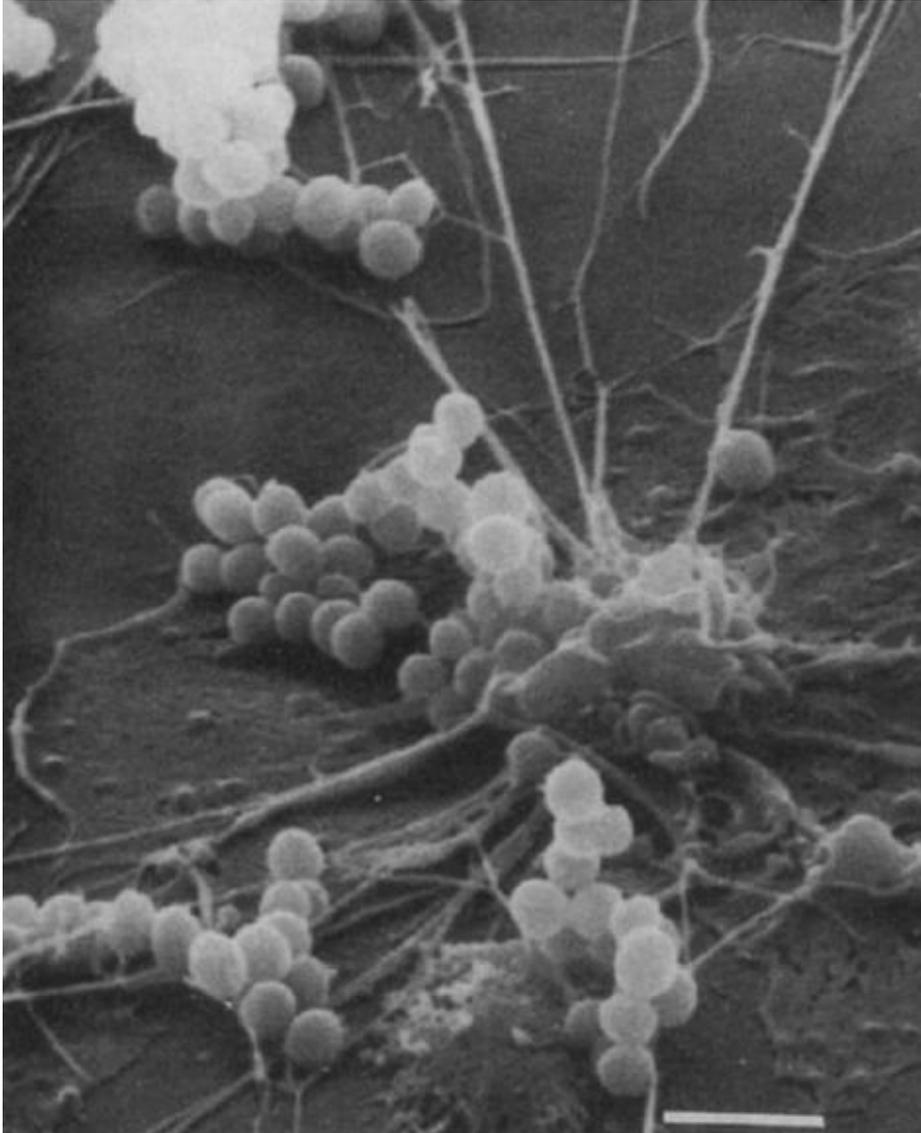


**Interference of *Staphylococcus aureus* virulence factors in the blood coagulation system**



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# Interference of *Staphylococcus aureus* virulence factors in the blood coagulation system

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May 2013

**Front page:** *Staphylococcus aureus* adheres to platelet-coated coverslips in the presence of plasma ([Herrmann \*et al.\* 1993](#))

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## Summary for laymen

The blood coagulation system is a process in the body which is mainly used for the repair of injured vessels. Activation of this system will result in the formation of a blood clot which will close the injured part of the vessel. However, this system can also be activated by the immune system in the case of infection. In this way the coagulation system can trap the invading bacteria in a blood clot and spreading of the bacteria is prevented. Unfortunately, many microorganisms are able to use the blood coagulation to their advantage. *Staphylococcus aureus* is one of these microorganisms.

*S. aureus* is a bacterium that causes a multitude of infections like wound infections but also lethal diseases such as meningitis and sepsis. Many people get infected with this bacteria. In fact, about 90% of people will carry the bacteria with them at some point during their lives. Since *S. aureus* has become resistant against antibiotics, treatment has become harder and the number of deaths in *Staphylococcus aureus* infected patients has increased.

