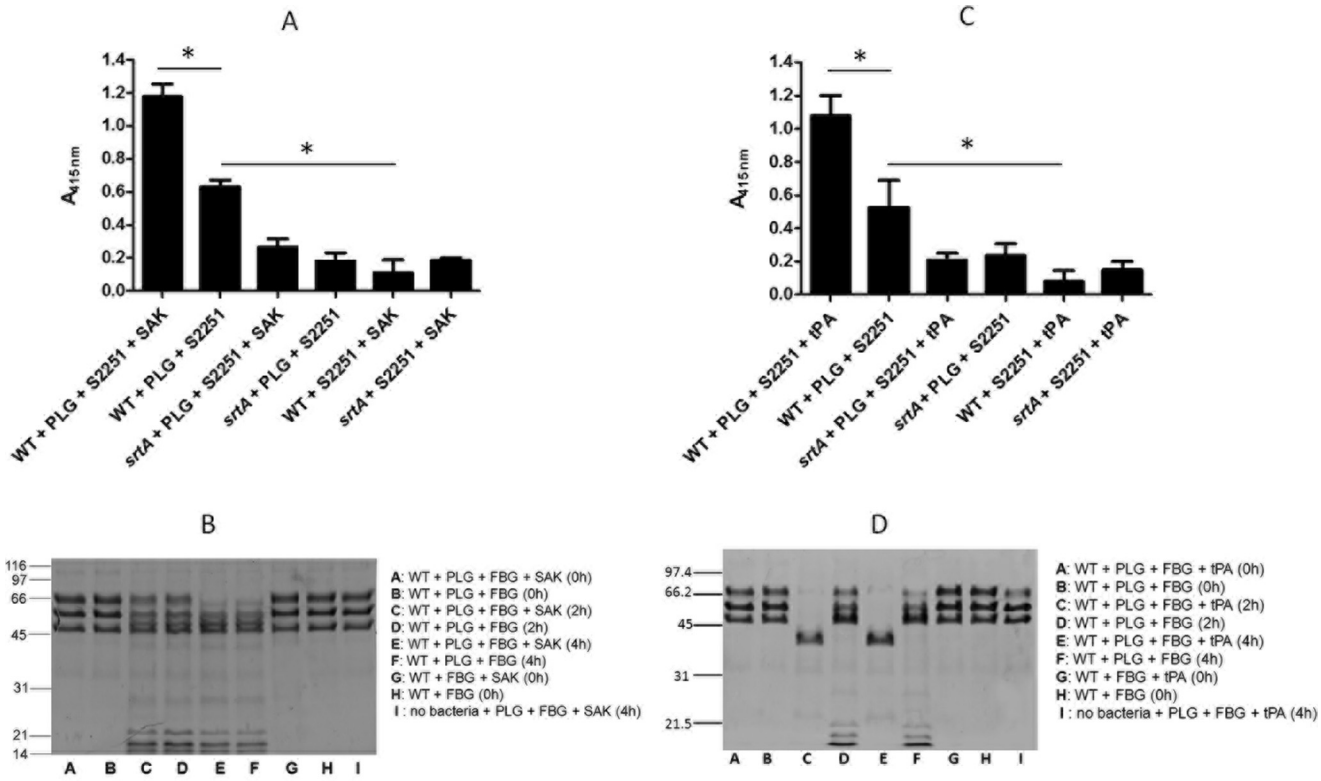

FIGURE 3. **PLG captured by *S. aureus* LAC is functionally active.** *S. aureus* LAC and the *srtA* mutant were immobilized on the surface of microtiter plates and then incubated with human PLG for 1 h at 37 °C. After washing, cells were incubated with recombinant SAK (*A*) or tPA (*C*) and S-2251 for 8 h, and the activity of plasmin was determined measuring the absorbance at 415 nm. Controls lacking PLG or activator were included. Data points are the means of three independent experiments each performed in triplicate ± S.D. Statistically significant differences are indicated (Student's *t* test; *, $p < 0.05$). *S. aureus* LAC wild-type cells were also immobilized on microtiter plates and incubated with human PLG for 1 h at 37 °C. After washing, PLG was activated by addition of rSAK (*B*) or tPA (*D*) and the plasmin substrate FBG. Samples were incubated for 2 or 4 h, and FBG in the fluid phase was subjected to SDS-PAGE and stained with Coomassie Blue. The figure is the representative of three independent experiments.

that CWA proteins are the dominant PLG-binding proteins on the bacterial cell surface and that other surface-located proteins contribute minimally. To compare the plasmin and plasminogen binding activity of *S. aureus*, equal quantities of the purified PLG and plasmin were incubated with LAC cells. After washing, the bacteria-bound plasmin(ogen) was extracted from the staphylococcal surface, and the PLG and plasmin in the extracts were evaluated by ELISA. The results show that bacteria captured the same level of PLG and plasmin (data not shown).

PLG Bound to the Surface of S. aureus Can Be Activated—The ability of PLG bound to the surface of *S. aureus* LAC to be activated by exogenous or endogenous PLG activators was tested (Fig. 3). *S. aureus* LAC is lysogenized by a bacteriophage that carries the gene for SAK, a known activator of the zymogen PLG. Human tissue PLG activator was also tested. Bacterial cells (LAC and LAC *srtA*) were immobilized on the surface of microtiter wells and incubated with PLG and then incubated for 8 h with exogenously purified recombinant SAK (rSAK) or tPA and the chromogenic plasmin substrate S-2251. The substrate was consumed following incubation with the wild type, but minimal substrate consumption was seen with LAC *srtA* (Fig. 3, *A* and *C*). Controls where PLG was omitted did not have the ability to cleave the substrate. Interestingly, incubation of wild-type LAC with PLG and S-2251 alone showed a significantly higher cleavage of the substrate as compared with the controls. Conversely, although the level of substrate consumption was significantly lower than that observed with samples with exogenous SAK or tPA added, the assays reached the same level if left for longer periods (data not shown).

These data show that PLG bound to the cell surface by CWA proteins can be activated efficiently by exogenously added rSAK or tPA (Fig. 3, *A* and *C*, respectively), and furthermore, the immobilized cells can release an endogenous PLG activator, presumably SAK. Plasmin is a central component of the fibrinolytic system and can actively cleave fibrin and FBG. To determine whether PLG captured by CWA proteins on the surface of *S. aureus* can be activated by SAK and cleave FBG, immobilized *S. aureus* LAC cells were incubated with PLG. After washing, rSAK and human FBG were added. The integrity of FBG was assessed by SDS-PAGE and staining with Coomassie Blue. After 2 and 4 h of incubation, FBG was progressively cleaved (Fig. 3*B*). Samples where rSAK was omitted also cleaved FBG, which was presumably due to secretion of endogenous SAK (Fig. 3*B*, *tracks D* and *F*). When cells were incubated with tPA rather than rSAK, FBG was digested more efficiently. In fact, all three FBG chains were hydrolyzed compared with the mild hydrolysis observed when PLG was activated by SAK. This is probably due to the different mechanisms of PLG activation promoted by tPA and SAK, respectively (Fig. 3*D*) (28).

Captured PLG Can Be Activated by Endogenously Expressed SAK—To determine whether cleavage of plasmin substrates S-2251 and whole FBG in the experiments described in Figs. 2

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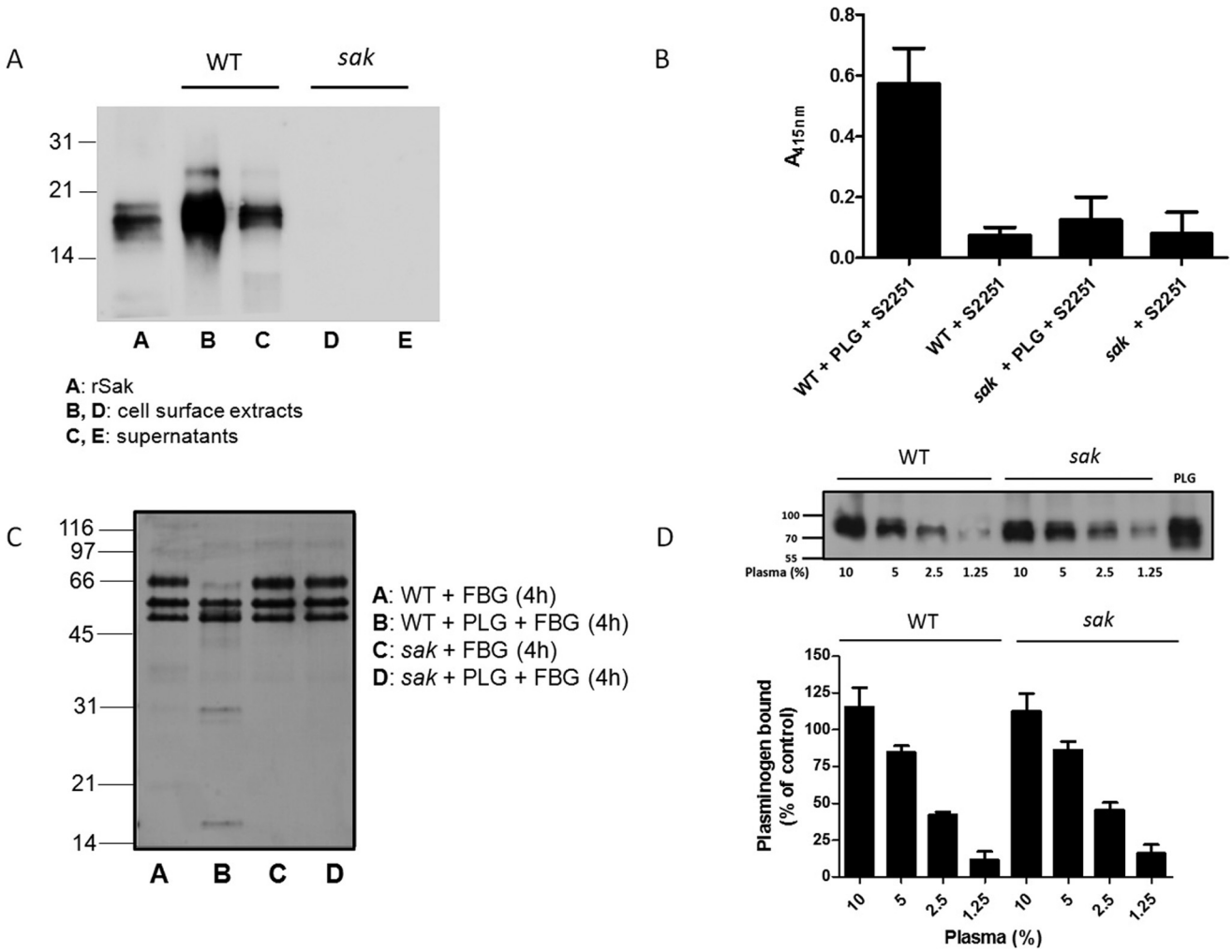

FIGURE 4. ***S. aureus* cells-bound PLG can be activated by endogenous SAK.** *A,* TCA-precipitated culture supernatants and proteins extracted from the cell surface were subjected to SDS-PAGE and Western immunoblot probing with mouse anti-rSAK IgG and HRP-conjugated rabbit anti-mouse IgG. *B, S. aureus* LAC and the *sak* mutant were immobilized on the surface of microtiter plates and then incubated with human PLG for 1 h at 37 °C. After washing, cells were incubated with S-2251 for 8 h, and the activity of plasmin was determined by measuring the absorbance at 415 nm. Controls lacking PLG were included. Data points are the means of three independent experiments each performed in triplicate ± the S.D. *C, S. aureus* LAC wild-type and *sak* mutant cells were immobilized on microtiter plates and incubated with human PLG for 1 h at 37 °C. After washing, the plasmin substrate FBG was added. Samples were incubated for 2 or 4 h, and FBG in the fluid phase was subjected to SDS-PAGE and stained with Coomassie Blue. The figure is the representative of three independent experiments. *D, S. aureus* strain LAC and *sak* mutant cells were mixed with different concentrations of human plasma for 60 min. *Upper panel,* proteins that were bound to the cell surface were released with extraction buffer and separated by SDS-PAGE under non-reducing conditions and analyzed by Western immunoblotting. The membranes were probed with rabbit anti-human PLG followed by HRP-conjugated mouse anti-rabbit IgG. *Lower panel,* densitometric analysis of released PLG. The band intensities were quantified relative to a sample of pure human PLG. The reported data are the mean values from three independent experiments.

and 3, where exogenous rSAK or tPA was not present, was due to SAK expressed by the bacterium, a null mutation in the chromosomal *sak* gene of LAC was constructed (LAC *sak*). Culture supernatants and cell surface extracts were examined by SDS-PAGE and Western immunoblotting using anti-SAK IgG. SAK was present in the supernatant and is associated with the cell surface of LAC, and no SAK was detected in fractions from LAC *sak* (Fig. 4*A*). To determine whether endogenously expressed SAK is responsible for activating captured PLG, wild-type and mutant cells were compared (Fig. 4*B*). Cleavage of the substrate S-2251 was evident with wild-type cells, whereas no cleavage occurred with the *sak* mutant. Similarly, wild-type cells could capture and activate PLG and cleave FBG (Fig. 4*C*), whereas the *sak* mutant was defective. To demonstrate directly that LAC *sak* could still capture PLG, bacteria were incubated with different concentrations of plasma, and the amount of PLG captured was quantified by Western immunoblotting and densitometry (Fig. 4*D*). No difference in PLG bound to the wild-type or *sak* mutant cells was observed. Thus, it can be concluded that SAK secreted by *S. aureus* LAC in the supernatant or bound to the cell surface is capable of activating captured PLG.

Fibronectin-binding proteins A and B Bind PLG—The inability of the *srtA* mutant of *S. aureus* LAC to capture PLG indicates that one or more sortase-anchored cell wall-associated protein(s) is involved. To begin the process of identifying which of the plethora of CWA proteins can bind PLG, purified recombinant ligand binding domains of several CWA proteins were tested by far Western blotting. Proteins were subjected to SDS-PAGE, and Western blottings were probed with purified human PLG and anti-PLG IgG. Subdomains N2 and N3 of the

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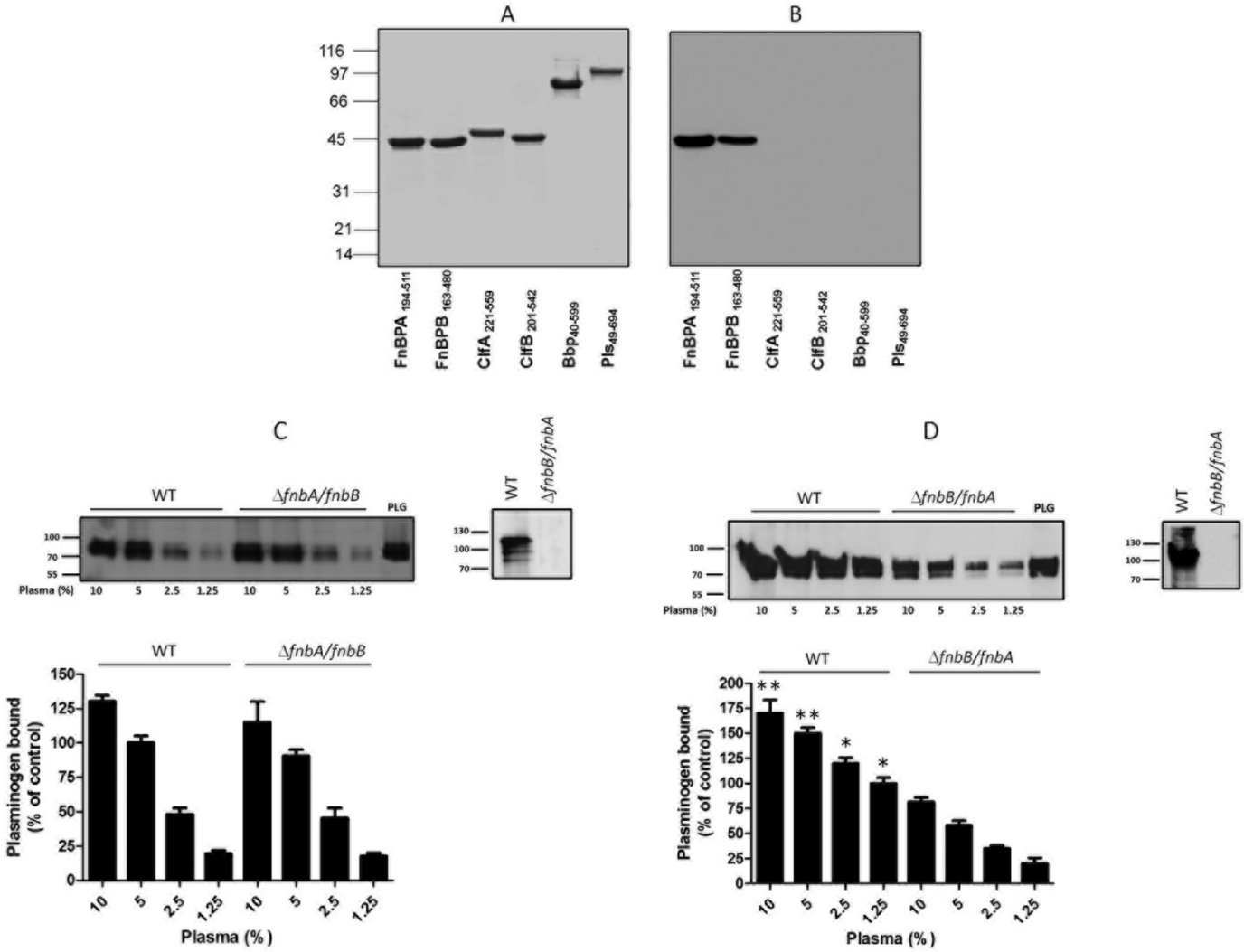

FIGURE 5. **Recombinant cell wall-anchored proteins binding to PLG.** Purified recombinant A domains of several CWA proteins of *S. aureus* were subjected to SDS-PAGE and either stained with Coomassie Blue (*A*) or transferred to a nitrocellulose membrane (*B*) and probed with human PLG followed by rabbit anti-PLG serum and then HRP-conjugated goat anti-rabbit IgG. *C, S. aureus* strains LAC and its *fnbA/fnbB* double mutant cells (*upper panel*) were mixed with different concentrations of human plasma for 60 min. Proteins that were bound to the cell surface were released by extraction buffer and separated by SDS-PAGE under non-reducing conditions and analyzed by Western immunoblotting. The membranes were probed with rabbit anti-human PLG followed by HRP-conjugated mouse anti-rabbit IgG and developed with the ECL Western blotting detection kit. *D,* similar experiment was performed with strain BH1CC and its *fnbA/fnbB* double mutant (*upper panel*). Densitometric analysis of PLG bound to the strains (*lower panels* of *C* and *D*). The band intensity was quantified relative to a sample of pure human PLG. The reported data are the mean values ± S.D. from three independent experiments. In the *insets* the material released by treatment of bacteria with lysostaphin was subjected to Western blotting and probed with FN. Statistically significant differences are indicated (Student's *t* test; *, $p < 0.05$; **, $p < 0.01$).

N-terminal A regions of both fibronectin-binding proteins FnBPA and FnBPB bound PLG, whereas the A domains of clumping factors A and B (ClfA and ClfB), the bone sialoprotein-binding protein, and the plasmin-sensitive protein did not (Fig. 5*B*). To investigate whether FnBPA and FnBPB are the sole PLG-binding proteins of *S. aureus* LAC, a mutant that lacks both proteins was tested for PLG capture. No statistically significant difference between the wild type and the *fnbA fnbB* mutant was seen (Fig. 5*C*), which indicates that one or more CWA protein(s) in addition to FnBPA and FnBPB can bind PLG. Strain LAC expresses low levels of FnBPs compared with the HA-associated strain BH1CC (12), so we decided to determine whether FnBPs contribute to PLG capture when FnBPs are highly expressed. Strain BH1CC showed higher PLG binding than LAC (Fig. 5*D*), and BH1CC-bound PLG that was activated by either SAK or tPA cleaved more efficiently the artificial substrate S2251 compared with the LAC strain (data not shown). The *fnbA fnbB* mutant of BH1CC captured significantly less PLG than the parental strain (Fig. 5*D*) showing the importance of FnBPs to the phenotype. Likewise, *fnbA fnbB* mutants of 8325-4 and LS1 showed a significantly lower ability to capture PLG from plasma. Furthermore, the *fnbA fnbB* deletion mutant of LAC that was transformed with a plasmid bearing either the *fnbA* or *fnbB* gene, which overexpressed FnBPA or FnBPB, respectively, showed a much higher potential to capture plasma PLG that the parental strain (Table 1). All together these data indicate that FnBPA and FnBPB represent critical PLG receptors in *S. aureus*. In contrast, as reported for LAC, FnBPA and FnBPB apparently do not seem to be the predominant PLG-binding proteins in

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TABLE 1

Capture of PLG from human plasma by *S. aureus* strains

S. aureus strains and their *fnbA*/*fnbB* mutants were mixed with 5% diluted human plasma for 60 min. Proteins that were bound to the cell surface were released by extraction buffer and separated by SDS-PAGE under non-reducing conditions and analyzed by Western immunoblotting. The membranes were probed with rabbit anti-human PLG followed by HRP-conjugated mouse anti-rabbit IgG and developed with the ECL Western blotting detection kit. The densitometric analysis of PLG bound to *S. aureus* strains was performed as reported under "Experimental Procedures." The band intensity was quantified relative to a sample of pure PLG (5 μg, 100% intensity). Statistically significant differences between WT strains and their corresponding mutants are indicated (Student's t test; **, $p < 0.01$).

Bacterial strain	Band intensity (% PLG bound, mean ± S.D.)
LAC	110 ± 10
LAC Δ*fnbA*/*fnbB*	95 ± 15
LAC Δ*fnbA*/*fnbB* pFNBA4	205 ± 12[a]
LAC Δ*fnbA*/*fnbB* pFNBB4	195 ± 20[a]
BH1CC	150 ± 15
BH1CC Δ*fnbA*/*fnbB*	62 ± 6[a]
P1	115 ± 12
P1 Δ*fnbA*/*fnbB*	96 ± 8
8325–4	106 ± 15
8325-4 Δ*fnbA*/*fnbB*	47 ± 4[a]
LS1	165 ± 13
LS1 Δ*fnbA*/*fnbB*	58 ± 9[a]
SH1000	108 ± 10
SH1000 Δ*fnbA*/*fnbB*	105 ± 12

[a] Statistically significant differences between WT strains and their corresponding mutants are indicated (Student's t test; $p < 0.01$).

strains P1 and SH1000, suggesting that additional CWA proteins are involved (Table 1).

Lactococcus lactis-expressing FnBPB Can Capture PLG—To study FnBPB in isolation from other *S. aureus* CWA proteins, a strain of *L. lactis*-expressing FnBPB from a gene cloned into the plasmid vector pNZ8037 was used. *L. lactis* pNZ8037::*fnbB* and the same strain carrying the empty vector were immobilized in microtiter wells and incubated with PLG, and following the washing steps, the chromogenic plasmin substrates S-2251 and rSAK (Fig. 6*A*) or tPA (Fig. 6*C*) were added. Cleavage of the substrate was observed, whereas the cells bearing the empty vector had no activity. It should be noted that this system was somewhat less active than *S. aureus* LAC cells. *L. lactis* (pNZ8037::*fnbB*) cells were also incubated with FBG and rSAK (Fig. 6*B*) or tPA (Fig. 6*D*). SDS-PAGE analysis showed that FBG was cleaved by the FnBPB-expressing cells and not by those carrying the empty vector. These experiments show that *L. lactis* cells normally lack the ability to capture PLG and that FnBPB expressed on the bacterial cell surface can capture PLG in a form that can be activated by exogenous PLG activators.

PLG and FBG Bind FnBPB at Distinct Sites—The A domain of FnBPB binds to FBG by the dock, lock, and latch mechanism. To determine whether PLG and FBG bind FnBPB at the same or distinct sites, rFnBPB(163–480) was immobilized onto the surface of microtiter wells and tested for binding to saturating concentrations of PLG in the presence of increasing amounts of FBG by ELISA-type assay. Bound PLG was detected with specific antibodies, and the amount of PLG bound did not change as the concentration of FBG added increased (Fig. 7*A*). A similar result was obtained when saturating concentrations of FBG were tested in the presence of increasing amounts of PLG (Fig. 7*B*). In both panels, dose-dependent binding of increasing amounts of either FBG or PLG to FnBPB was also reported. Together, these data indicate that PLG and FBG bind to distinct sites on the A domain of FnBPB without steric hindrance. The failure of FBG to interfere with rFnBPB binding to PLG suggested that the ligands bind to different sites within the A domain of the protein. To investigate whether the dock, lock, and latch mechanism is involved in PLG binding and to further localize its binding site, variants of the recombinant A domain were tested for ligand binding in solid phase ELISA-type assays. A truncate lacking 17 residues at the C terminus of subdomain N3 that is involved in the locking and latching steps of the dock, lock, and latch mechanism (rFnBPB(163–463)) and a protein carrying amino acid substitutions in residues Asn-312 and Phe-314 of the FnBPB fibrinogen-binding trench (rFnBPB(163–480)-N312A/F314A) were tested for binding to FBG and PLG (Fig. 8, *A–D*). Neither the C-terminal truncate nor the trench mutant could bind FBG, but each bound PLG dose-dependently and saturably in a manner that was indistinguishable from rFnBPB(163–480). This confirms that PLG does not bind to the FnBPB A domain using the dock, lock, and latch mechanism. Finally, subdomains N2 and N3 were expressed separately (N2 subdomain rFnBPB(163–308) and N3 subdomain rFnBPB(309–480)) and tested for binding to PLG and FBG (Fig. 8, *E* and *F*). Neither subdomain N2 nor N3 bound FBG detectably, whereas subdomain N3 bound PLG dose-dependently and saturably with the same profile as N2N3 (rFnBPB(163–480)), which is also consistent with the two ligands binding to FnBPB at different non-overlapping sites. This suggests that the PLG-binding site of FnBPB is contained within the N3 subdomain.

Measurement of the Dissociation Constant of FnBPB Binding to PLG by Surface Plasmon Resonance—The affinity of the FnBPB for PLG was measured by surface plasmon resonance. Purified rFnBPB(163–480) was immobilized onto the surface of a dextran chip, and PLG was passed over the chip in concentrations ranging from 0.78 to 5 μM (Fig. 9). The K_D for the interaction was 0.532 ± 0.028 μM, an affinity that is nearly 4-fold higher than the interaction between FnBPB and FBG (33).

Localization of the Binding Sites within PLG and FnBPB—PLG binds to the N3 subdomain of FnBPB that was cloned from *S. aureus* 8325-4. There are seven isoforms of FnBPB N2N3 subdomains with amino acid sequence identities ranging from 61 to 85% (33). FnBPBs from strains 8325-4, LAC, and BH1CC have identical amino acid sequences and belong to isoform I. Each of the seven isoforms retains the ability to bind FBG, fibronectin, and elastin (33). The recombinant N2N3 subdomains of isoforms II–VII were purified and tested for their ability to bind to PLG in an ELISA-type assay. Each isoform bound PLG similarly to isoform I (Fig. 10*A*).

PLG binding to its natural ligands is attributed to strong affinity of kringle domain(s) for lysine-rich regions of target proteins. To investigate whether lysine residues are important in binding to FnBPB isoform I, PLG binding assays were performed with the recombinant bacterial protein in the presence of lysine and the lysine analog ϵ-aminocaproic acid (EACA). Both molecules caused dose-dependent and complete inhibition of the interaction, with EACA being far more potent (Fig 10*B*).

The structure of the N3 subdomain of FnBPB isoform I was modeled on the x-ray crystal structure of the related protein

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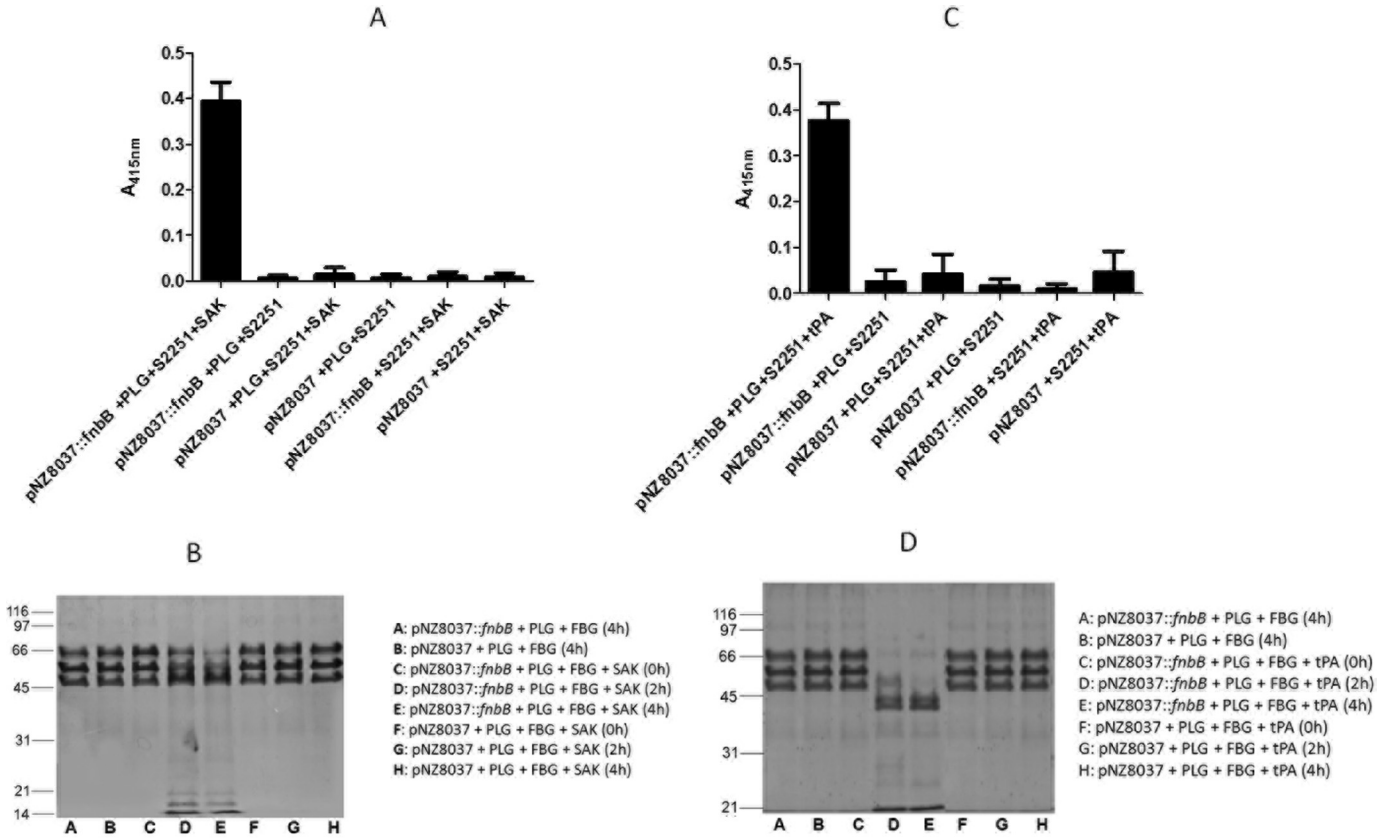

FIGURE 6. **Activation of PLG bound to *L. lactis*-expressing FnBPB.** *L. lactis*-expressing FnBPB (pNZ8037*fnbB*) and cells carrying the empty vector were immobilized in microtiter plates and incubated with PLG for 1 h at 37 °C. After washing, cells were incubated with rSAK (*A*) or tPA (*C*) and the chromogenic plasmin substrate S-2251 for 8 h. The activity of plasmin was determined measuring the absorbance at 415 nm. The data points are the means (±S.D.) of three independent experiments each performed in triplicate. Immobilized *L. lactis*-expressing FnBPB and cells carrying the empty vector were also incubated with PLG for 1 h at 37 °C. After washing, cells were incubated with FBG and rSAK (*B*) or tPA (*D*) for 2 and 4 h. Controls lacking rSAK or tPA were included (*tracks A* and *B*). The fluid was analyzed by SDS-PAGE followed by staining with Coomassie Blue.

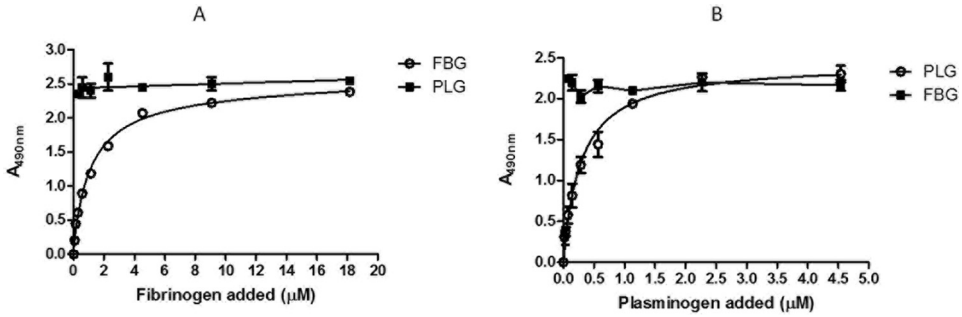

FIGURE 7. **Binding of FnBPB to PLG in the presence of FBG.** *A,* rFnBPB was immobilized on the surface of microtiter wells. Saturating concentrations of PLG were added along with increasing concentrations of FBG. Bound PLG was detected with rabbit anti-PLG followed by HRP-conjugated goat anti-rabbit IgG (*full squares*). In the same panel, binding of increasing amounts of FBG to the wells is also reported (*open dots*). Bound FBG was detected with mouse anti-FBG serum followed by HRP-conjugated rabbit anti-mouse IgG. The data points are the means ± S.D. of three independent experiments each performed in triplicate. *B,* ELISA-type assay with rFnBPB immobilized on the surface of microtiter wells. Saturating concentrations of FBG were added along with increasing concentrations of PLG. Bound FBG was detected with mouse anti-FBG followed by HRP-conjugated rabbit anti-mouse IgG (*full squares*). The panel shows binding of increasing amounts of PLG to the wells (*open dots*). Bound proteins were detected with specific antibodies as in *A*. The data points are the means (±S.D.) of three independent experiments each performed in triplicate.

FnBPA. Amino acid sequence alignments of isotypes I–VII of FnBPB were used to identify conserved lysine residues in subdomain N3. The conserved lysine residues were visualized using the molecular model, and two conserved lysine-rich surface regions were identified. Alanine substitutions were isolated in site 1 (Lys-330, Lys-334, and Lys-362) and site 2 (Lys-342, Lys-374, and Lys-423) and then combined to form a double site 1-site 2 mutant. The variants were tested for their ability to bind to PLG and FBG. Both site 1 and site 2 mutants showed a significant reduction in binding to PLG, a difference that was even greater when site 1 and site 2 substitutions were combined. In contrast, FBG binding was not reduced significantly (Fig. 10*C*). This is consistent with PLG and FBG binding to FnBPB A domain at different sites.

To determine which part of the PLG protein binds FnBPB, truncates comprising kringles 1–3 and 1–4, and mini-PLG,

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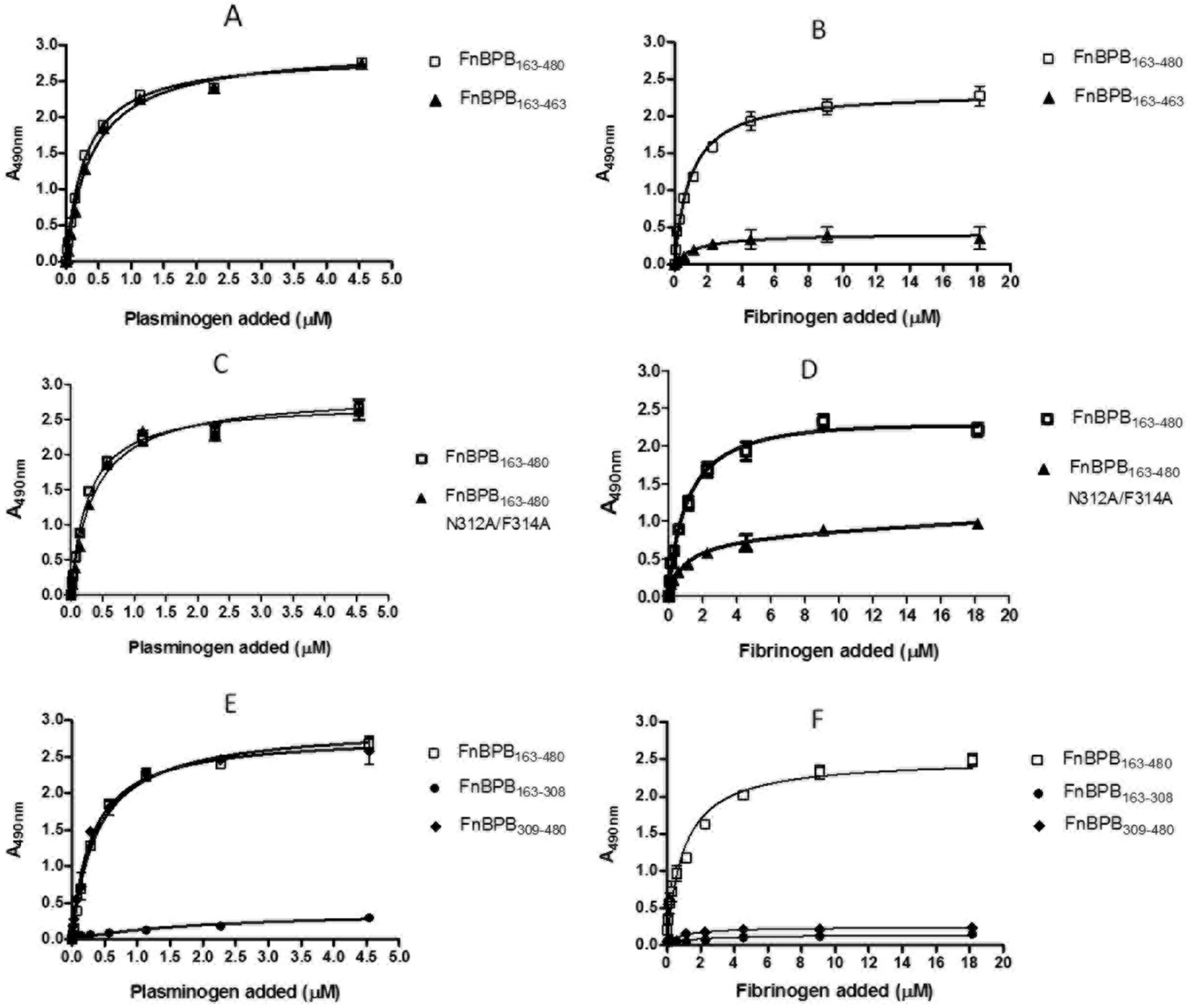

FIGURE 8. **PLG binding to FnBPB(163–480) and its derivatives.** Truncated variants of rFnBPB A domain were immobilized in microtiter plate wells and tested for binding to PLG (*A, C,* and *E*) or FBG (*B, D,* and *E*). Bound PLG was detected with rabbit antibodies to human PLG followed by HRP-conjugated goat anti-rabbit IgG. Bound FBG was detected with mouse antibodies to human FBG followed by HRP-conjugated rabbit anti-mouse IgG. Immobilized proteins: *A* and *B,* C-terminal truncate lacking the lock and latch regions (rFnBPB(163–463)); *C* and *D,* amino acid substitution mutant in the ligand-binding trench (rFnBPB(163–480) N321A/F314A); *E* and *F,* N2 and N3 subdomains rFnBPB(163–308) and rFnBPB(309–480). The data points are the means (±S.D.) of three independent experiments each performed in triplicate.