Another things that makes *S. aureus* very hard to eliminate, is its ability to escape the immune system. The bacterium produces many factors that can contribute to this, whether is it to shield off the bacterium or simply break down parts of the hosts immune system. The blood coagulation system is also used by *S. aureus* to ensure its survival. By activating this system a clot and eventually an abscess is formed. This abscess poses a shield for the bacteria that protects it from the immune attack of the host. In this way, *S. aureus* is able to survive and multiply within the body of the host.

*S. aureus* produces two main factors that are able to activate blood coagulation and contribute to the formation of abscesses; Coagulase and von Willebrand binding factor. Although other factors are not able to activate the coagulation system on their own, they can influence certain parts of the system. Clumping factor and Fibronectin binding factor for example, bind to platelets and activate them. This results in clumping together of the bacteria with platelets, which makes it easier for the bacteria to colonize in host tissue. Many other factors play some role in influencing the blood coagulation system, however a complete model of these interactions is not fully established yet.

Since factors that influence the blood coagulation system have been shown to be important for *S. aureus* infections, many of these factors have been targeted for the production of a vaccine.

## List of abbreviations

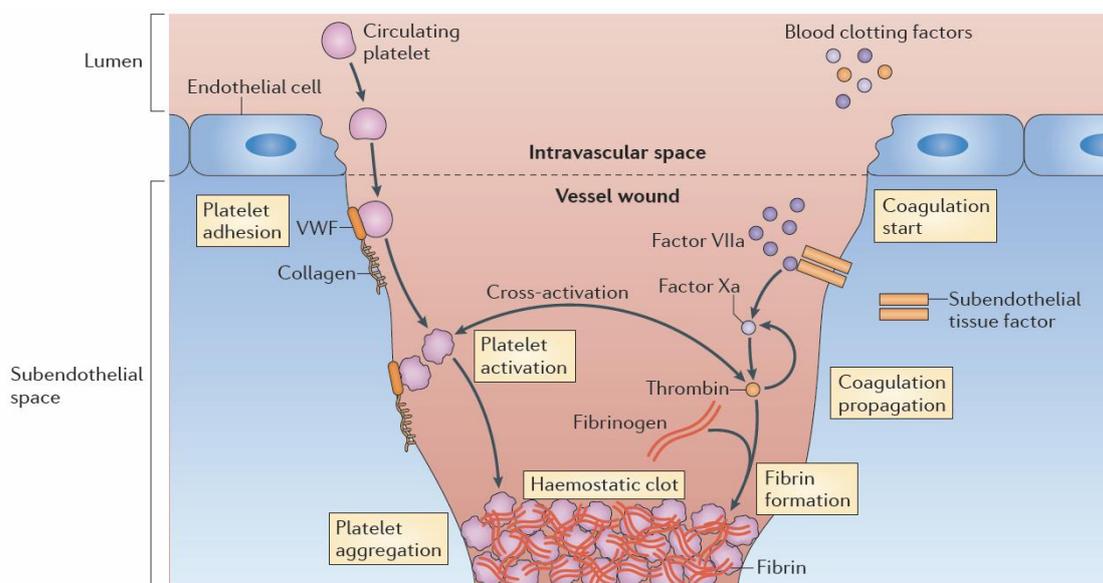
C5aR	C5a receptor
CHIPS	Chemotaxis inhibitory protein of staphylococci
Clf	Clumping factor
Can	Collagen binding adhesin
Coa	Coagulase
CR	Complement receptor
Eap	Extracellular adherence protein
Efb	Extracellular fibrinogen-binding protein
Fg	Fibrinogen
Fnbp	Fibronectin binding protein
FPR	Formyl peptide receptor
ICAM-1	Intercellular adhesion molecule 1
<i>L. lactis</i>	<i>Lactococcus lactis</i>
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
PAMP	Pattern associated molecular patterns
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SAK	Staphylokinase
SdrE	Serine aspartate repeat protein
SERAM	Secretable expanded repertoire adhesive molecule
SpA	Protein A
TF	Tissue factor
vWF	von Willebrand factor
vWbp	von Willebrand factor binding protein

## Introduction

The innate immune system uses coagulation as a defense mechanism against pathogens. In this way, the invading bacteria is trapped and immobilized in a blood clot (McAdow *et al.* 2012). However, bacteria also use coagulation as an immune evasive strategy (McAdow *et al.* 2012). *Staphylococcus aureus* is one of these bacteria, which produces virulence factors that enable blood clotting. This results in the formation of abscesses where *S. aureus* can replicate freely and the depletion of clotting factors from the blood (McAdow 2012). The fact that *S. aureus* causes blood clotting has been described as early as 1903 (Loeb 1903). Since then, this phenomenon has mainly been attributed to Coagulase and von Willebrand factor (Hartleib *et al.* 2000). However, many more virulence factors have the ability to influence hemostasis in staphylococcal infections. In this review the effects of *S. aureus* on several aspects of the blood coagulation system are described.