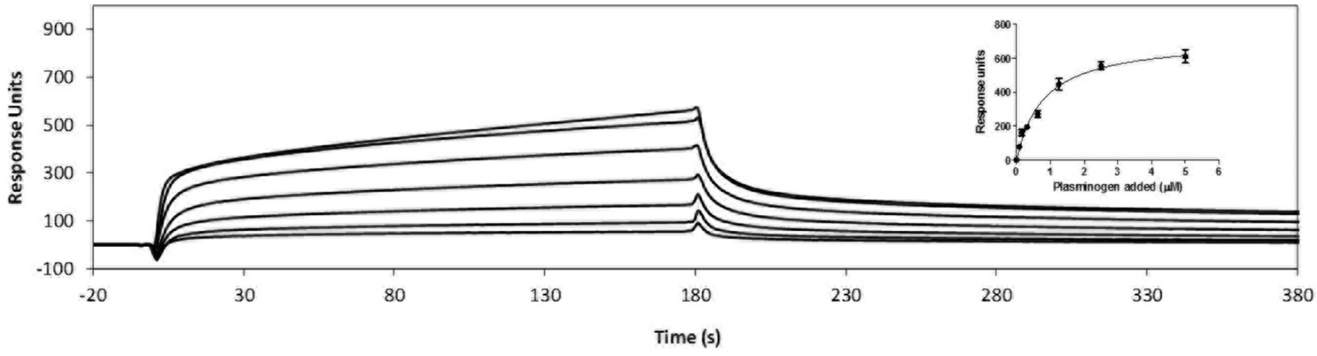

FIGURE 9. **FnBPB binds PLG with high affinity.** Surface plasmon resonance analysis of the interaction of rFnBPB(163–480) with PLG. Representative sensorgrams display binding of PLG to and dissociation from rFnBPB(163–480). The affinity was calculated from curve fitting to a plot of the response unit values against concentrations of PLG (*inset*). The figure shows one representative of three experiments.

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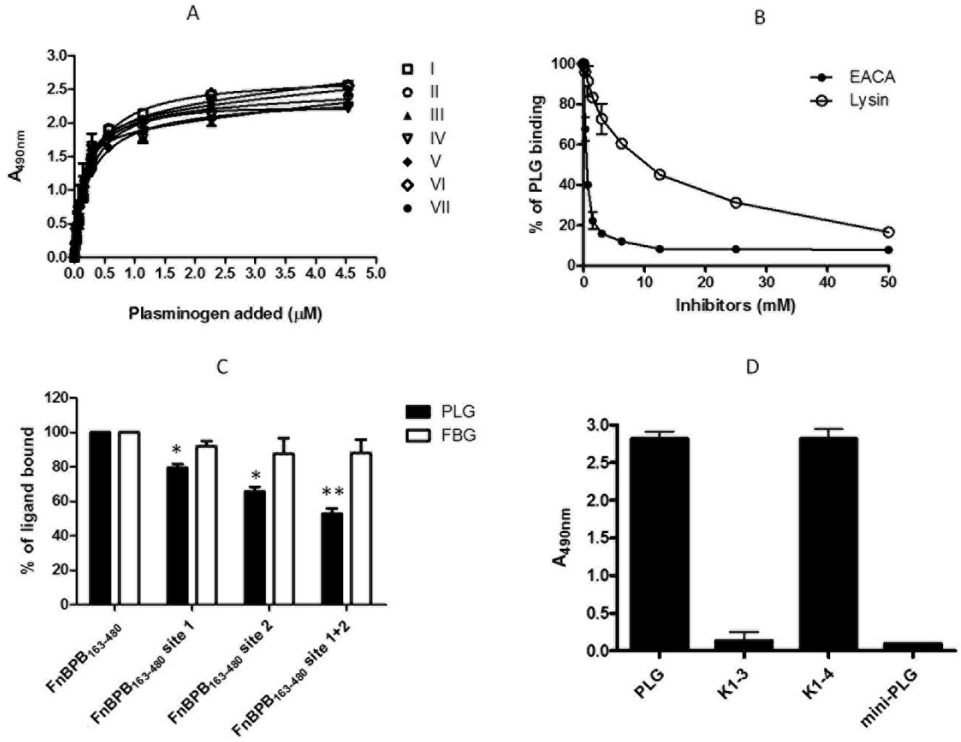

FIGURE 10. *A,* recombinant N2N3 subdomains of isoforms I–VII of FnBPB were immobilized in microtiter plate wells and tested for binding to PLG. Bound PLG was detected with rabbit antibodies to human PLG followed by HRP-conjugated goat anti-rabbit IgG. The data points are the means ± S.D. of three independent experiments each performed in triplicate. *B,* rFnBPB(163–480) was immobilized onto microtiter plates and incubated with PLG in the presence of increasing concentrations of lysine and the lysine analog ϵ-aminocaproic acid. PLG bound to FnBPB was detected with a mouse polyclonal PLG antiserum and HRP-conjugated anti-rabbit IgG. Binding of PLG by FnBPB in the absence of inhibitor was set to 100%. Values represent the means of three separate experiments performed in triplicate, and *error bars* show S.D. *C,* microtiter plates coated with FnBPB(163–480) and its isoforms mutated at site 1, site 2, or both sites were incubated with PLG or FBG. Bound PLG was detected with rabbit antibodies followed by HRP-conjugated goat anti-rabbit IgG. Bound FBG was detected with mouse antibody followed by HRP-conjugated rabbit anti-mouse IgG. Binding of each ligand to the variants was expressed as percentage of the controls where binding of PLG or FBG to FnBPB(163–480) was set to 100%. The data points are the means ± S.D. of three independent experiments each performed in triplicate. Statistically significant differences are indicated (Student's *t* test; *, $p < 0.05$; **, $p < 0.01$). *D,* microtiter plates coated with FnBPB(163–480) were incubated with equimolar concentrations of PLG or PLG fragments K1–K3, K1–K4, and mini-PLG. PLG and the fragments bound to FnBPB were detected by incubation of the plates with a rabbit PLG antibody followed by goat HRP-conjugated secondary IgG. Three independent experiments were conducted in triplicate, and *graphs* represent means ± S.D.

which is composed of kringle 5 along with C-terminal residues, were tested for binding to the bacterial protein. Kringles 1–3 and kringle 5 failed to bind FnBPB, whereas kringles 1–4 bound similarly to full-length PLG. Thus, kringle 4 seems to be the sole binding domain within PLG for FnBPB (Fig. 10*D*).

Discussion

S. aureus, like other invasive pathogens such as *S. pneumoniae* (13, 14), *S. pyogenes* (15, 16), and *S. agalactiae* (17, 34, 35) can capture PLG from human plasma. Staphylococcal proteins that bind the human protein have been described previously, the secreted and wall-associated protein Sbi (29), the MntC (30), and the moonlighting cytoplasmic protein enolase (31), but the biological significance of these interactions is not clear because experiments were performed either with purified protein *in vitro* or with cell lysates.

Here, we have shown for the first time that sortase A-anchored cell wall-associated proteins are the dominant PLG-binding proteins on the *S. aureus* cell surface. A sortase A mutant of *S. aureus* LAC bound at least a 10-fold lower level of PLG than the wild type. It is noteworthy that PLG captured from human plasma could be activated by exogenously added tissue PLG activator t-PA and SAK.

Two CWA proteins, FnBPA and FnBPB, were shown to bind PLG *in vitro* and in the case of FnBPB when expressed on the surface of the surrogate host *L. lactis*. However, deletion mutants of *S. aureus* USA300 LAC, P1, and SH1000 lacking FnBPs bound the same level of PLG as the parental strain, although mutants of BH1CC, 8325-4, and LS1 showed at least a 50% reduction in PLG binding, indicating that one or more of the other CWA proteins are also important. Thus, the contribution of FnBPs to PLG capture will vary depending on strain background and the level of *fnbA fnbB* gene expression, something that is influenced by the growth conditions of the bacterium. To identify the repertoire of PLG binding CWA proteins and to determine their relative importance in the capacity of the bacterium to bind PLG, it would be necessary to express recombinantly all 23 sortase-anchored proteins and to test them by far Western blotting and/or ELISA. This would need to be backed up by the isolation of mutants defective in the candidate proteins, both singly and in combination.

The A domain of FnBPB was chosen for detailed analysis of PLG binding. This domain can bind to FBG by the dock, lock, and latch mechanism (8). We have shown that PLG and FBG bind FnBPB at distinct non-overlapping sites and that PLG

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TABLE 2
Bacterial strains
Abbreviations used are as follows: Em^r, erythromycin resistance; Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance.

Bacterial strains	Relevant properties	Reference
S. aureus		
LAC	Community-associated MRSA of USA300 lineage	5
LAC Δ*fnbA fnbB*	Deletion of *fnbA* and *fnbB* genes isolated by allelic exchange	12
LAC Δ*fnbA fnbB* (pFNBA4)	Mutant transformed with plasmid-expressing FnBPA	12
LAC Δ*fnbA fnbB* (pFNBB4)	Mutant transformed with plasmid-expressing FnBPB	12
LAC *srtA*	Mutant of LAC defective in sortase A. Transduction of *srtA*::Em^r mutation from strain Newman into an erythromycin-sensitive derivative of LAC	This study
LAC *sak*	Mutant of LAC defective in staphylokinase. Transduction of *sak*::Tn Em^r from the Nebraska transposon mutant library strain JE2 *sak*::Tn into an erythromycin-sensitive derivative of LAC	This study
8325-4	NCTC8325 cured of three prophages	39
8325-4 *fnbA fnbB*	*fnbA*::Tc^r *fnbB*::Em^r mutations isolated by allelic exchange	40
BH1CC	Hospital-associated MRSA	3
BH1CC *fnbA fnbB*	*fnbA*::Tc^r *fnbB*::Em^r mutations transduced from 8325-4 *fnbA fnbB*	3
P1	Rabbit passaged strain derived from ATCC25923	41
P1 *fnbA fnbB*	*fnbA*::Tc^r *fnbB*::Em^r mutations transduced from 8325-4 *fnbA fnbB*	42
LS1	Isolate from spontaneous outbreak of septic arthritis in mouse colony	43
LS1 *fnbA fnbB*	*fnbA*::Tc^r *fnbB*::Em^r mutations transduced from 8325-4 *fnbA fnbB*	44
SH1000	*rbsU*I restored in 8325-4	45
SH1000 *fnbA fnbB*	*fnbA*::Tc^r *fnbB*::Em^r mutations transduced from 8325-4 *fnbA fnbB*	This study
L. lactis		
L. lactis NZ9800 (pNZ8037)	Expression vector with nisin-inducible promoter, Cm^r	46
L. lactis NZ9800 (pNZ8037*fnbB*)	*fnbB* gene cloned in pNZ8037 Cm^r	47

binding does not involve the dock, lock, and latch mechanism. This is important because PLG and FBG are both components of blood plasma. FnBPB can simultaneously capture both PLG and FBG (a substrate for plasmin, once plasminogen becomes activated).

The PLG-binding site is confined to subdomain N3, and it does not overlap the FBG-binding site, and variants of the recombinant A domain with substitutions at two distinct conserved lysine-rich sites in subdomain N3 bound PLG with reduced affinity. X-ray crystal structure analysis of the FnBPB-PLG complex will provide molecular details of the interaction. The seven distinct isoforms of FnBPB each bound PLG with similar affinities in solid phase assays. This attests to the idea that PLG binding is an important property that has been retained during diversification of the N3 subdomain surface residues. It will be interesting to compare PLG binding by FnBPA and its seven isoforms.

Our data indicate that kringle 4 probably comprises the only binding domain within PLG for FnBPB and that this binds the two lysine-rich patches within subdomain N3 of FnBPB. Structural analysis of streptokinase binding to PLG showed that the bacterial zymogen activator bound in such a way as to avoid the kringle domains. SAK and streptokinase belong to the same protein superfamily even though they share little amino acid sequence similarity (36), so it seems likely that they interact with PLG at the same or a similar site leaving kringle 4 free to bind to FnBPB. It would be interesting to determine whether SAK can bind to and displace PLG from the FnBPB and stimulate the release of active plasmin or whether the host protease remains attached to the cell-bound MSCRAMM.

The presence of active plasmin on the surface of *S. aureus* cells most likely contributes to the pathogenesis of different infections. For example, degradation of host- or SAK-promoted fibrin clots could help bacteria to spread (37). Damage to subcutaneous tissue could contribute to the pathogenesis of skin soft tissue infections caused by CA-MRSA (38). Degradation of opsonins could contribute to the avoidance of neutrophil-mediated phagocytosis and killing (20–22). To investigate this further requires the employment of appropriate animal models, and to test the role of SAK in these processes requires the use of a transgenic mouse expressing human PLG.

In summary, we have shown that CWA proteins are the dominant bacterial receptors for capturing host PLG by *S. aureus,* and we have investigated in detail the mechanism of PLG binding by one such CWA protein FnBPB.

Experimental Procedures

Bacterial Strains and Culture Conditions—All strains are listed in Table 2 (39–47). *S. aureus* was grown in brain heart infusion broth (VWR International Srl, Milan, Italy) at 37 °C with shaking. *L. lactis* cells carrying the expression vector (pNZ8037) alone or harboring the *fnbB* gene (pNZ8037::*fnbB*) were grown overnight in M17 medium (Difco, Detroit, MI) supplemented with 10% lactose, 0.5% glucose, and chloramphenicol (10 μg/ml) at 30 °C without shaking. Cultures were diluted 1:100 in the above medium and allowed to reach exponential phase. Nisin (6.4 ng/ml) was added, and cultures were allowed to grow overnight at 30 °C without shaking. In those experiments, where a defined number of cells were used, bacteria were harvested from the cultures by centrifugation, washed and suspended in PBS, and counted in a Petroff-Hausser chamber. *Escherichia coli* TOPP3 transformed with vector pQE30 (Stratagene, La Jolla, CA) or derivatives were grown in Luria agar and Luria broth (VWR) containing 100 μg/ml ampicillin.

Plasmid and DNA Manipulation—Plasmid DNA (Table 3) was isolated using the Wizard®Plus SV miniprep kit (Promega, Madison, WI), according to the manufacturer's instructions, and finally transformed into *E. coli* TOPP3 cells using standard procedures (48). Transformants were screened by restriction analysis and verified by DNA sequencing (Eurofins Genomics, Milan, Italy). Chromosomal DNA was extracted using the bacterial genomic DNA purification kit (Edge Biosystems, Gaithersburg, MD). DNA encoding residues 163–480 of FnBPB incorporating alanine substitutions in site 1 (residues Lys-330,

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TABLE 3
Plasmids
Abbreviation used is as follows: Amp^R ampicillin resistance.

Plasmid	Features	Marker	Source/ Reference
pQE30	*E. coli* vector for the expression of hexa-His-tagged recombinant proteins	Amp^R	Qiagen
pQE30::rFnBPB(163–480)	pQE30 derivative encoding the N2N3 subdomain of FnBPB from *S. aureus* 8325–4	Amp^R	8
pQE30::rFnBPB(163–480) site 1	pQE30 derivative encoding the N2N3 incorporating alanine substitutions in site1 (residues Lys-330, Lys-334, and Lys-362)	Amp^R	This study
pQE30::rFnBPB(163–480) site 2	pQE30 derivative encoding the N2N3 incorporating alanine substitutions in site2 (residues Lys-342, Lys-374, and Lys-423)	Amp^R	This study
pQE30::rFnBPB(163–480) site 3	pQE30 derivative encoding the N2N3 incorporating alanine substitutions site 1 and 2 substitutions combined	Amp^R	This study
pQE30::rFnBPB(163–463)	pQE30 derivative encoding residues 163–463 of FnBPB from *S. aureus* 8325–4	Amp^R	8
pQE30::rFnBPB(163–480) N312A/F314A	pQE30 derivative encoding the N2N3 subdomain of FnBPB from *S. aureus* 8325-4 with mutations encoding the changes N312A and F314A	Amp^R	8
pQE30::rFnBPB(163–308)	pQE30 derivative encoding the N2 subdomain of FnBPB from *S. aureus* 8325–4	Amp^R	This study
pQE30::rFnBPB(309–480)	pQE30 derivative encoding the N3 subdomain of FnBPB from *S. aureus* 8325–4	Amp^R	This study

Lys-334, and Lys-362), site 2 (Lys-342, Lys-374, and Lys-423), or site 1 and 2 substitutions combined was synthesized as g-Block® double-stranded DNA fragments by Integrated DNA Technologies (Coralville, IA). Primers rFnBPB(163–480)F and rFnBPB(163–480)R (Table 4) were used to amplify the sequence for cloning into pQE30. Restriction digests and ligations were carried out using enzymes from New England Biolabs (Ipswich, MA) according to the manufacturer's protocols. Oligonucleotides were purchased from Integrated DNA Technologies (Leuven, Belgium) and are listed in Table 4. DNA purification was carried out using the Wizard®SV gel and PCR clean-up system (Promega).

Expression and Purification of Recombinant Proteins—Recombinant proteins FnBPB(163–308), FnBPB(309–480), and FnBPB(163–463) latch truncated were expressed from pQE30 (Qiagen, Chatsworth, CA) in *E. coli* TOPP3 (Stratagene). Overnight starter cultures were diluted 1:50 in Luria broth containing ampicillin (100 μg/ml) and incubated with shaking until the culture reached an optical density at 600 nm (A_{600}) of 0.4–0.6. Recombinant protein expression was induced by addition of isopropyl 1-thio-β-D-galactopyranoside (0.5 mM) and continued for 2 h. Bacterial cells were harvested by centrifugation and frozen at −80 °C and purified from cell lysates by Ni^{+2}-affinity chromatography on a HiTrap chelating column (GE Healthcare). Recombinant FnBPB isotypes (33), FnBPB(163–480) N312A/F314A trench mutant (8), FnBPA(194–511) (49), ClfA(221–559) (50), ClfB(201–542) (51), bone sialoprotein-binding protein(40–599) (52), and plasmin-sensitive protein(49–694) (53), were each previously expressed with His_6 N-terminal affinity tags using *E. coli* vectors and purified on a HiTrap chelating column (GE Healthcare) by Ni^{2+}-chelate chromatography as described above. The *E. coli* clone expressing recombinant SAK protein was generously provided by Dr. S. H. M. Rooijakkers, University Medical Center Utrecht, The Netherlands, and the SAK protein was purified according to the methods described for isolation of CHIPS by De Haas *et al.* (54). Protein purity was assessed to be 98% by SDS-PAGE, Coomassie Brilliant Blue staining, and densitometry analysis. A bicinchoninic acid protein assay (Pierce) was used to measure concentrations of purified proteins.

Proteins—Human PLG was purified from plasma by affinity chromatography on a Lys-Sepharose column (55). Human FBG was obtained from Calbiochem. FBG was further purified on a gelatin-Sepharose column to remove contaminating fibronectin (FN). Human FN was purified from plasma by a combination of gelatin- and arginine-Sepharose affinity chromatography. To exclude the presence of trace amounts of contaminants, affinity-purified FN was spotted onto nitrocellulose membranes at different concentrations and overlaid with anti-FBG and anti-PLG antibodies (56). The purity of the proteins was assessed by 7.5–12.5% SDS-PAGE and Coomassie Brilliant Blue staining. Kringle 1–3 and Kringle 1–4 were purchased from Sigma and MyBiosource (San Diego), respectively. The mini-PLG (residues Val^{442}-Asn^{790}) was obtained by digestion of PLG with porcine pancreatic elastase (Sigma), as described previously (57, 58). Unless stated otherwise, all other reagents were purchased from Sigma.

Human Plasma—Normal human plasma was prepared from freshly drawn blood obtained from healthy volunteers with informed consent and permission of the ethical board of the University of Pavia (permit no. 19092013). After centrifugation, the plasma fraction was frozen in aliquots and stored at −20 °C.

Antibodies—Polyclonal antiserum against purified PLG was raised in a rabbit by routine immunization procedures using purified human PLG as antigen. Polyclonal antisera against human FBG or recombinant SAK were raised in mice using purified or recombinant SAK as antigens. Purification of rabbit or mouse antibodies from sera was performed by affinity chromatography using protein G-Sepharose columns (GE Healthcare, Buckinghamshire, UK). The anti-human FN rabbit polyclonal IgG was purchased from Pierce. Rabbit anti-mouse or goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Dako Cytomation (Glostrup, Denmark).

ELISA-type Solid-phase Binding Assays—The ability of immobilized proteins (FnBPB(163–480) and its derivatives) to

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TABLE 4
Primers
F is forward, and R is reverse.

Primer	Sequence (5′–3′)[a]	5′-Restriction site
rFnBPB(163–480) F	CGGGGATCCGCATCGGAACAAAACAATAC	BamHI
rFnBPB(163–480) R	AATCCCGGGTTACTTTAGTTTATCTTTGCC	SmaI
rFnBPB(163–308) F	GGGGGATCCGGTACAGATGTAACAAATAAAG	BamHI
rFnBPB(163–308) R	CTCCCCGGGCTATTGAATATTAAATATTTTGCTAA	SmaI
rFnBPB(309–480) F	CCCGGATCCTATTTAGGTGGAGTTAGAGATAAT	BamHI
rFnBPB(309–480) R	AATCCCGGGTTACTTTAGTTTATCTTTGCCG	SmaI

[a] Restriction sites used for cloning are underlined.

bind to soluble ligands (PLG or FBG) was determined using ELISA. Microtiter wells were coated overnight at 4 °C with 0.5 μg/well of the appropriate protein in 0.1 M sodium carbonate, pH 9.5. The plates were washed with 0.5% (v/v) Tween 20 in PBS (PBST). To block additional protein-binding sites, the wells were treated for 1 h at 22 °C with bovine serum albumin (BSA, 2% v/v) in phosphate-buffered saline (PBS). The plates were then incubated for 1 h with the appropriate amounts of the ligand. After several washings with PBST, 0.5 μg of the specific anti-ligand rabbit/mouse IgG in BSA (1% v/v) was added to the wells and incubated for 90 min. The plates were washed incubated for 1 h with HRP-conjugated anti-rabbit or anti-mouse IgG diluted 1:1000. After washing, *o*-phenylenediamine dihydrochloride was added, and the absorbance at 490 nm was determined using an ELISA plate reader.

To identify PLG kringle(s) involved in FnBPB(163–480) binding, microtiter plates were coated with 0.5 μg of FnBPB(163–480) per well and incubated with PLG, PLG fragments K1-K3, K1-K4, or mini-PLG (1 μM). Proteins bound to FnBPB(163–480) were detected by mouse PLG antibody followed by goat HRP-conjugated secondary IgG.

The effect of lysine and lysine analog EACA on FnBPB(163–480)/PLG interaction was examined incubating recombinant FnBPB(163–480) immobilized onto microtiter plates (0.5 μg/well) with 1 μg of purified PLG in the presence of increasing concentrations of lysine or EACA. PLG bound to FnBPB was detected with mouse polyclonal PLG antiserum and HRP-conjugated anti-rabbit IgG.

SDS-PAGE and Western Immunoblotting and Far Western Immunoblotting—Samples for analysis by SDS-PAGE were boiled for 3 min in sample buffer (0.125 M Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.002% (w/v) bromphenol blue) and separated by 10–15% (w/v) PAGE. The gels were stained with Coomassie Brilliant Blue (Bio-Rad). For Western immunoblotting, proteins were subjected to SDS-PAGE and electroblotted onto a nitrocellulose membrane (GE Healthcare), and the membrane was blocked overnight at 4 °C with 5% (w/v) skim milk (Sigma) in PBS. Blotted proteins were probed with rabbit polyclonal antibody against human PLG (1:5000) or mouse polyclonal antibody against SAK (5 μg/ml) for 1 h at 22 °C. Following washes with PBST, the membrane was incubated for 1 h with either HRP-conjugated goat anti-rabbit IgG (1:10,000) or rabbit anti-mouse IgG (1:10,000). Finally, blots were developed using the ECL Advance Western blotting detection kit (GE Healthcare), and an ImageQuant™ LAS 4000 mini-biomolecular imager (GE Healthcare) was used to capture images of the bands. When recombinant staphylococcal proteins were subjected to SDS-PAGE and far Western blotting, membranes were incubated with 1 μg/ml PLG in PBST for 1 h at 22 °C and washed, and the complexes were detected with anti-PLG antibody as reported above.

Preparation of Cell Surface-bound Proteins and TCA-precipitated Culture Supernatants—*S. aureus* USA300 was grown to exponential phase and washed, and ~1×10^8 cfu were incubated for 1 h with the indicated concentrations of human plasma. Cells were harvested by centrifugation, washed with PBS, treated with extraction buffer (125 mM Tris-HCl, pH 7.0, containing 2% SDS) (10 μl/mg wet weight of pellet) (59), for 3 min at 95 °C, and then centrifuged at 10,000 × *g* for 3 min. The supernatants were subjected to 10% PAGE under non-reducing conditions and transferred to a nitrocellulose membrane. The membrane was incubated with rabbit anti-PLG IgG followed by HRP-conjugated secondary antibody. The band intensities were quantified relative to the PLG sample (5 μg, 100% intensity) with the Quantity One software (Bio Rad, Milan, Italy).

To detect SAK on the surface of *S. aureus* USA300 LAC and LAC *sak*, bacteria were treated with extraction buffer, heated at 95 °C for 3 min, and subjected to 15% SDS-PAGE and Western blotting. SAK was detected by incubation with mouse anti-SAK IgG followed by a rabbit HRP-conjugated secondary antibody. Bacteria grown to exponential phase were removed from cultures by centrifugation at 4000 × *g* for 5 min, and the supernatant was concentrated by treatment with 20% trichloroacetic acid (TCA) (v/v) at 4 °C for 30 min. The precipitates were collected by centrifugation at 10,000 × *g* for 10 min, washed with acetone, dissolved in the sample buffer, boiled for 3 min and finally subjected to 15% SDS-PAGE and Western blotting. SAK was detected as reported above.

Release of Cell Wall-anchored Proteins from S. aureus and Detection of Fibronectin Binding Activity—To release cell wall-anchored proteins from *S. aureus*, bacteria were grown to an A_{600} of 0.4 to 0.6, harvested by centrifugation at 7000 × *g* at 4 °C for 15 min, washed three times with PBS, and resuspended to an A_{600} of 40 in lysis buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.5) supplemented with 30% raffinose. Cell wall proteins were solubilized from *S. aureus* by incubation with lysostaphin (200 μg/ml) at 37 °C for 20 min in the presence of protease inhibitors (Complete Mini; Roche Applied Science). Protoplasts were recovered by centrifugation at 6000 × *g* for 20 min, and the supernatants were taken as the wall fraction. The material was adsorbed on IgG-Sepharose column to remove protein A and subjected to SDS-PAGE and Western blotting incubating the membrane with human FN (5 μg/ml) and a rabbit anti-FN antibody (2 μg/ml).

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Surface Plasmon Resonance Analysis of PLG Binding to FnBPB(163–480)—To estimate the affinity of the interaction between PLG and FnBPB(163–480), surface plasmon resonance was conducted using a BIAcore X-100 instrument (GE Healthcare). FnBPB(163–480) was covalently immobilized on dextran matrix CM5 sensor chip surface by using an FnBPB(163–480) solution (30 μg/ml in 50 mM sodium acetate buffer, pH 5) in a 1:1 dilution with *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. The excess of active groups on the dextran matrix was blocked using 1 M ethanolamine, pH 8.5. On another flow cell, the dextran matrix was treated as described above but without any ligand to provide an uncoated reference flow cell. The running buffer used was PBS containing 0.005% (v/v) Tween 20. A 2-fold linear dilution series (0.078–5 μM) of PLG in running buffer was passed over the ligand at the flow rate of 10 μl/min, and all the sensorgrams were recorded at 22 °C. Assay channel data were subtracted from reference flow cell data. The response units at steady state were plotted as a function of PLG concentration and fitted to the Langmuir equation to yield the K_D of the PLG-FnBPB(163–480) interaction.

Effect of Bacteria Bound PLG on the Cleavage of Chromogenic Substrate S-2251 and Degradation of Human Fibrinogen—*S. aureus* LAC (5×10^7 cells) isolated from exponential phase or *L. lactis*-expressing FnBPB were suspended in PBS and immobilized onto microtiter wells at 37 °C overnight. After several washings with PBST, the cells were incubated for 1 h with human PLG (10 μg/well) in PBS at 37 °C. After washing with PBST, bacterially bound PLG was activated by addition of recombinant SAK (15 μg/ml) or 27 nM tPA, incubated with 0.6 mM chromogenic substrate S-2251 (H-D-valyl-L-leucyl-L-lysine-*p*-nitroaniline dihydrochloride; Chromogenix), dissolved in 100 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mg/ml PEG 8000 for 8 h, and the reaction followed spectrophotometrically at 415 nm.

Alternatively, surface-coated cells of *S. aureus* LAC or *L. lactis* were incubated for 90 min at 37 °C with PLG (1 μg/well) diluted in PBS. After several washings with PBST, FBG (10 μg/well) together with tPA (27 nM) or rSAK (15 μg/ml) in 100 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mg/ml PEG 8000 were added to the wells, and the mixtures were incubated at 22 °C for the indicated times. The reaction was stopped by the addition of reducing sample buffer and boiling for 3 min. Thereafter, the samples were separated by 12.5% SDS-PAGE, and degradation of FBG was evaluated by Coomassie Blue staining.

Statistical Methods—Continuous data were expressed as means and standard deviations. Two group comparisons were performed by Student's *t* test. One-way analysis of variance, followed by Bonferroni's post hoc tests, was exploited for comparison of three or more groups. Analyses were performed using Prism 4.0 (GraphPad). Two-tailed *p* values of 0.05 were considered statistically significant.

Author Contributions—G. P., T. J. F., J. A. G., and P. S. designed the study; G. P., G. N., V. G., and M. Z., performed the research; G. P., G. N., V. G., M. Z., T. J. F., J. A. G., and P. S., analyzed the data; G. P., T. J. F., J. A. G., and P. S., wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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References

1. Lowy, F. D. (1998) *Staphylococcus aureus* infections. *N. Engl. J. Med.* **339,** 520–532
2. van Belkum, A., Melles, D. C., Nouwen, J., van Leeuwen, W. B., van Wamel, W., Vos, M. C., Wertheim, H. F., and Verbrugh, H. A. (2009) Co-evolutionary aspects of human colonisation and infection by *Staphylococcus aureus*. *Infect. Genet. Evol.* **9,** 32–47
3. O'Neill, E., Pozzi, C., Houston, P., Humphreys, H., Robinson, D. A., Loughman, A., Foster, T. J., and O'Gara, J. P. (2008) A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. *J. Bacteriol.* **190,** 3835–3850
4. Vergara-Irigaray, M., Valle, J., Merino, N., Latasa, C., García, B., Ruiz de Los Mozos, I., Solano, C., Toledo-Arana, A., Penadés, J. R., and Lasa, I. (2009) Relevant role of fibronectin-binding proteins in *Staphylococcus aureus* biofilm-associated foreign-body infections. *Infect. Immun.* **77,** 3978–3991
5. Diep, B. A., Gill, S. R., Chang, R. F., Phan, T. H., Chen, J. H., Davidson, M. G., Lin, F., Lin, J., Carleton, H. A., Mongodin, E. F., Sensabaugh, G. F., and Perdreau-Remington, F. (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* **367,** 731–739
6. Foster, T. J., Geoghegan, J. A., Ganesh, V. K., and Höök, M. (2014) Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. *Nat. Rev. Microbiol.* **12,** 49–62
7. Loughman, A., Sweeney, T., Keane, F. M., Pietrocola, G., Speziale, P., and Foster, T. J. (2008) Sequence diversity in the A domain of *Staphylococcus aureus* fibronectin-binding protein A. *BMC Microbiol.* **8,** 74
8. Burke, F. M., Di Poto, A., Speziale, P., and Foster, T. J. (2011) The A domain of fibronectin-binding protein B of *Staphylococcus aureus* contains a novel fibronectin binding site. *FEBS J.* **278,** 2359–2371
9. Deivanayagam, C. C., Wann, E. R., Chen, W., Carson, M., Rajashankar, K. R., Höök, M., and Narayana, S. V. (2002) A novel variant of the immunoglobulin fold in surface adhesins of *Staphylococcus aureus*: crystal structure of the fibrinogen-binding MSCRAMM, clumping factor A. *EMBO J.* **21,** 6660–6672
10. Geoghegan, J. A., Monk, I. R., O'Gara, J. P., and Foster, T. J. (2013) Subdomains N2N3 of fibronectin-binding protein A mediate *Staphylococcus aureus* biofilm formation and adherence to fibrinogen using distinct mechanisms. *J. Bacteriol.* **195,** 2675–2683
11. Pozzi, C., Waters, E. M., Rudkin, J. K., Schaeffer, C. R., Lohan, A. J., Tong, P., Loftus, B. J., Pier, G. B., Fey, P. D., Massey, R. C., and O'Gara, J. P. (2012) Methicillin resistance alters the biofilm phenotype and attenuates virulence in *Staphylococcus aureus* device-associated infections. *PLoS Pathog.* **8,** e1002626
12. McCourt, J., O'Halloran, D. P., McCarthy, H., O'Gara, J. P., and Geoghegan, J. A. (2014) Fibronectin-binding proteins are required for biofilm formation by community-associated methicillin-resistant *Staphylococcus aureus* strain LAC. *FEMS Microbiol. Lett.* **353,** 157–164
13. Agarwal, V., Kuchipudi, A., Fulde, M., Riesbeck, K., Bergmann, S., and Blom, A. M. (2013) *Streptococcus pneumoniae* endopeptidase O (PepO) is a multifunctional plasminogen- and fibronectin-binding protein, facilitating evasion of innate immunity and invasion of host cells. *J. Biol. Chem.* **288,** 6849–6863
14. Bergmann, S., Schoenen, H., and Hammerschmidt, S. (2013) The interaction between bacterial enolase and plasminogen promotes adherence of *Streptococcus pneumoniae* to epithelial and endothelial cells. *Int. J. Med. Microbiol.* **303,** 452–462
15. Berge, A., and Sjöbring, U. (1993) PAM, a novel plasminogen-binding protein from *Streptococcus pyogenes*. *J. Biol. Chem.* **268,** 25417–25424
16. Sanderson-Smith, M. L., Dowton, M., Ranson, M., and Walker, M. J. (2007) The plasminogen-binding group A streptococcal M protein-related protein Prp binds plasminogen via arginine and histidine residues. *J.*