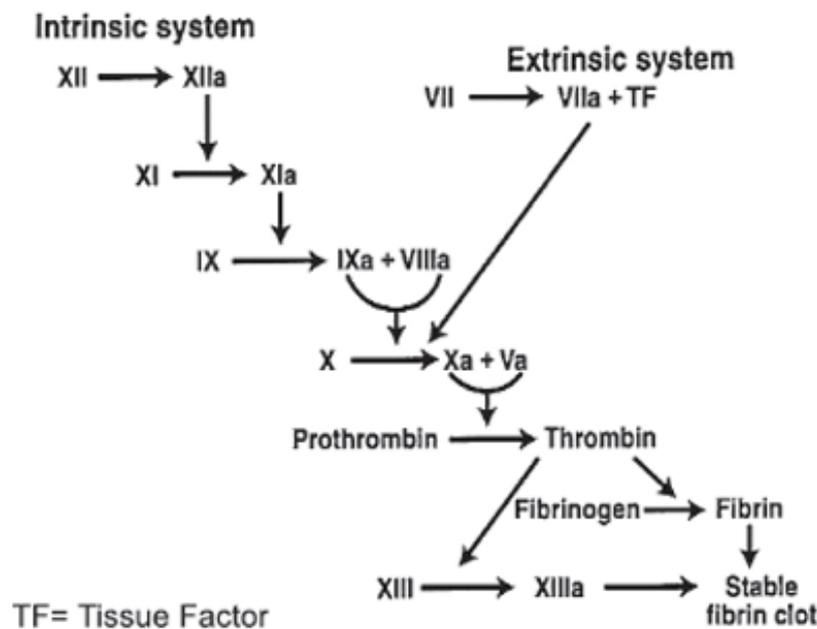
## The blood coagulation system

Coagulation is the process in which fluid blood forms a gelatinous clot, most often in the event of vessel wall injury. Unless steps are taken, blood will be lost. The first step in the blood coagulation system is vasoconstriction. This causes a decrease in blood flow and pressure in the vessel (Silverthorn *et al.* 2007). Second, von Willebrand factor (vWf) is secreted by the endothelial cells and then bound to exposed collagen fibers. This causes platelet aggregation, as platelets adhere to the wound via their interaction with von Willebrand factor. Next to von Willebrand factor, platelets also bind soluble fibrinogen via their glycoproteins (Cook *et al.* 1992). The platelets become activated and start releasing cytokines, which attract other platelets through a process known as platelet aggregation. The resulting platelet plug stops immediate bleeding, but must be consolidated into a more stable plug. Exposed collagen and tissue factor (TF), which is released from subendothelial cells and platelets, initiate a series of reactions known as the coagulation cascade, which is the last step in the blood coagulation system (Figure 1) (Silverthorn *et al.* 2007).



**Figure 1: The blood coagulation system.** Platelets are recruited to the site of injury and bind to collagen and von Willebrand Factor. Inactive coagulation enzymes are converted into active enzymes in the coagulation cascade, starting with factor XII and resulting in fibrin formation. Platelet aggregation in combination with the coagulation cascade results in the formation of a stable clot (Engelmann & Massberg, 2013)

In the coagulation cascade, two pathways have been described – the extrinsic and the intrinsic activation pathway (**Figure 2**)([Johari & Loke 2012](#)). The intrinsic pathway is important for the amplification of the cascade and starts with the activation of factor XII to XIIa by collagen or negatively charged surfaces. XIIa causes the conversion of XI to XIa which in turn converts IX to IXa. Factor IXa converts X into Xa with the help of factor VIIIa. The extrinsic pathway is important for the initializing of the cascade. When TF is released from the damaged tissue it interacts with factor VIIa and converts factor X to Xa. It is at this point where the pathways are united at the common pathway to create thrombin from prothrombin by Xa and other factors. Thrombin converts fibrinogen to fibrin which forms a cross-linked polymer that stabilizes the clot ([Silverthorn et al. 2007](#); [Hoffbrand et al. 2006](#); [McAdow et al. 2012](#)). As the site of injury heals, fibrinolysis helps remove the clot. This is done by plasmin. During clot formation, plasmin molecules are also incorporated in the clot. Plasmin is converted from plasminogen by thrombin, and breaks down fibrin polymers into fibrin fragments. ([Silverthorn et al. 2007](#)) An imbalance between coagulation and anticoagulation can cause a myriad of diseases and disorders like hemophilia and thrombocytopenia.