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Bacteriol. **189,** 1435–1440
17. Six, A., Bellais, S., Bouaboud, A., Fouet, A., Gabriel, C., Tazi, A., Dramsi, S., Trieu-Cuot, P., and Poyart, C. (2015) Srr2, a multifaceted adhesin expressed by ST-17 hypervirulent Group B Streptococcus involved in binding to both fibrinogen and plasminogen. *Mol. Microbiol.* **97,** 1209–1222
18. Fuchs, H., Wallich, R., Simon, M. M., and Kramer, M. D. (1994) The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 12594–12598
19. Önder, Ö., Humphrey, P. T., McOmber, B., Korobova, F., Francella, N., Greenbaum, D. C., and Brisson, D. (2012) OspC is potent plasminogen receptor on surface of *Borrelia burgdorferi*. *J. Biol. Chem.* **287,** 16860–16868
20. Attali, C., Durmort, C., Vernet, T., and Di Guilmi, A. M. (2008) The interaction of *Streptococcus pneumoniae* with plasmin mediates transmigration across endothelial and epithelial monolayers by intercellular junction cleavage. *Infect. Immun.* **76,** 5350–5356
21. Fulde, M., Rohde, M., Hitzmann, A., Preissner, K. T., Nitsche-Schmitz, D. P., Nerlich, A., Chhatwal, G. S., and Bergmann, S. (2011) SCM, a novel M-like protein from *Streptococcus canis*, binds (mini)-plasminogen with high affinity and facilitates bacterial transmigration. *Biochem. J.* **434,** 523–535
22. Rooijakkers, S. H., van Wamel, W. J., Ruyken, M., van Kessel, K. P., and van Strijp, J. A. (2005) Anti-opsonic properties of staphylokinase. *Microbes Infect.* **7,** 476–484
23. Law, R. H., Caradoc-Davies, T., Cowieson, N., Horvath, A. J., Quek, A. J., Encarnacao, J. A., Steer, D., Cowan, A., Zhang, Q., Lu, B. G., Pike, R. N., Smith, A. I., Coughlin, P. B., and Whisstock, J. C. (2012) The x-ray crystal structure of full-length human plasminogen. *Cell Rep.* **1,** 185–190
24. Chavakis, T., Athanasopoulos, A., Rhee, J. S., Orlova, V., Schmidt-Wöll, T., Bierhaus, A., May, A. E., Celik, I., Nawroth, P. P., and Preissner, K. T. (2005) Angiostatin is a novel anti-inflammatory factor by inhibiting leukocyte recruitment. *Blood* **105,** 1036–1043
25. Duboscq, C., Genoud, V., Parborell, M. F., and Kordich, L. C. (1997) Impaired clot lysis by rt-PA catalyzed mini-plasminogen activation. *Thromb. Res.* **86,** 505–513
26. Castellino, F. J., and Powell, J. R. (1981) Human plasminogen. *Methods Enzymol.* **80,** 365–378
27. Bhattacharya, S., Ploplis, V. A., and Castellino, F. J. (2012) Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. *J. Biomed. Biotechnol.* **2012,** 482096
28. Peetermans, M., Vanassche, T., Liesenborghs, L., Lijnen, R. H., and Verhamme, P. (2015) Bacterial pathogens activate plasminogen to breach tissue barriers and escape from innate immunity. *Crit. Rev. Microbiol.* **2015,** 1–17
29. Koch, T. K., Reuter, M., Barthel, D., Böhm, S., van den Elsen, J., Kraiczy, P., Zipfel, P. F., and Skerka, C. (2012) *Staphylococcus aureus* proteins Sbi and Efb recruit human plasmin to degrade complement C3 and C3b. *PLoS ONE* **7,** e47638
30. Salazar, N., Castiblanco-Valencia, M. M., da Silva, L. B., de Castro, Í., Monaris, D., Masuda, H. P., Barbosa, A. S., and Arêas, A. P. (2014) *Staphylococcus aureus* manganese transport protein C (MntC) is an extracellular matrix- and plasminogen-binding protein. *PLoS ONE* **9,** e112730
31. Antikainen, J., Kuparinen, V., Lähteenmäki, K., and Korhonen, T. K. (2007) Enolases from Gram-positive bacterial pathogens and commensal lactobacilli share functional similarity in virulence-associated traits. *FEMS Immunol. Med. Microbiol.* **51,** 526–534
32. Furuya, H., and Ikeda, R. (2011) Interaction of triosephosphate isomerase from *Staphylococcus aureus* with plasminogen. *Microbiol. Immunol.* **55,** 855–862
33. Burke, F. M., McCormack, N., Rindi, S., Speziale, P., and Foster, T. J. (2010) Fibronectin-binding protein B variation in *Staphylococcus aureus*. *BMC Microbiol.* **10,** 160
34. Wiles, K. G., Panizzi, P., Kroh, H. K., and Bock, P. E. (2010) Skizzle is a novel plasminogen- and plasmin-binding protein from *Streptococcus agalactiae* that targets proteins of human fibrinolysis to promote plasmin generation. *J. Biol. Chem.* **285,** 21153–21164
35. Buscetta, M., Firon, A., Pietrocola, G., Biondo, C., Mancuso, G., Midiri, A., Romeo, L., Galbo, R., Venza, M., Venza, I., Kaminski, P. A., Gominet, M., Teti, G., Speziale, P., Trieu-Cuot, P., and Beninati, C. (2016) PbsP, a cell wall-anchored protein that binds plasminogen to promote hematogenous dissemination of Group B *Streptococcus*. *Mol. Microbiol.* **101,** 27–41
36. Parry, M. A., Zhang, X. C., and Bode, I. (2000) Molecular mechanisms of plasminogen activation: bacterial cofactors provide clues. *Trends Biochem. Sci.* **25,** 53–59
37. Loof, T. G., Goldmann, O., Naudin, C., Mörgelin, M., Neumann, Y., Pils, M. C., Foster, S. J., Medina, E., and Herwald, H. (2015) *Staphylococcus aureus*-induced clotting of plasma is an immune evasion mechanism for persistence within the fibrin network. *Microbiology* **161,** 621–627
38. Wang, X., Towers, S., Panchanathan, S., and Chowell, G. (2013) A population based study of seasonality of skin and soft tissue infections: implications for the spread of CA-MRSA. *PLoS ONE* **8,** e60872
39. Novick, R. (1967) Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. *Virology* **33,** 155–166
40. Greene, C., McDevitt, D., Francois, P., Vaudaux, P. E., Lew, D. P., and Foster, T. J. (1995) Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of *fnb* genes. *Mol. Microbiol.* **17,** 1143–1152
41. Sherertz, R. J., Carruth, W. A., Hampton, A. A., Byron, M. P., and Solomon, D. D. (1993) Efficacy of antibiotic-coated catheters in preventing subcutaneous *Staphylococcus aureus* infection in rabbits. *J. Infect. Dis.* **167,** 98–106
42. Roche, F. M., Downer, R., Keane, F., Speziale, P., Park, P. W., and Foster, T. J. (2004) The N-terminal A domain of fibronectin-binding proteins A and B promotes adhesion of *Staphylococcus aureus* to elastin. *J. Biol. Chem.* **279,** 38433–38440
43. Bremell, T., Lange, S., Svensson, L., Jennische, E., Gröndahl, K., Carlsten, H., and Tarkowski, A. (1990) Outbreak of spontaneous staphylococcal arthritis and osteitis in mice. *Arthritis Rheum.* **33,** 1739–1744
44. Palmqvist, N., Foster, T., Fitzgerald, J. R., Josefsson, E., and Tarkowski, A. (2005) Fibronectin-binding proteins and fibrinogen-binding clumping factors play distinct roles in staphylococcal arthritis and systemic inflammation. *J. Infect. Dis.* **191,** 791–798
45. Horsburgh, M. J., Aish, J. L., White, I. J., Shaw, L., Lithgow, J. K., and Foster, S. J. (2002) σB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from *Staphylococcus aureus* 8325-4. *J. Bacteriol.* **184,** 5457–5467
46. de Ruyter, P. G., Kuipers, O. P., and de Vos, W. M. (1996) Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl. Environ. Microbiol.* **62,** 3662–3667
47. Fitzgerald, J. R., Loughman, A., Keane, F., Brennan, M., Knobel, M., Higgins, J., Visai, L., Speziale, P., Cox, D., and Foster, T. J. (2006) Fibronectin-binding proteins of *Staphylococcus aureus* mediate activation of human platelets via fibrinogen and fibronectin bridges to integrin GPIIb/IIIa and IgG binding to the FcγRIIa receptor. *Mol. Microbiol.* **59,** 212–230
48. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 175–177, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
49. Keane, F. M., Loughman, A., Valtulina, V., Brennan, M., Speziale, P., and Foster, T. J. (2007) Fibrinogen and elastin bind to the same region within the A domain of fibronectin-binding protein A, an MSCRAMM of *Staphylococcus aureus*. *Mol. Microbiol.* **63,** 711–723
50. O'Connell, D. P., Nanavaty, T., McDevitt, D., Gurusiddappa, S., Höök, M., and Foster, T. J. (1998) The fibrinogen-binding MSCRAMM (clumping factor) of *Staphylococcus aureus* has a Ca^{2+}-dependent inhibitory site. *J. Biol. Chem.* **273,** 6821–6829
51. Mulcahy, M. E., Geoghegan, J. A., Monk, I. R., O'Keeffe, K. M., Walsh, E. J., Foster, T. J., and McLoughlin, R. M. (2012) Nasal colonisation by *Staphylococcus aureus* depends upon clumping factor B binding to the squamous epithelial cell envelope protein loricrin. *PLoS Pathog.* **8,** e1003092
52. Vazquez, V., Liang, X., Horndahl, J. K., Ganesh, V. K., Smeds, E., Foster, T. J., and Hook, M. (2011) Fibrinogen is a ligand for the *Staphylococcus aureus* microbial surface components recognizing adhesive matrix molecules (MSCRAMM) bone sialoprotein-binding protein (Bbp). *J. Biol. Chem.* **286,** 29797–29805
53. Roche, F. M., Meehan, M., and Foster, T. J. (2003) The *Staphylococcus aureus* surface protein SasG and its homologues promote bacterial adher-

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ence to human desquamated nasal epithelial cells. *Microbiology* **149,** 2759-2767
54. de Haas, C. J., Veldkamp, K. E., Peschel, A., Weerkamp, F., Van Wamel, W. J., Heezius, E. C., Poppelier, M. J., Van Kessel, K. P., and van Strijp, J. A. (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J. Exp. Med.* **199,** 687–695
55. Deutsch, D. G., and Mertz, E. T. (1970) Plasminogen: purification from human plasma by affinity chromatography. *Science* **170,** 1095–1096
56. Speziale, P., Visai, L., Rindi, S., and Di Poto, A. (2008) Purification of human plasma fibronectin using immobilized gelatin and Arg affinity chromatography. *Nat. Protoc.* **3,** 525–533
57. Christensen, U., and Mølgaard, L. (1992) Positive co-operative binding at two weak lysine-binding sites governs the Glu-plasminogen conformational change. *Biochem. J.* **285,** 419–425
58. Váli, Z., and Patthy, L. (1982) Location of the intermediate and high affinity ω-aminocarboxylic acid-binding sites in human plasminogen. *J. Biol. Chem.* **257,** 2104–2110
59. Hussain, M., Becker, K., von Eiff, C., Schrenzel, J., Peters, G., and Herrmann, M. (2001) Identification and characterization of a novel 38.5-kilodalton cell surface protein of *Staphylococcus aureus* with extended-spectrum binding activity for extracellular matrix and plasma proteins. *J. Bacteriol.* **183,** 6778–6786

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Molecular Interactions of Human Plasminogen with Fibronectin-binding Protein B (FnBPB), a Fibrinogen/Fibronectin-binding Protein from *Staphylococcus aureus*

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Fibronectin Binding Proteins SpsD and SpsL Both Support Invasion of Canine Epithelial Cells by *Staphylococcus pseudintermedius*

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In this study, we investigated the cell wall-anchored fibronectin-binding proteins SpsD and SpsL from the canine commensal and pathogen *Staphylococcus pseudintermedius* for their role in promoting bacterial invasion of canine progenitor epidermal keratinocytes (CPEK). Invasion was examined by the gentamicin protection assay and fluorescence microscopy. An Δ*spsD* Δ*spsL* mutant of strain ED99 had a dramatically reduced capacity to invade CPEK monolayers, while no difference in the invasion level was observed with single mutants. *Lactococcus lactis* transformed with plasmids expressing SpsD and SpsL promoted invasion, showing that both proteins are important. Soluble fibronectin was required for invasion, and an RGD-containing peptide or antibodies recognizing the integrin $\alpha_5\beta_1$ markedly reduced invasion, suggesting an important role for the integrin in this process. Src kinase inhibitors effectively blocked internalization, suggesting a functional role for the kinase in invasion. In order to identify the minimal fibronectin-binding region of SpsD and SpsL involved in the internalization process, recombinant fragments of both proteins were produced. The $SpsD_{520-846}$ and $SpsL_{538-823}$ regions harboring the major fibronectin-binding sites inhibited *S. pseudintermedius* internalization. Finally, the effects of staphylococcal invasion on the integrity of different cell lines were examined. Because SpsD and SpsL are critical factors for adhesion and invasion, blocking these processes could provide a strategy for future approaches to treating infections.

The Gram-positive bacterium *Staphylococcus pseudintermedius* is a common commensal of dogs (1, 2). The bacterium is also the most common pathogen associated with canine otitis externa and pyoderma as well as surgical wound infections and urinary tract infections (3). Sporadic cases of human infection have also been reported, including some in individuals exposed to colonized household pets (4–7). Genome sequence analysis (8, 9) indicates that *S. pseudintermedius* could encode many potential virulence factors, including toxins, enzymes, and surface proteins, some of which can promote adhesion of the bacterium to the surface of epithelial cells (10–13) and to components of the extracellular matrix (14, 15).

Two cell wall-anchored surface proteins that are likely to be important in host tissue colonization and pathogenesis are SpsD and SpsL (Fig. 1) (15). The primary translation product of SpsD from strain ED99 has an N-terminal secretory signal sequence and a C-terminal cell wall-anchoring domain (the sorting signal) comprising an LPXTG motif, a hydrophobic transmembrane domain, and a short sequence rich in positively charged residues. Residues at the N terminus of SpsD are 40% identical to the fibrinogen-binding A domain of FnBPB from *Staphylococcus aureus* and are predicted to fold into three subdomains: N1, N2, and N3. This domain is followed by a connecting region, region C, and a repeat region, region R. SpsL includes a signal sequence at the N terminus followed by an A domain with two IgG-like folds (N2 and N3), a domain containing seven tandem repeats with weak homology to the fibronectin binding repeats of FnBPA from *S. aureus*, and a C-terminal sorting signal.

SpsD and SpsL mediate bacterial adherence to fibrinogen (15) and fibronectin (Fn) (15), while SpsD also binds to cytokeratin 10 and elastin (16). The binding site in fibrinogen for SpsD was mapped to residues 395 to 411 in the γ-chain, while a binding site for SpsD in Fn was localized to the N-terminal region. SpsD also binds to glycine- and serine-rich omega loops within the C-terminal tail region of cytokeratin 10 (16).

Another important Sps protein involved in host colonization is SpsO, which has been demonstrated to mediate adherence to *ex vivo* canine keratinocytes (12). However, the host ligand(s) interacting with SpsO remains to be determined (15).

The SpsO protein of *S. pseudintermedius* is also likely to be involved in colonization of the canine host. It promotes adhesion to *ex vivo* canine corneocytes, as does SpsD, although the ligand(s) recognized by SpsO remains to be identified. Invasive bacteria actively induce their own uptake by phagocytosis into normally nonphagocytic cells, where they establish a protected niche within which they can replicate (17). For example, *S. aureus*, usually considered an extracellular pathogen, can invade a variety of nonpro-

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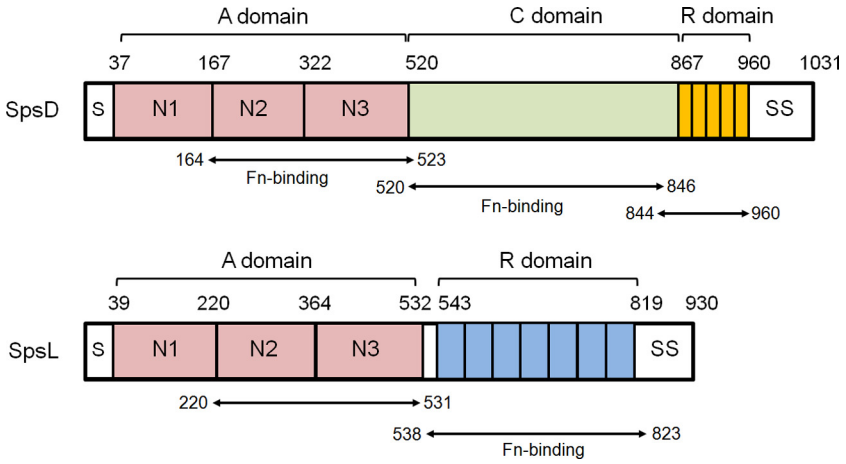

FIG 1 Schematic diagram of SpsD and SpsL proteins from *S. pseudintermedius* ED99. The A domain of SpsD spans residues 37 to 519 following the secretory signal sequence (S), followed by a connecting domain (C; residues 520 to 866) and a repeat region (R). A sorting signal (SS; LPXTG motif, hydrophobic domain, and positively charged residues) occurs at the extreme C terminus. SpsL includes a signal sequence (S) at the N terminus, followed by an A domain (residues 39 to 531), a domain containing seven tandem repeats (R; residues 543 to 818), and a C-terminal sorting signal (SS). The A domains of both proteins align with A domains of the MSCRAMM family of *S. aureus* surface proteins, and each comprises three subdomains, N1, N2, and N3, with N2 and N3 being predicted to form IgG-like folds. The A domains of SpsD and SpsL from ED99 have 30% identity and 50% similarity. The recombinant proteins are indicated, along with ability of each truncated form to bind to fibronectin.

fessional phagocytic cells, explaining its capacity to colonize mucosa and its persistence in tissue after bacteremia. The underlying major molecular mechanism of invasion involves the Fn-binding adhesins FnBPA and FnBPB (18, 19). Fn bridging between FnBPs and $\alpha_5\beta_1$ integrins on the host cell surface is sufficient to induce zipper-type uptake of staphylococci (18–20). The ternary complex promotes integrin clustering and a relay of signals that result in cytoskeletal rearrangements. The rearrangements are accompanied by endocytosis of *S. aureus* and internalization (17).

In this study, we wished to investigate whether *S. pseudintermedius* shares with *S. aureus* the ability to invade nonprofessional phagocytic cells and to determine the bacterial and host components that are involved. We reasoned that both SpsD and SpsL could be involved in the internalization of *S. pseudintermedius* by host cells. The objective of this study was to investigate internalization and its mechanistic basis. The analysis of this process will provide insights into the potential of a vaccine comprising components of SpsD and SpsL for the prevention of canine pyoderma.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. pseudintermedius* strain ED99 (formerly M732/99) was isolated from a canine bacterial pyoderma case presented to the Dermatology Service of The Hospital for Small Animals, Division of Veterinary Clinical Sciences, The Royal (Dick) School of Veterinary Studies, The University of Edinburgh. *S. pseudintermedius* strains 264, 324, 326, 327, 328, and 329 were isolated from cases of canine pyoderma and were a kind gift from Neil McEwan, University of Liverpool. *S. pseudintermedius* strains 81852, 91180, 253834, 237425, and 235214/1 were isolated from cases of canine pyoderma and were donated from Istituto Zooprofilattico Sperimentale della Lombardia e della Emilia Romagna, Pavia, Italy. The strains were classified as *S. pseudintermedius* using standard phenotypic tests (21). *S. pseudintermedius* ED99 and its mutants were grown in brain heart infusion (BHI) broth (VWR International Srl, Milan, Italy) at 37°C with shaking. Transformants of *Lactococcus lactis* harboring plasmid pOri23, pOri23::*spsD*, or pOri23::*spsL* (15) were grown in M17 medium (Difco, Detroit, MI, USA) supplemented with 10% lactose, 0.5% glucose, and 10 μg ml^{-1} erythromycin at 30°C without shaking. *Escherichia coli* DC10B (22) and TOPP3 (Stratagene, La Jolla, CA) were grown in Luria agar (LA) and Luria broth (LB) (VWR International Srl).

Reagents, proteins, and antibodies. Human fibronectin was purified from plasma by a combination of gelatin- and arginine-Sepharose affinity chromatography. The purity of the protein was assessed by 7.5% SDS-PAGE and Coomassie brilliant blue staining. To exclude the possibility of trace amounts of contaminants, affinity-purified fibronectin was spotted onto nitrocellulose membranes at different concentrations and overlaid with antifibrinogen and antiplasminogen antibodies (23). The N-terminal fragment of Fn (N29), containing the five N-terminal type I modules, and the gelatin-binding domain (GBD), consisting of four type I modules and two type II modules, were isolated as previously reported (24). Unless stated otherwise, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The anti-human Fn rabbit polyclonal IgG was purchased from Pierce (Rockford, IL, USA). The mouse monoclonal antibody JBS5 against the human integrin $\alpha_5\beta_1$ was purchased from Merck-Millipore (Darmstadt, Germany). The rabbit polyclonal antibody against the α_5 chain of the $\alpha_5\beta_1$ integrin and the mouse monoclonal antibodies BV7 against the human β_1 chain and B212 against the human β_3 chain of the integrin $\alpha_v\beta_3$ were a generous gift from G. Tarone (University of Turin, Italy). Mouse polyclonal antibodies against region A of SpsD and SpsL were prepared as previously reported (16).

DNA manipulation. DNA encoding regions $SpsD_{164-523}$, $SpsD_{520-846}$, $SpsD_{844-960}$, $SpsL_{220-531}$, and $SpsL_{538-823}$ were amplified by PCR using *S. pseudintermedius* ED99 genomic DNA as the template. Oligonucleotides were purchased from Integrated DNA Technologies (Leuven, Belgium) (see Table S1 in the supplemental material). Restriction enzyme cleavage sites (see Table S1) were incorporated at the 5′ ends of the primers to facilitate cloning into plasmid pQE30 (Qiagen, Chatsworth, CA, USA). Restriction enzymes were purchased from New England BioLabs (Hertfordshire, United Kingdom). The integrity of cloned DNA was confirmed by sequencing (Primmbiotech, Milan, Italy).

Expression and purification of recombinant proteins. Recombinant proteins were expressed from pQE30 in *E. coli* TOPP3 (Stratagene). Overnight starter cultures were diluted 1:50 in LB containing ampicillin (100 μg ml^{-1}) and incubated with shaking until the culture reached an optical density at 600 nm (OD_{600}) of 0.4 to 0.6. Recombinant protein expression was induced by addition of isopropyl 1-thio-β-D-galactopyranoside (0.5 mM) and continued for 2 h. Bacterial cells were harvested by centrifuga-

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TABLE 1 Plasmids used for the construction of *spsD*- and *spsL*-null mutants

Plasmid	Description	Reference
pIMAY	Thermosensitive plasmid for allelic exchange	21
pIMAY Δ*spsD*	pIMAY with fragments flanking *spsD*	This paper
pIMAY-Z	pIMAY derivative with a constitutive *lacZ* marker	25
pIMAY-Z Δ*spsL*	pIMAY-Z with fragments flanking *spsL*	This paper

tion and frozen at −80°C. Recombinant proteins were purified from cell lysates by Ni^{2+} affinity chromatography on a HiTrap chelating column (GE Healthcare, Buckinghamshire, United Kingdom). Protein purity was assessed to be 98% by SDS-PAGE, Coomassie brilliant blue staining, and densitometric analysis.