**Figure 2: Overview of the coagulation cascade** ([Sander & Giles 2004](#))

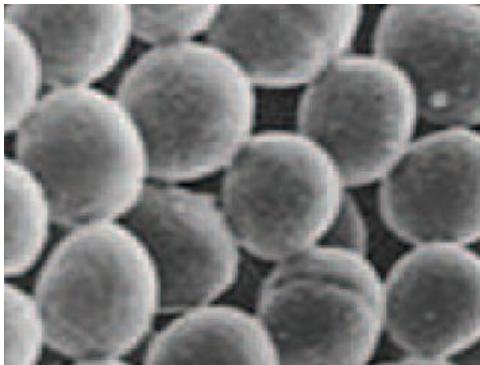
## Coagulation and immunity

The activation of hemostatic mediators is one of the results of inflammation and the interactions between coagulation and inflammatory mediators are essential for a successful host defense against infectious agents ([Bergman & Hammerschmidt 2007](#)). The formation of a fibrin clot can serve as a physical barrier whereby invading bacteria are immobilized and spreading of the bacteria is inhibited. Also, it has been shown that components of the coagulation system are able to activate complement ([Oikonomopoulou et al. 2012](#)). However, coagulation is not only used by the host as a defense mechanism. Microorganisms interfere with components of the blood coagulation system to establish immune evasion and infection ([Bergman & Hammerschmidt 2007](#)). Some bacteria actually use the formation of fibrin clots for their own benefit to escape from innate immune response. In the case of *S. aureus*, the interaction with fibrinogen and fibronectin leads to the formation of abscesses. The

bacterium is able to replicate within these abscesses, protected from immune cells (Cheng *et al.* 2010). In addition, many bacteria are able to affect fibrinolysis by activating plasmin. Since plasmin not only breaks down clots, but also extracellular matrix (ECM), basal membrane and host tissue, bacteria can use this process to facilitate spreading and to evade the innate immune response (Bergmann & Hammerschmidt 2007). One of these plasmin activators is Staphylokinase, which is produced by *S. aureus* (Bergmann & Hammerschmidt 2007). *S. aureus* not only interacts with blood and matrix proteins, but also with platelets (Hartleib *et al.* 2000).

## Staphylococcus aureus

Staphylococci are gram-positive bacteria which divide in two planes and, therefore, grow in clusters (Figure 3). They have a genome size of between 2000 and 3000 kbp. *S. aureus* are very adaptable pathogenic bacteria. Because of immune evasion and interference they are difficult to eliminate. Also, *S. aureus* is able to survive in almost any environment including inanimate objects for long



**Figure 3. High resolution electron micrograph of *S. aureus*.** The spherical bacteria form grape-like clusters (Proctor *et al.*, 2006).

periods (Engleberg *et al.* 2006). Up to 90% of people are colonized with *S. aureus* in the nostrils at some point in their lives. The emergence of a *Staphylococcus aureus* strain that is resistant to methicillin (MRSA) has increased mortality from infection and MRSA has even become the most frequent cause of skin and soft tissue infections in the US (O'Brien *et al.* 2002, Klevens *et al.* 2007). Next to skin and soft tissue infections, *S. aureus* can cause more severe and even lethal diseases such as abscesses of various organs, meningitis and sepsis (Krishna & Miller 2012).

*S. aureus* produces a large number of virulence factors like enzymes and toxins that help them evade the immune system and survive phagocytosis. A few examples of these

factors are Protein A, which binds to the Fc terminus of antibodies and reduces its availability and thereby opsonization; pore-forming toxins, which damage phagocytotic and other cells; and teichoic acid, a cell wall constituent which appears to be involved in complement activation (Engleberg *et al.*, 2006). Another virulence factor is coagulase (Coa). This factor makes *S. aureus* easy to distinguish from other staphylococcus genus, since *S. aureus* is the only one that produces this enzyme.