ELISA-type solid-phase binding assays. The ability of immobilized recombinant proteins to interact with soluble human Fn was determined using enzyme-linked immunosorbent assays (ELISAs). Microtiter wells were coated overnight at 4°C with 100 μl of 10 μg ml^{-1} of bacterial protein in 50 mM sodium carbonate, pH 9.5. To block additional protein-binding sites, the wells were treated for 1 h at 22°C with 200 μl of 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The plates were then incubated for 1 h with increasing amounts of Fn. One microgram of the specific anti-Fn rabbit IgG (1:2,000) in PBS with 0.1% BSA was added to the wells, followed by incubation for 90 min. After washing, the plates were incubated for 1 h with peroxidase-conjugated secondary anti-rabbit IgG diluted 1:1,000. After washing, *o*-phenylenediamine dihydrochloride was added and the absorbance at 490 nm was determined. To calculate the relative affinity association constant (K_A) values of each bacterial protein for Fn the following equation was employed: $A = A_{max}[L]K_A/(1 + K_A[L])$, where [L] is the molar concentration of ligand. The dissociation constants (K_D values) were calculated as reciprocals of the K_A values. The assays were performed at least 3 times for each protein.

Construction of *spsD*- and *spsL*-null mutants. Allele replacement mutagenesis of *spsD* and *spsL* was performed using the thermosensitive plasmids pIMAY and pIMAY-Z (Table 1). For generation of the *spsD*-null mutation, approximately 500-bp fragments of DNA flanking the gene were PCR amplified using the AB and CD primers, spliced together, and cloned into the blunt-end ligation pSC-B vector (StrataClone, Agilent Technologies, Santa Clara, CA) before subcloning into pIMAY to produce the pIMAY Δ*spsD* construct. The plasmid was transformed into *E. coli* DC10B before being electrotransformed into *S. pseudintermedius* ED99 at 28°C with selection on 10 μg ml^{-1} chloramphenicol, as previously described for *S. aureus* (25). For generation of the *spsL*-null mutation, a sequence ligase-independent cloning (SLIC) protocol was performed (26) using pIMAY-Z, a derivative of pIMAY with a constitutive *lacZ* marker, to construct pIMAY-Z Δ*spsL*. Once the plasmids were transformed into ED99 at 28°C, growth at the restrictive temperature of 37°C selected for integrants. OUT primers, located outside the flanking regions and gene of interest, were used to determine if integration had occurred upstream or downstream of the chromosomal gene (22). A single colony from each site of integration was inoculated into broth and grown at 28°C and then diluted and grown at 37°C. The *S. aureus* antisense *secY* mechanism within pIMAY (27) was nonfunctional in *S. pseudintermedius*, and the *lacZ* marker was ineffective because plasmid-free cells expressed endogenous β-galactosidase activity. Allele exchange was confirmed by using OUT primer PCR and sequencing the resultant fragment (see Table S2 in the supplemental material).

Release of surface proteins from *S. pseudintermedius* and *L. lactis*. *S. pseudintermedius* and *L. lactis* cells were grown to an OD_{600} of 0.4 to 0.6. Cells were harvested by centrifugation at 7,000 × *g* at 4°C for 15 min, washed 3 times with PBS, and resuspended at an OD_{600} of 40 in lysis buffer (50 mM Tris-HCl, 20 mM $MgCl_2$ [pH 7.5]) supplemented with 30% raffinose. Cell wall proteins were solubilized from *S. pseudintermedius* by incubation with lysostaphin (200 μg ml^{-1}) and from *L. lactis* with mutanolysin (1,000 U/ml) and lysozyme (900 μg ml^{-1}) at 37°C for 20 min in the presence of protease inhibitors (Complete Mini; Roche Molecular Biochemicals, Indianapolis, IN, USA). Protoplasts were recovered by centrifugation at 6,000 × *g* for 20 min, and the supernatants were taken as the wall fractions. The material obtained from *S. pseudintermedius* ED99 and its mutants was adsorbed on IgG-Sepharose columns before Western immunoblotting analysis to remove IgG-binding proteins that would otherwise interfere with the specific antibody staining.

SDS-PAGE and Western immunoblotting. Samples for analysis by SDS-PAGE were boiled for 5 min in sample buffer (0.125 M Tris-HCl, 4% [wt/vol] SDS, 20% [vol/vol] glycerol, 10% [vol/vol] β-mercaptoethanol, 0.002% [wt/vol] bromophenol blue) and separated on 10% (wt/vol) polyacrylamide gels. The gels were stained with Coomassie brilliant blue (Bio-Rad, Hercules, CA, USA). For Western immunoblotting, material was subjected to SDS-PAGE and then electroblotted onto a nitrocellulose membrane (GE Healthcare). The membrane was blocked overnight at 4°C with 5% (wt/vol) skim milk in PBS, washed, and incubated with mouse polyclonal antibody against region A of SpsD or SpsL (1 μg ml^{-1}) for 1 h at 22°C. Following additional washings with 0.5% (vol/vol) Tween 20 in PBS (PBST), the membrane was incubated for 1 h with horseradish peroxidase-conjugated rabbit anti-mouse IgG. Finally, blots were developed using the ECL Advance Western blotting detection kit (GE Healthcare) and an ImageQuantTM LAS 4000 mini-biomolecular imager (GE Healthcare).

Mammalian cell lines and culture conditions. Canine progenitor epidermal keratinocytes (CPEK) were cultured in CnT-0.9 medium (CELLnTEC, Bern, Switzerland), without antibiotics at 37°C in 5% CO_2. The spontaneously immortalized keratinocytes (HaCaT) and the human epithelial cell line HEp-2 were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (EuroClone, Milan, Italy), 2% penicillin and streptomycin, 2% sodium pyruvate, and 2% L-glutamine at 37°C in 5% CO_2. Cells were cultured in T75 flasks to approximately 95% confluence, liberated with trypsin-EDTA (EuroClone), resuspended in invasion medium (growth medium without antibiotics), and plated as reported below for the cell invasion assay.

Cell invasion assay. Cell invasion assays were performed essentially as described previously (28). Briefly, cells were plated at 5 × 10^5 (in 0.4 ml invasion medium) into 24-well plates (Corning) and allowed to attach for 24 h at 37°C. Staphylococcal cultures were grown overnight in BHI at 37°C with shaking. *L. lactis* was grown overnight in M17 broth at 30°C without shaking. The following day, cultures were diluted 1:40 in fresh BHI or M17 medium, respectively, grown to an OD_{600} of 0.4, washed 3 times in PBS, and diluted to obtain 10^7 cells/ml in CnT-BM.2 medium supplemented with 10% FBS plus 2 mM L-glutamine. Bacterial suspensions (1 ml) were added to each well and the plates incubated for 2 h at 37°C. Monolayers were then washed 3 times in PBS to remove unattached bacteria. Medium containing antibiotics (200 μg ml^{-1} gentamicin plus 2% penicillin and streptomycin) was added, and the plate was incubated for an additional 2 h to kill extracellular bacteria. The wells were washed again, and internalized bacteria were released by incubating with 200 μl of H_2O containing 0.1% (vol/vol) Triton X-100. Serial dilutions of the cell lysates were plated in duplicate on BHI agar, and CFU were counted after incubation. All assays were carried out in triplicate. Samples of monolayers were lysed prior to inoculation and plated on BHI agar, and the absence of staphylococcal colonies was noted.

Inhibition of invasion. The Src-family kinase inhibitors PP2, PP3, and CGP77675 (25 μM) were dissolved in dimethyl sulfoxide (DMSO), added to the cell medium at the appropriate concentrations, and preincubated with monolayers for 1 h at 37°C in 5% CO_2 before addition of bacteria. Likewise, wortmannin (20 nM), genistein (200 μM), and cy-

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tochalasin D (50 μM) were dissolved in PBS and incubated with cells for 60 min prior to the addition of bacteria. Gentamicin protection assays were then performed as described above, except that no intermediate washing was carried out. To test cell viability during exposure to the Src inhibitors, the compounds were added to cell monolayers for 3 h at 37°C. Then the cells were gently washed with DMEM, trypsinized, and mixed with an equal volume of trypan blue (0.5% [vol/vol] in PBS) for 5 min. Ten microliters of the mixture was placed on a Neubauer chamber, and stained cells were counted by light microscopy. The percentage of dead cells was calculated by dividing the mean number of dead (stained) cells by the total number of cells in 50 microscopic fields and multiplying the result by 100.

Fluorescence microscopy. Bacteria were grown to an OD_{600} of 0.3 (*S. pseudintermedius*) or 0.4 (*L. lactis*), centrifuged, and resuspended in 100 μl PBS. Then, 0.5 μl 10 mM calcein-AM (Molecular Probes, Eugene, OR, USA) was added and incubated for 1 h at 37°C (*S. pseudintermedius*) or 2 h at 30°C (*L. lactis*). Stained bacteria were washed 3 times with PBS and resuspended in 1 ml PBS. Suspensions (100 μl) were added to CPEK monolayers and incubated for 2 h at 37°C to allow internalization. Cells were washed with PBS, counterstained for 1 to 3 min with ethidium bromide (10 μg ml^{-1}), and washed again. Fluorescence microscopy (Olympus BX51; Olympus, Segrate, Italy) was performed using a green filter, a red filter, and white light. Images were captured with a charge-coupled device (CCD) camera and assembled using Adobe Photoshop Creative Suite 2.

Staining of monolayers. Mammalian cells were stained with Giemsa stain modified solution (Sigma) according to the manufacturer's instructions and observed under a light microscope at a magnification of ×20.

Invasion assays with formaldehyde-fixed staphylococci. To perform invasion assays with killed bacteria, staphylococci were fixed in 0.5% formaldehyde in PBS for 1 h, stained with calcein-AM, and subjected to fluorescence microscopy. Alternatively, to analyze the effect of formaldehyde-fixed staphylococci on cell survival, monolayers were stained with Giemsa and observed as reported above.

MTT assay. The MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] tetrazolium reduction assay was performed according to the manufacturer's instructions (Sigma).

Statistical methods. Continuous data were expressed as means and standard deviations. Two-group comparisons were performed by Student's *t* test. One-way analysis of variance, followed by Bonferroni's *post hoc* tests, was exploited for comparison of three or more groups. Analyses were performed using Prism 4.0 (GraphPad). Two-tailed *P* values of 0.001 were considered statistically significant.

RESULTS

SpsD and SpsL binding to fibronectin. To localize the Fn-binding sites in SpsD and SpsL, recombinant domains were obtained following PCR amplification of genomic DNA from strain ED99. The cloned SpsD domains included the minimum fibrinogen-binding region (residues 164 to 523) ($SpsD_{164–523}$), a connecting region (region C, residues 520 to 846) ($SpsD_{520–846}$), and a repeat region (region R, residues 844 to 960) ($SpsD_{844–960}$). Two recombinant SpsL domains were expressed: the N-terminal region encompassing residues 220 to 531 ($SpsL_{220–531}$) and the repetitive domain spanning residues 538 to 823 ($SpsL_{538–823}$). As shown in ELISA-type solid-phase binding assays (Fig. 2), recombinant $SpsD_{520–846}$ and $SpsL_{538–823}$ regions bound Fn dose dependently and saturably, while no binding was exhibited by $SpsL_{220–531}$ or $SpsD_{844–960}$. $SpsD_{520–846}$ and $SpsL_{538–823}$ bound Fn in the low nanomolar range ($SpsD_{520–846}$ $K_D = 1.7 \pm 0.38$, $SpsL_{538–823}$ $K_D = 0.81 \pm 0.02$ nM), while $SpsD_{164–523}$ gave a half-maximal binding of 2.19 ± 0.47 μM (data not shown).

Invasion of mammalian cells by *S. pseudintermedius*. Several *S. pseudintermedius* isolates were found to invade canine keratinocyte-derived CPEK. The magnitude of invasion was very similar to or even higher than that of the archetypal invasive *S. aureus* strain Cowan 1 (Fig. 3). Thus, invasion of CPEK is a general property of *S. pseudintermedius*.

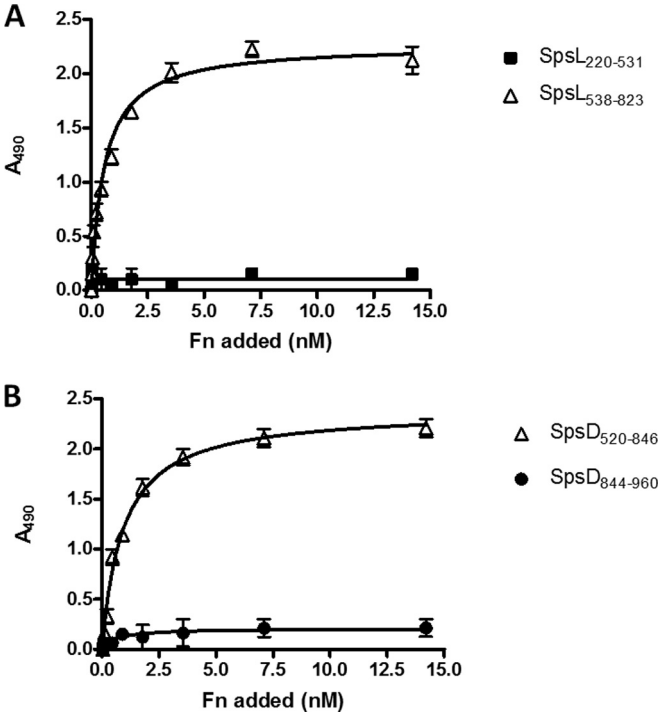

FIG 2 Dose-dependent binding of fibronectin to SpsD and SpsL fragments in an ELISA-type assay. Microtiter wells were coated with $SpsD_{520–846}$, $SpsD_{844–960}$, $SpsL_{220–531}$, and $SpsL_{538–823}$. The wells were probed with increasing amounts of Fn, followed by incubation with rabbit anti-Fn IgG and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. The graph is representative of three experiments, with each point representing the average for triplicate wells.

Requirement for fibronectin for efficient invasion by *S. pseudintermedius* ED99. To investigate the role of soluble plasma-derived Fn in invasion, FBS was passed over a gelatin-Sepharose column to remove soluble Fn before being used in the invasion assay. An 85% reduction in the level of invasion of CPEK was observed when the Fn-depleted FBS was used in the invasion medium compared to unadsorbed FBS. The addition of human Fn to a final concentration of 1 μg ml^{-1} was sufficient to restore the level of invasion to that observed in the presence of whole FBS (Fig. 4A). This suggests that *S. pseudintermedius* strain ED99 can use soluble plasma-derived Fn in the invasion process. Removal of FBS from the assay reduced the invasion level by 95%, suggesting that additional minor components in the FBS other than Fn might contribute. However, although removal of Fn from the gelatin-adsorbed FBS was shown by ELISA and Western blotting, residual internalization can be due to trace amounts of Fn remaining in the invasion medium.

When bacteria were preincubated with increasing amounts of the N29 fragment of Fn and then tested for adherence to or invasion of CPEK, we observed an almost complete inhibition of bacterial internalization. Conversely, no effect was observed when the invasion assay was performed with a high concentration of the GBD of Fn (Fig. 4B). Together, these findings indicate that the N29 domain is specifically involved in adhesion to and invasion of CPEK.

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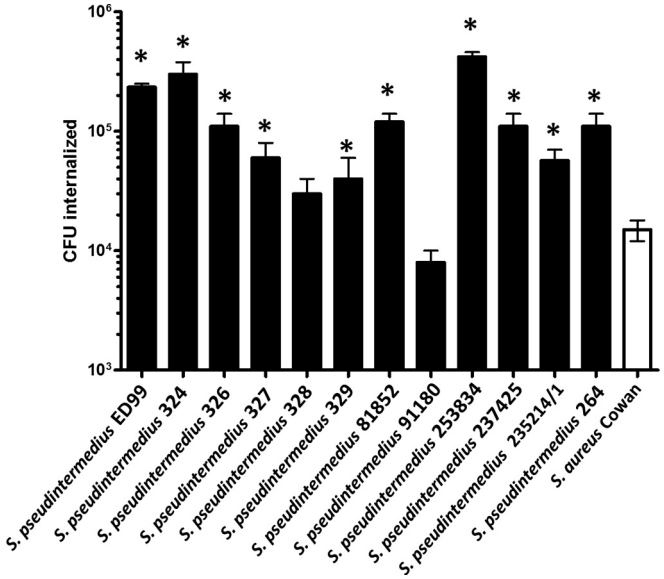

FIG 3 Invasion of CPEK monolayers by *S. pseudintermedius* strains. *S. pseudintermedius* cells were incubated with CPEK monolayers. Extracellular bacteria were killed with gentamicin, and internalized bacteria were quantified by plating lysates on BHI agar. The assay was performed three times. Each point represents the average value for three replicas, and error bars show the standard deviations (SD). Statistically significant ($P < 0.05$; Student's *t* test) differences in values compared with the control value (*S. aureus* Cowan 1) are indicated by an asterisk. The absence of intracellular bacteria in cell cultures was established by lysing samples of the confluent monolayers and plating on BHI agar prior to staphylococcal inoculation.

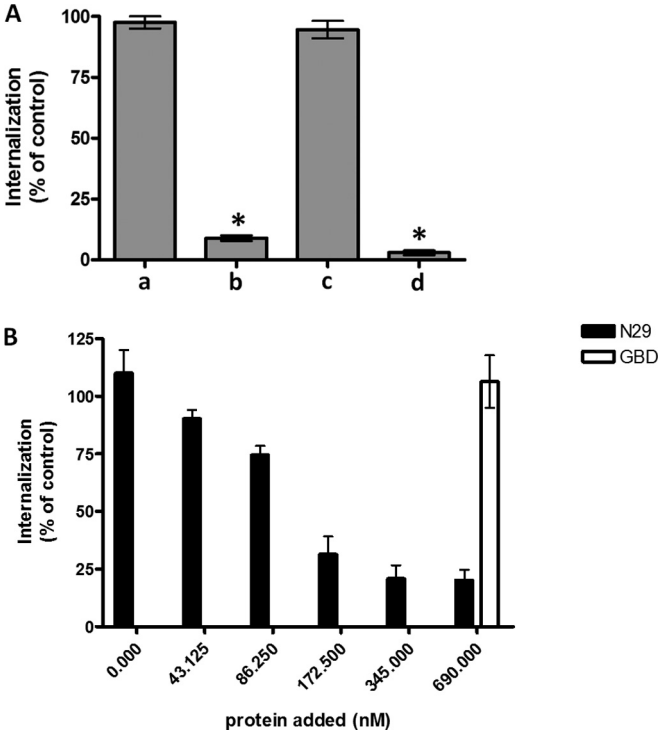

FIG 4 Role of soluble fibronectin on invasion by *S. pseudintermedius*. (A) To evaluate the effect of endogenous fibronectin, the invasion assay was performed in the presence of 10% FBS (a), 10% Fn-depleted FBS (b), and 10% Fn-depleted FBS plus 1 μg/ml soluble human plasma Fn (c) and in the absence of FBS (d). Bacteria were incubated with CPEK monolayers, and internalized bacteria were quantified as described in the legend to Fig. 3. Invasion is expressed as a percentage of that observed in the presence of 10% whole FBS (control; 2×10^5 CFU). Each point represents the average value for three replicates, and error bars represent SD from three independent experiments performed in triplicate. Statistically significant ($P < 0.01$; Student's *t* test) differences in values compared with control value in the presence of FBS are indicated by an asterisk. (B) To determine the effect of the N29 fragment, staphylococci were incubated with increasing concentrations of N29 or 650 nM GBD for 30 min at 22°C. Bacteria were then added to CPEK monolayers and incubated at 37°C for 2 h, and internalized bacteria were quantified as described in the legend to Fig. 3. Invasion is expressed as a percentage of that observed in the absence of inhibitors (control; 2.5×10^5 CFU). Values and error bars represent means and SD from three independent experiments performed in triplicate.