Whether it is during primary infection or when the bacteria spreads to other tissues and forms abscesses, at some point during *S. aureus* infection, staphylococcal cells will enter the bloodstream (Lowy 1998). Here, Coa can activate prothrombin and may help prevent the organisms from being phagocytized, since it is difficult for white cells to penetrate fibrin clots (Engleberg *et al.*, 2006). In fact, the area in which *S. aureus* colonizes may become surrounded with a thick-walled fibrin capsule in response to coagulases and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) in combination with the host's immune response (Engleberg *et al.*, 2006). When this capsule is stabilized it becomes an abscess which protects the bacterium from the host's immune attack. The bacteria are able to multiply within the center of these abscesses without infiltrating immune cells. Eventually the lesion can rupture, spreading even more of the pathogen into the blood circulation and to uninfected tissues (Cheng *et al.* 2010).

*S.aureus* is responsible for many diseases, such as TSS and endocarditis. The consequences of colonization of *S.aureus* depend on the location. Colonization of the heart, for example, is the leading cause of infective endocarditis (Fowler *et al.* 2005; Engleberg *et al.* 2006).

## Host response

The innate immune system, which represents the first line of defense against pathogens, consists of three effector mechanisms: antimicrobial peptides, the complement system and phagocytes (Rooijackers *et al.* 2005; Zeconni & Scali 2013). Antimicrobial peptides recognize and bind to microbial pathogens. This promotes killing of the bacterium by lysing their membrane. (Peschel 2002; Zeconni & Scali 2013). Antimicrobial peptides are conserved in their structure and function; however in gram-positive bacteria they are only effective at high concentrations (Rooijackers *et al.* 2005; Zeconni & Scali 2013).

The complement system is composed of over 30 proteins and divided in three main pathways. After activation, the three pathways converge on cleavage of complement component C3. The end product is opsonization by C3b and C3bi to support uptake by phagocytes, and generation of anaphylotoxins C5a which attracts phagocytes to the site of infection (Rooijackers *et al.* 2005; Serruto *et al.* 2010). Activation of the complement system is accompanied by the regulation of the coagulation system. The activation of MASPs by the lectin pathway can for example convert prothrombin to thrombin. Another consequence of the complement system is enhanced expression of TF by C5a. As mentioned, the blood coagulation system may be used as a strategy to immobilize bacteria and prevent them from spreading (Bergmann & Hammerschmidt, 2007).

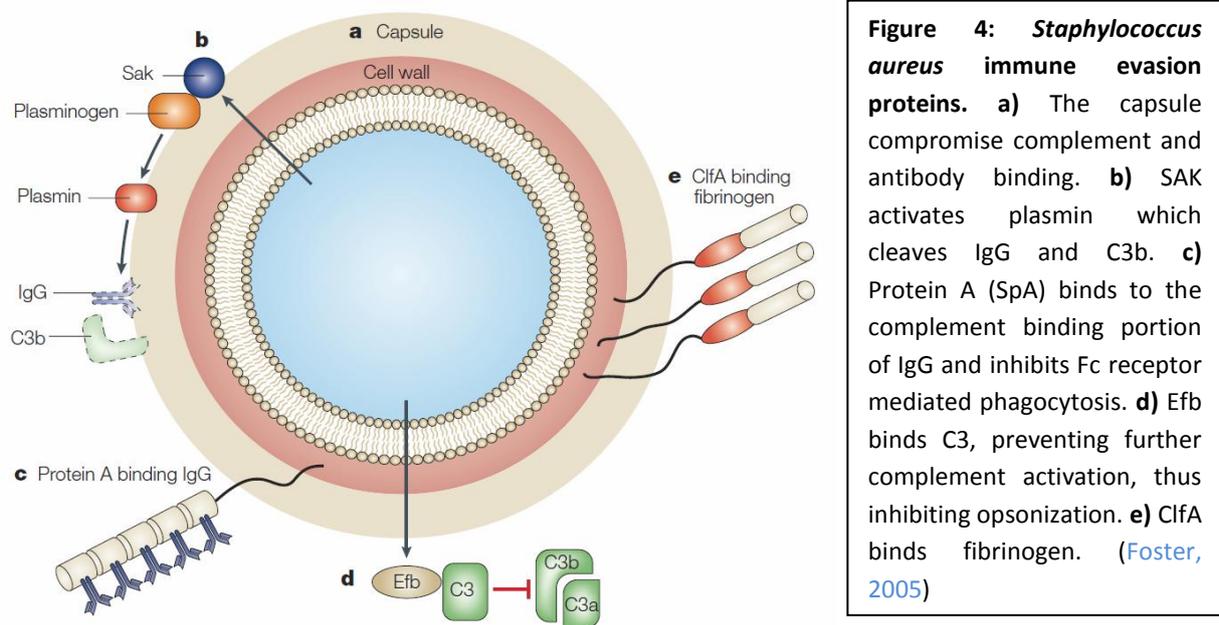
Phagocytes are recruited to the site of infection by cytokines and chemokines to attempt to engulf and dispose of the bacteria (Foster, 2005). Neutrophils are the most important type of phagocytic cells. They recognize pathogen associated molecular patterns (PAMPs) with specific receptors like Toll-like receptors (TLRs) (Fournier & Philpott, 2005). TLR-2 and TLR-6 are activated by these PAMPs and cause activation of reactive oxygen species and the secretion of several cytokines, chemokines and antimicrobial peptides (Zeconni & Scali, 2013).