Invasion of CPEK by ED99 mutants and *L. lactis* expressing SpsD or SpsL. Mutants of *S. pseudintermedius* ED99 deficient in SpsD and SpsL were tested for their ability to attach to surface-coating Fn. Mutants defective in either SpsD or SpsL alone adhered as well as the parental strain, while the double mutant defective in both proteins did not bind at all (data not shown). The absence of SpsD or SpsL proteins was confirmed by testing material solubilized from the cell wall with lysostaphin by Western blotting and probing with antibodies against region A of SpsD or SpsL. Both the proteins were absent from the double mutant (Fig. 5A). Conversely, SpsL was expressed normally by the SpsD mutant and *vice versa*.

The SpsD- and SpsL-defective mutants were also tested for invasiveness. Single mutants retained the ability to invade CPEK at the same level as the wild type, while the double mutant lacking both SpsD and SpsL invaded at a much lower level (Fig. 5B).

In order to test whether coreceptors are required for SpsD- or SpsL-mediated invasion, we expressed both *S. pseudintermedius* proteins individually in *Lactococcus lactis* (Fig. 6A). Transformants of *L. lactis* carrying plasmid pOri23::*spsD* and pOri23::*spsL* (Fig. 6A) showed invasiveness similar to that of the wild-type strain ED99 (Fig. 5B), while very low internalization by CPEK was observed with *L. lactis* harboring the empty plasmid (Fig. 6B).

Reduced invasion by the double mutant of ED99 was also assessed by visualizing uptake into CPEK by fluorescent imaging. Bacteria were stained with calcein-AM (green) prior to CPEK invasion, and at the assay endpoint, the fluorescence of external bacteria was visualized with ethidium bromide (red). As shown in Fig. 5C, the wild type and the single mutant strains were observed inside CPEK, while no green fluorescence, indicative of the internalized bacteria, was detected when the double mutant was tested. *L. lactis* expressing SpsL or SpsD behaved similarly (Fig. 6C). Together these results demonstrate that expression of a single adhesin (SpsD or SpsL) is sufficient to confer efficient uptake of bacteria into CPEK.

Localization of Sps domains promoting invasion of CPEK. To identify the domains of SpsD and SpsL that are involved in invasion, recombinant fragments were assessed for inhibition of *S. pseudintermedius* ED99 uptake into CPEK. We found that $SpsD_{520-846}$ (Fig. 7A) and $SpsL_{538-823}$ (Fig. 7B) strongly inhibited internalization, whereas $SpsD_{164-523}$ showed a weak inhibitory effect. The inhibitory effects exhibited by these proteins correlate with their affinities for fibronectin. $SpsL_{220-631}$ and $SpsD_{844-960}$ used at the same concentrations did not interfere with staphylococcal invasion.

Dependence of invasion on integrin $\alpha_5\beta_1$. Immunofluores-

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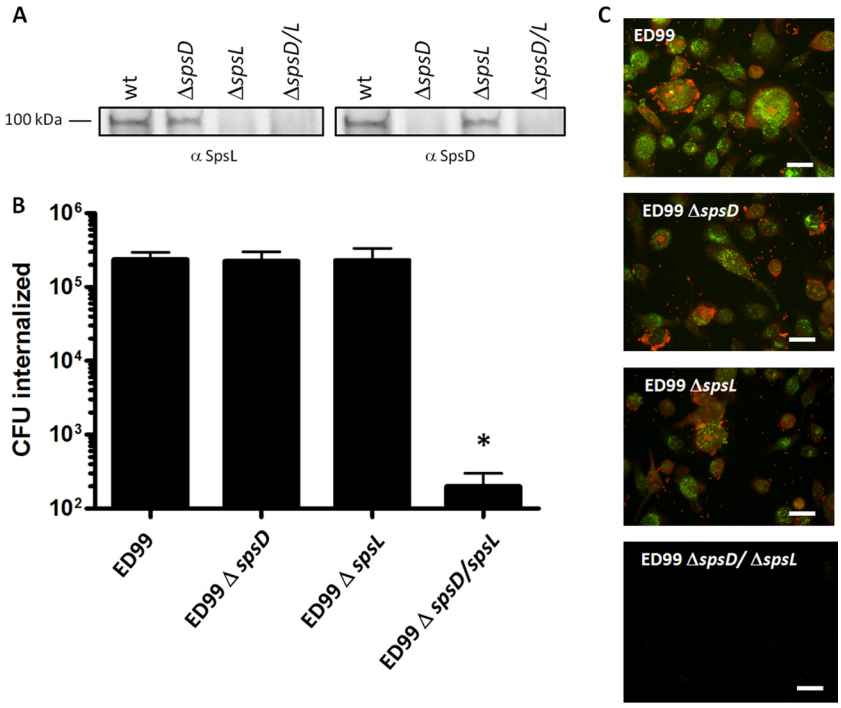

FIG 5 Invasion of CPEK monolayers by *S. pseudintermedius* and its mutants. (A) Expression of SpsD and SpsL proteins. Cell wall proteins from the wild type and mutants were solubilized with lysostaphin, separated by SDS-PAGE and analyzed by Western immunoblotting using mouse anti-SpsD or anti-SpsL and HRP-labeled rabbit anti-mouse IgG. (B) *S. pseudintermedius* wild-type and the Δ*spsD* Δ*spsL* mutant were incubated with CPEK monolayers, and internalized bacteria were quantified as described in the legend to Fig. 3. Bars and error bars show means and SD from three independent determinations performed in triplicate. An asterisk indicates a significant difference ($P < 0.05$; Student's *t* test) compared with the control (invasion with the wild-type strain). (C) Fluorescence microscopy investigating the contribution of SpsD or SpsL to interactions with CPEK. Confluent CPEK monolayers were incubated with calcein-AM-labeled *S. pseudintermedius* to allow internalization, washed with PBS, and counterstained with ethidium bromide. Green fluorescence represents intracellular staphylococci, and red fluorescence represents extracellular bacteria. Bar, 40 μm.

cent antibodies that specifically bind to the α_5 subunit of the human Fn-binding $\alpha_5\beta_1$ integrin stained CPEK, suggesting that the canine cells express an $\alpha_5\beta_1$ integrin that is closely related to the human integrin (Fig. 8A, inset). To test the role of the $\alpha_5\beta_1$ integrin in invasion, CPEK were preincubated with function-blocking anti-$\alpha_5\beta_1$ IgG prior to the addition of *S. pseudintermedius*. Antibodies recognizing the α_5 and the β_1 chains both reduced internalization of *S. pseudintermedius* by more than 80%, whereas antibodies against the β_3 chain of the human $\alpha_v\beta_3$ integrin did not alter invasion (Fig. 8A). This indicates that the $\alpha_5\beta_1$ integrin on canine CPEK is responsible for Fn-mediated bacterial invasion.

Inhibition of invasion by an RGD-containing peptide. The $\alpha_5\beta_1$ integrin recognizes the tripeptide sequence RGD within the cell-binding domain of Fn (29, 30). To investigate the role of this interaction in invasion of *S. pseudintermedius*, the effect of the RGDS peptide was analyzed. Incubation of CPEK with the RGDS peptide reduced the level of invasion by strain ED99 in a dose-dependent manner, while a control peptide RGES had no inhibitory effect (Fig. 8B). This suggests that the interaction of $\alpha_5\beta_1$ with Fn is necessary for efficient invasion of CPEK.

Protein phosphorylation during *S. pseudintermedius* invasion. To identify changes in host cell signaling associated with staphylococcal invasion, the assay was performed in the presence of protein tyrosine phosphorylation inhibitors. Genistein, a tyrosine kinase inhibitor, strongly inhibited internalization, whereas wortmannin, an inhibitor of the phosphatidylinositol-3-phosphate kinase, did not (Fig. 8C). We also tested Src kinase inhibitors and found that both CGP77675 and PP-2 inhibited *S. pseudintermedius* internalization into CPEK. While both inhibitors effectively blocked internalization, CGP77675 appeared to be a more potent inhibitor than PP-2. PP-3, a compound similar to PP-2 but with no significant Src inhibitory activity, had no effect on internalization (Fig. 8C). At the concentrations used, the inhibitors did not affect bacterial adhesion to the CPEK or cause loss of viability, as shown by trypan blue staining (data not shown). To investigate a possible role for actin cytoskeleton rearrangements in *S. pseudintermedius* invasion, we tested cytochalasin D, which interferes with F-actin polymerization. Pretreatment of CPEK with 1 μg/ml cytochalasin D almost completely abolished invasion, demonstrating the involvement of actin cytoskeletal rearrangements (Fig. 8C).

Invasion of Hep-2 and HaCaT cell lines by *S. pseudintermedius*. Human-derived Hep-2 and HaCaT cells were efficiently invaded by *S. pseudintermedius*, and invasion was dependent on Sps proteins. In addition, as reported for CPEK, internalization required the presence of Fn and involved the $\alpha_5\beta_1$ integrin (see Fig. S1 and S2 in the supplemental material).

Alterations to cell monolayers following internalization by *S. pseudintermedius*. To investigate alterations to CPEK, Hep-2, and HaCaT cells following *S. pseudintermedius* invasion, cell monolayers were infected with *S. pseudintermedius* strain ED99 for 2 h prior to gentamicin treatment. Then the cells were incubated for 4 h and 36 h, fixed, and analyzed for morphological changes by light microscopy. A remarkable difference in mor-

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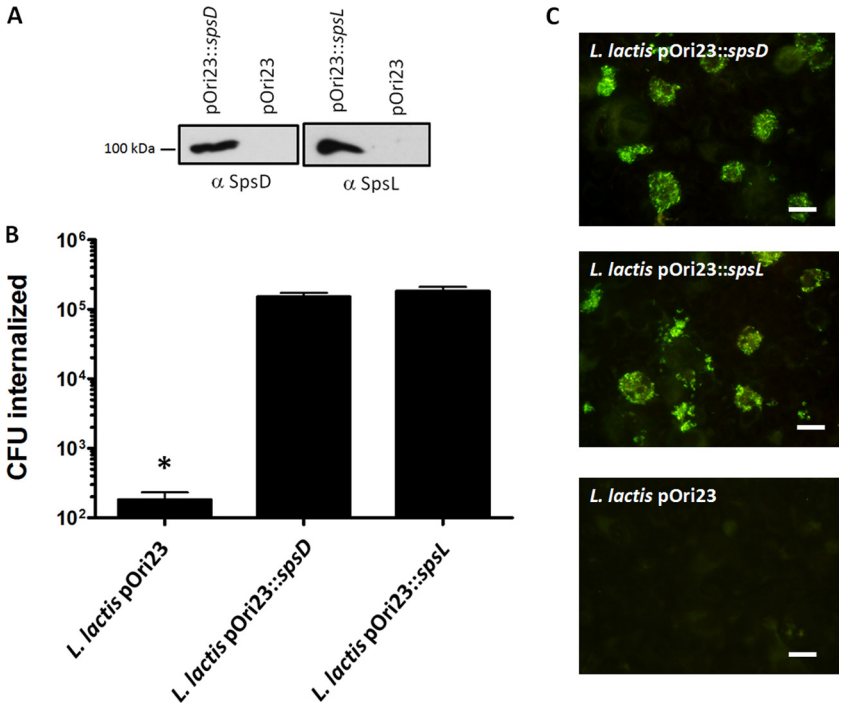

FIG 6 Invasion of CPEK monolayers by *L. lactis*. (A) Expression of SpsD and SpsL by *L. lactis*. Cell wall proteins were solubilized with mutanolysin and lysostaphin, separated by SDS-PAGE, and analyzed by Western immunoblotting using mouse anti-SpsD or anti-SpsL as the primary antibody and HRP-labeled rabbit anti-mouse IgG. (B) CPEK invasion by *L. lactis* expressing SpsD or SpsL. *L. lactis* were incubated with CPEK monolayers, and internalized bacteria were measured as indicated above. Error bars show SD of the means from three independent determinations performed in triplicate. An asterisk indicates a significant difference ($P < 0.05$; Student's *t* test) compared with *L. lactis* pOri23::*spsD* or pOri23::*spsL*. (C) Fluorescent imaging investigating the contribution of SpsD or SpsL to interactions with CPEK monolayers. Confluent CPEK monolayers were incubated with calcein-AM-labeled *L. lactis* to allow internalization. Fluorescence was visualized as in Fig. 5. Bar, 40 μm.

phology was observed between infected and uninfected cell monolayers. Internalization of bacteria by CPEK caused cell detachment and a reduction of the cell density (Fig. 9A). Incubation of Hep-2 cells with strain ED99 for 36 h resulted in rounding and detachment of the cells (see Fig. S3A in the supplemental material). In contrast, HaCaT cells showed a pattern similar to that exhibited by uninfected cells (see Fig. S3C).

The assessment of the cell growth and survival of infected cells by the MTT assay showed that staphylococcal invasion substantially reduced the viability of CPEK (Fig. 9B) and Hep-2 (see Fig. S3B in the supplemental material) cells, whereas HaCaT cells survived to a level comparable to that of the uninfected cells (see Fig. S3D). To further investigate the contribution of bacterial invasion to cell damage, all the cell lines were incubated with the Δ*spsD* Δ*spsL* mutant. Both morphological observations and MTT assays showed that the double mutant did not affect the viability of cells.

DISCUSSION

In this study, we analyzed the molecular mechanism by which *S. pseudintermedius* adheres to and invades canine keratinocytes (CPEK) and the effects of internalization on the viability of the mammalian cells. We found that all strains of *S. pseudintermedius* tested invaded CPEK efficiently. Importantly, we found that both the cell wall-anchored surface proteins SpsD and SpsL efficiently promoted invasion of strain ED99. Single mutants defective in either SpsD or SpsL alone showed no reduction in invasion. Only the double mutant lacking both proteins was defective. Conversely, both SpsD and SpsL promoted efficient uptake of the non-invasive surrogate host *L. lactis* when expressed ectopically from recombinant plasmids. Subdomains within SpsD and SpsL were expressed as recombinant proteins, which allowed identification of regions with a high affinity for Fn and which also strongly inhibited bacterial invasion. Invasion of CPEK was dependent on the presence of Fn, as demonstrated by the markedly reduced uptake upon removal of Fn from the cell culture medium (fetal bovine serum) and the restoration of invasion by supplementation with purified human Fn. We recognize the limitation of using human and bovine Fn to assess the role of this protein in bacterial invasion of a canine cell line. However, it should be noted that there is a high level of similarity between human, bovine, and canine Fn (93 to 94% identity, 98% similarity), so that the use of human or bovine Fn is valid.

In *S. aureus* Fn-binding proteins FnBPA and FnBPB both promote invasion of mammalian cells, where Fn acts as a bridge between the bacterial surface protein which binds to the N-terminal N29 domain by the tandem β zipper mechanism and the $\alpha_5\beta_1$ integrin, which recognizes an RGD motif within C-terminal repeat 10 of Fn (18, 31). The finding that the N-terminal region of Fn and an RGD-containing peptide inhibited *S. pseudintermedius* invasion of CPEK strongly suggests that the same mechanism is employed and involves the Fn binding domains of SpsD and SpsL. Inhibition of invasion of CPEK by monoclonal antibodies recognizing epitopes in the human $\alpha_5\beta_1$ integrin strongly suggests that the canine CPEK express an immuno-cross-reactive integrin that is responsible for bacterial adhesion and invasion.

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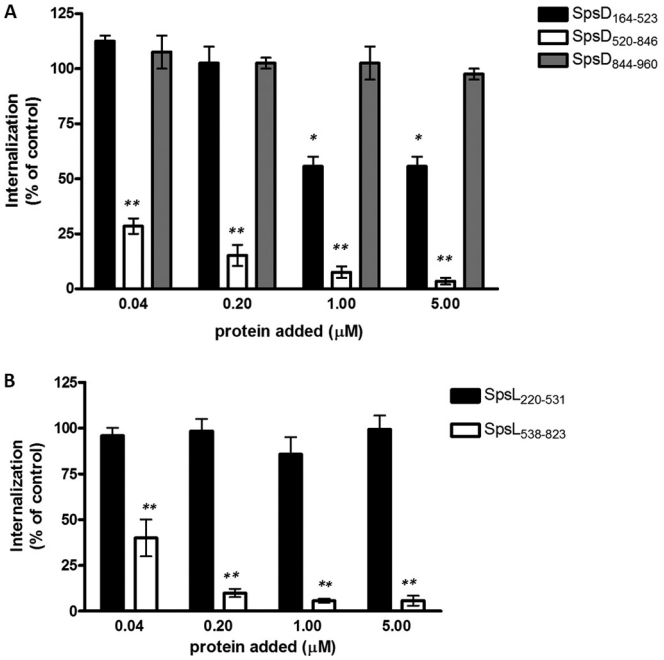

FIG 7 Effect of SpsD and SpsL fragments on invasion of CPEK by *S. pseudintermedius*. CPEK monolayers were incubated with increasing concentrations of $SpsD_{164-523}$, $SpsD_{520-846}$, or $SpsD_{844-960}$ (A) or $SpsL_{220-531}$ or $SpsL_{538-823}$ (B) prior to addition of bacteria. Invasion is expressed as a percentage of that observed in the absence of potential inhibitors (control; 2.2×10^5 CFU). Error bars show SD of the means from three independent determinations performed in triplicate. Statistically significant differences are indicated (Student's two-tailed *t* test; *, $P < 0.05$; **, $P < 0.001$).

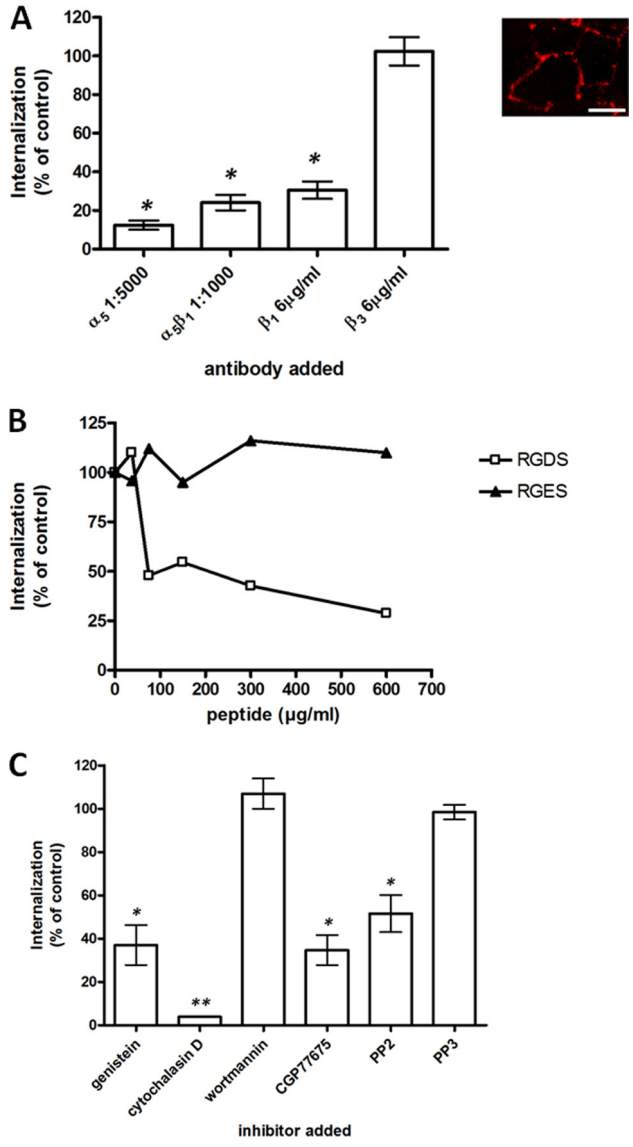

FIG 8 (A) Effect of antiintegrin antibodies on invasion of CPEK by *S. pseudintermedius*. CPEK monolayers were incubated with antibodies against $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins prior to the addition of bacteria. After incubation, internalized bacteria were quantified as described above. Invasion is expressed as a percentage of that observed in the absence of antibodies (control; 2.8×10^5 CFU). Bars and error bars represent the means and SD from three independent determinations performed in triplicate. Statistically significant differences are indicated (Student's two-tailed *t* test; *, $P < 0.05$). The inset shows expression of $\alpha_5\beta_1$ integrin by CPEK by staining with immunofluorescent antibodies that specifically bind to the α_5 subunit of the $\alpha_5\beta_1$ integrin. Bar, 40 μm. (B) Effect of an RGD-containing peptide on invasion of CPEK by *S. pseudintermedius*. CPEK were incubated with increasing concentrations of the RGDS or RGES peptide prior to addition of bacteria. After incubation, internalized bacteria were quantified as described above. Invasion is expressed as a percentage of that observed in the absence of peptides (control; 2.3×10^5 CFU). Values are means from three independent determinations performed in triplicate. (C) Effect of kinase inhibitors on invasion of CPEK by *S. pseudintermedius*. CPEK were exposed to genistein, CGP77675, PP2, PP3, wortmannin, and cytochalasin D for 1 h before addition of bacteria. Invasion assays were performed on inhibitor-treated cells three times with similar results. Invasion is expressed as a percentage of that observed in the absence of inhibitors (control; 2.1×10^5 CFU). Bars and error bars represent the means and SD from three independent determinations performed in triplicate. Statistically significant differences are indicated (Student's two-tailed *t* test; *, $P < 0.05$; **, $P < 0.001$).

The Fn bridging mechanism for attachment to and invasion of mammalian cells results from integrin-initiated actin polymerization stimulated by receptor clustering and cell signaling events involving Src (32, 33). Here, invasion of *S. pseudintermedius* was strongly reduced by the Src-specific inhibitors CGP77675 and PP-2, implying that similar mechanisms are responsible. Similar results were obtained when human Hep-2 epithelial cells and HaCaT keratinocytes were tested for invasion by *S. pseudintermedius* ED99. However, certain differences were observed compared to invasion of CPEK. A 10-fold-smaller inoculum was needed for efficient invasion of Hep-2 cells than for HaCaT and CPEK. We speculate that the differences in invasion efficiencies might be due to variations in the density of the $\alpha_5\beta_1$ integrins, although other factors could be involved. Together, these data demonstrate that *S. pseudintermedius* employs a mechanism of host cell invasion similar to that used by *S. aureus* that involves bacterial surface proteins binding to Fn and uptake mediated by integrin $\alpha_5\beta_1$.

Invasion of CPEK and Hep-2 cells resulted in cells detaching and losing viability, whereas HaCaT cells remain unchanged. Thus, in the first two cell lines, invasion by *S. pseudintermedius* triggers a reduction in cell viability. Formaldehyde-killed bacterial cells were actively internalized by the mammalian cells, suggesting that no active expression of invasion-promoting factors was necessary to achieve invasion. Conversely, the lack of effects on host cell survival by killed bacterial cells indicates that additional factors such as secreted toxins are required to induce cell death.

Membrane-damaging toxins that are expressed by intracellular *S. aureus* are major factors in promoting apoptosis (34). *S. pseud-*

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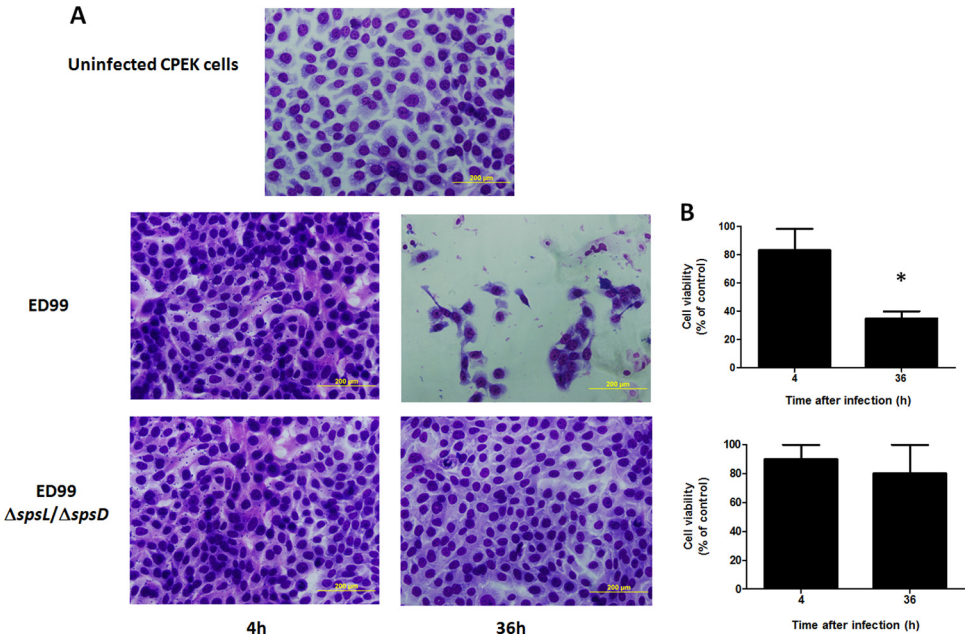

FIG 9 Effects on CPEK following invasion. (A) Alterations to CPEK monolayers after infection with *S. pseudintermedius* and the Δ*spsD* Δ*spsL* mutant. Monolayers were infected with *S. pseudintermedius* prior to gentamicin treatment. Then, the cells were incubated for 4 h and 36 h, stained with Giemsa, and observed for morphological changes by light microscopy. Bars, 200 μm. (B) Assessment of survival of infected cells. Confluent CPEK were infected for 2 h with *S. pseudintermedius* ED99 or the Δ*spsD* Δ*spsL* mutant and then examined at 4 h and 36 h after infection for viability by the MTT assay. Viability was assessed as the percentage of absorbance at 575 nm of treated cells relative to that of solvent-treated controls. Bars and error bars represent the means and SD from three independent determinations performed in triplicate. Statistically significant differences are indicated (Student's two-tailed *t* test; *, $P < 0.05$).

intermedius has the potential to express a bicomponent leukotoxin, Luk-I, which is similar to the Panton-Valentine leucocidin (PVL) of *S. aureus* (35), as well as a homologue of β-toxin and a putative hemolysin (hemolysin III) (8). It can be hypothesized that at least one of these factors is responsible for inducing cell death in CPEK. Indeed, PVL facilitates escape of *S. aureus* from human keratinocyte endosomes and induces apoptosis (34), which might indicate a role here for Luk-I. Studies with mutants lacking one or more of the toxins will help clarify this point.

The initiation of the skin infection canine pyoderma is probably related to the ability of *S. pseudintermedius* to adhere to corneocytes on the surface of the stratum corneum as well as to invade the underlying keratinocytes. *S. pseudintermedius* adheres more strongly to corneocytes from regions of inflamed skin of dogs with atopic dermatitis than to noninflamed areas, suggesting that ligands for bacterial surface protein adhesins are present at higher levels (36). Both SpsD and SpsO mediate bacterial adherence to canine corneocytes, but the host ligands involved are not known (12). In addition, fibronectin is present in the stratum corneum of atopic human skin, where it could provide an abundant ligand, whereas it was not detected in healthy skin (37). Thus, Fn could promote colonization of the stratum corneum as well as invasion of keratinocytes.

In conclusion, we have identified and characterized two fibronectin-binding proteins of *S. pseudintermedius* which are required for adhesion to and invasion of keratinocytes. An appropriate animal model will be required to assess the significance of SpsD and SpsL in the pathogenesis of canine pyoderma and to establish whether these antigens are suitable candidates for a multicomponent vaccine to combat the disease.

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REFERENCES

1. **Perreten V, Kadlec K, Schwarz S, Grönlund Andersson U, Finn M, Greko C, Moodley A, Kania SA, Frank LA, Bemis DA, Franco A, Iurescia M, Battisti A, Duim B, Wagenaar JA, van Duijkeren E, Weese JS, Fitzgerald JR, Rossano A, Guardabassi L.** 2010. Clonal spread of methicillin-resistant *Staphylococcus pseudintermedius* in Europe and North America: an international multicentre study. J Antimicrob Chemother **65:**1145–1154. http://dx.doi.org/10.1093/jac/dkq078.
2. **Weese JS, van Duijkeren E.** 2010. Methicillin-resistant Staphylococcus aureus and *Staphylococcus pseudintermedius* in veterinary medicine. Vet Microbiol **140:**418–429. http://dx.doi.org/10.1016/j.vetmic.2009.01.039.
3. **Maluping RP, Paul NC, Moodley A.** 2014. Antimicrobial susceptibility of methicillin-resistant *Staphylococcus pseudintermedius* isolated from veterinary clinical cases in the UK. Br J Biomed Sci **71:**55–57.
4. **Gerstadt K, Daly JS, Mitchell M, Wessolossky M, Cheeseman SH.** 1999. Methicillin-resistant *Staphylococcus intermedius* pneumonia following coronary artery bypass grafting. Clin Infect Dis **29:**218–219. http://dx.doi.org/10.1086/520168.
5. **Campanile F, Bongiorno D, Borbone S, Venditti M, Giannella M, Franchi C, Stefani S.** 2007. Characterization of a variant of the SCCmec element in a bloodstream isolate of *Staphylococcus intermedius*. Microb Drug Resist **13:**7–10. http://dx.doi.org/10.1089/mdr.2006.9991.
6. **Kempker R, Mangalat D, Kongphet-Tran T, Eaton M.** 2009. Beware of the pet dog: a case of *Staphylococcus intermedius* infection. Am J Med Sci **338:**425–427. http://dx.doi.org/10.1097/MAJ.0b013e3181b0baa9.
7. **Stegmann R, Burnens A, Maranta CA, Perreten V.** 2010. Human infection associated with methicillin-resistant *Staphylococcus pseudintermedius*

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ST71. J Antimicrob Chemother **65:**2047–2048. http://dx.doi.org/10.1093/jac/dkq241.
8. **Ben Zakour NL, Bannoehr J, van den Broek AH, Thoday KL, Fitzgerald JR.** 2011. Complete genome sequence of the canine pathogen *Staphylococcus pseudintermedius*. J Bacteriol **193:**2363–2364. http://dx.doi.org/10.1128/JB.00137-11.
9. **Tse H, Tsoi HW, Leung SP, Urquhart IJ, Lau SK, Woo PC, Yuen KY.** 2011. Complete genome sequence of the veterinary pathogen *Staphylococcus pseudintermedius* strain HKU10-03, isolated in a case of canine pyoderma. J Bacteriol **193:**1783–1784. http://dx.doi.org/10.1128/JB.00023-11.
10. **McEwan NA.** 2000. Adherence by *Staphylococcus intermedius* to canine keratinocytes in atopic dermatitis. Res Vet Sci **68:**279–283. http://dx.doi.org/10.1053/rvsc.2000.0378.
11. **McEwan NA, Kalna G, Mellor D.** 2005. A comparison of adherence by four strains of *Staphylococcus intermedius* and *Staphylococcus hominis* to canine corneocytes collected from normal dogs and dogs suffering from atopic dermatitis. Res Vet Sci **78:**193–198. http://dx.doi.org/10.1016/j.rvsc.2004.09.002.
12. **Bannoehr J, Brown JK, Shaw DJ, Fitzgerald RJ, van den Broek AH, Thoday KL.** 2012. *Staphylococcus pseudintermedius* surface proteins SpsD and SpsO mediate adherence to *ex vivo* canine corneocytes. Vet Dermatol **23:**119–124, e26. http://dx.doi.org/10.1111/j.1365-3164.2011.01021.x.
13. **Latronico F, Moodley A, Nielsen SS, Guardabassi L.** 2014. Enhanced adherence of methicillin-resistant *Staphylococcus pseudintermedius* sequence type 71 to canine and human corneocytes. Vet Res **45:**70. http://dx.doi.org/10.1186/1297-9716-45-70.
14. **Geoghegan JA, Smith EJ, Speziale P, Foster TJ.** 2009. *Staphylococcus pseudintermedius* expresses surface proteins that closely resemble those from *Staphylococcus aureus*. Vet Microbiol **138:**345–352. http://dx.doi.org/10.1016/j.vetmic.2009.03.030.
15. **Bannoehr J, Ben Zakour NL, Reglinski M, Inglis NF, Prabhakaran S, Fossum E, Smith DG, Wilson GJ, Cartwright RA, Haas J, Hook M, van den Broek AH, Thoday KL, Fitzgerald JR.** 2011. Genomic and surface proteomic analysis of the canine pathogen *Staphylococcus pseudintermedius* reveals proteins that mediate adherence to the extracellular matrix. Infect Immun **79:**3074–3086. http://dx.doi.org/10.1128/IAI.00137-11.
16. **Pietrocola G, Geoghegan JA, Rindi S, Di Poto A, Missineo A, Consalvi V, Foster TJ, Speziale P.** 2013. Molecular characterization of the multiple interactions of SpsD, a surface protein from *Staphylococcus pseudintermedius*, with host extracellular matrix proteins. PLoS One **8:**e66901. http://dx.doi.org/10.1371/journal.pone.0066901.
17. **Fraunholz M, Sinha B.** 2012. Intracellular *Staphylococcus aureus*: live-in and let die. Front Cell Infect Microbiol **2:**43. http://dx.doi.org/10.3389/fcimb.2012.00043.
18. **Sinha B, François PP, Nüsse O, Foti M, Hartford OM, Vaudaux P, Foster TJ, Lew DP, Herrmann M, Krause KH.** 1999. Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin alpha5beta1. Cell Microbiol **1:**101–117. http://dx.doi.org/10.1046/j.1462-5822.1999.00011.x.
19. **Peacock SJ, Foster TJ, Cameron BJ, Berendt AR.** 1999. Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells. Microbiology **145:**3477–3486.
20. **Fowler T, Wann ER, Joh D, Johansson S, Foster TJ, Höök M.** 2000. Cellular invasion by *Staphylococcus aureus* involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. Eur J Cell Biol **79:**672–679. http://dx.doi.org/10.1078/0171-9335-00104.
21. **Bannoehr J, Ben Zakour NL, Waller AS, Guardabassi L, Thoday KL, van den Broek AH, Fitzgerald JR.** 2007. Population genetic structure of the *Staphylococcus intermedius* group: insights into agr diversification and the emergence of methicillin-resistant strains. J Bacteriol **189:**8685–8692. http://dx.doi.org/10.1128/JB.01150-07.
22. **Monk IR, Shah IM, Xu M, Tan MW, Foster TJ.** 2012. Transforming the untransformable: application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. mBio **3**(2): e00277-11. http://dx.doi.org/10.1128/mBio.00277-11.
23. **Speziale P, Visai L, Rindi S, Di Poto A.** 2008. Purification of human plasma fibronectin using immobilized gelatin and Arg affinity chromatography. Nat Protoc **3:**525–533. http://dx.doi.org/10.1038/nprot.2008.12.
24. **Zardi L, Carnemolla B, Balza E, Borsi L, Castellani P, Rocco M, Siri A.** 1985. Elution of fibronectin proteolytic fragments from a hydroxyapatite chromatography column. A simple procedure for the purification of fibronectin domains. Eur J Biochem **146:**571–579.
25. **Monk IR, Tree JJ, Howden BP, Stinear TP, Foster TJ.** 2015. Complete bypass of restriction systems for major *Staphylococcus aureus* lineages. mBio **6**(3):e00308-15. http://dx.doi.org/10.1128/mBio.00308-15.
26. **Li MZ, Elledge SJ.** 2007. Harnessing homologous recombination *in vitro* to generate recombinant DNA via SLIC. Nat Methods **4:**251–256. http://dx.doi.org/10.1038/nmeth1010.
27. **Bae T, Schneewind O.** 2006. Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. Plasmid **55:**58–63. http://dx.doi.org/10.1016/j.plasmid.2005.05.005.
28. **Edwards AM, Massey RC.** 2011. Invasion of human cells by a bacterial pathogen. J Vis Exp **2011**(49):2693. http://dx.doi.org/10.3791/2693.
29. **Akiyama SK, Yamada KM.** 1985. The interaction of plasma fibronectin with fibroblastic cells in suspension. J Biol Chem **260:**4492–4500.
30. **Pytela R, Pierschbacher MD, Ruoslahti E.** 1985. Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. Cell **40:**191–198. http://dx.doi.org/10.1016/0092-8674(85)90322-8.
31. **Edwards AM, Potter U, Meenan NA, Potts JR, Massey RC.** 2011. *Staphylococcus aureus* keratinocyte invasion is dependent upon multiple high-affinity fibronectin-binding repeats within FnBPA. PLoS One **6:**e18899. http://dx.doi.org/10.1371/journal.pone.0018899.
32. **Agerer F, Michel A, Ohlsen K, Hauck CR.** 2003. Integrin-mediated invasion of *Staphylococcus aureus* into human cells requires Src family protein-tyrosine kinases. J Biol Chem **278:**42524–42531. http://dx.doi.org/10.1074/jbc.M302096200.
33. **Fowler T, Johansson S, Wary KK, Höök M.** 2003. Src kinase has a central role in in vitro cellular internalization of *Staphylococcus aureus*. Cell Microbiol **5:**417–426. http://dx.doi.org/10.1046/j.1462-5822.2003.00290.x.
34. **Chi CY, Lin CC, Liao IC, Yao YC, Shen FC, Liu CC, Lin CF.** 2014. Panton-Valentine leukocidin facilitates the escape of *Staphylococcus aureus* from human keratinocyte endosomes and induces apoptosis. J Infect Dis **209:**224–235. http://dx.doi.org/10.1093/infdis/jit445.
35. **Prevost G, Bouakham T, Piemont Y, Monteil H.** 1996. Characterisation of a synergohymenotropic toxin produced by *Staphylococcus intermedius*. FEBS Lett **381:**272.
36. **McEwan NA, Mellor D, Kalna G.** 2006. Adherence by *Staphylococcus intermedius* to canine corneocytes: a preliminary study comparing noninflamed and inflamed atopic canine skin. Vet Dermatol **17:**151–154. http://dx.doi.org/10.1111/j.1365-3164.2006.00503.x.
37. **Cho SH, Strickland I, Boguniewicz M, Leung DY.** 2001. Fibronectin and fibrinogen contribute to the enhanced binding of *Staphylococcus aureus* to atopic skin. J Allergy Clin Immunol **108:**269–274. http://dx.doi.org/10.1067/mai.2001.117455.

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