Phagocytes that have taken up the bacterium are translocated to the lymphnodes where they stimulate B cells to secrete specific antibodies. This, in order to neutralize bacteria and promote efficient phagocytosis. Although antibodies against *S. aureus* can be detected in humans, protective immunity is not observed and recurrence occurs frequently. This suggests that antibody levels are insufficient to prevent subsequent infections (Foster 2005; Kim *et al.* 2012).

## Immune evasion

In order to survive and cause disease, *S. aureus* is able to escape the host immune response via immune evasion strategies (Bardoel *et al.* 2012). To do this, *S. aureus* uses an array of immune evasive molecules that interfere with the different aspects of the immune attack. In this way *S. aureus* can create a microenvironment that is shielded from the immune attack where bacteria can survive and multiply (Rooijackers *et al.* 2005). Adhesion of *S. aureus* to host tissue is essential to its colonization and pathogenesis (Zeconni & Scali 2013). Most adhesion proteins are microbial surface recognizing adhesive matrix molecules (MSCRAMMs). They bind to extracellular matrix, endothelial cells and other host substrates and often play a role in immune evasion as well (Zeconni & Scali, 2013). Protein A (SpA) is an MSCRAMM which binds to vWf, immunoglobulin and platelets. Binding to platelets has actually been shown to determine *S. aureus* virulence (Nyugen *et al.* 2000). SpA also

interferes in opsonization by complement. The N-terminal part of SpA binds the Fc fragment of IgG. This is the part of IgG that complement subcomponent C1q usually binds, to promote an activation cascade resulting in the opsonization of the bacterium by binding of complement factors. By binding to the FC fragment of IgG, SpA blocks opsonization but also inhibits Fc receptor mediated phagocytosis (Rooijakkers *et al.* 2005; Kim *et al.* 2012). SpA is actually one of the most researched virulence factors of *S. aureus*. Its role in immune evasion spans many molecules that are important in the immune response. SpA not only binds IgG, but also B-cell associated IgM, promoting proliferation and apoptosis of B cells (Sasso *et al.* 1989; Kim *et al.* 2012). Furthermore, SpA has recently been shown to bind to TNFR1, which is the receptor for TNF- $\alpha$ . Doing so SpA reduces the TNF- $\alpha$  pro-inflammatory response (Rooijakkers *et al.* 2005; Zeconni & Scali 2013). Taken together, SpA is clearly an important player in immune evasion. Indeed, *S. aureus* mutants that do not express protein A show reduced virulence for staphylococcal disease (Kim *et al.* 2012).



Most of the immune evasion molecules are directed against opsonization by the complement system (Figure 4). Extracellular fibrinogen-binding protein (Efb) and Clumping factor A (ClfA) both compromise opsonization by C3b. Efb prevents opsonization by C3b by binding to complement factor C3 thereby blocking further activation of complement (Foster, 2005). ClfA on the other hand, is mostly known for its role in immune evasion via the conversion of C3b to inactive iC3b (Zeconni & Scali 2013). ClfA also has an antiphagocytic effect. Since it binds to fibrinogen, it is thought that this prevents phagocyte access to opsonins because the bacteria become covered with fibrinogen (Foster, 2005). Staphylokinase (SAK) interferes with complement opsonization by activating plasminogen into plasmin which is able to cleave opsonins IgG and C3b. SAK also provides resistance against  $\alpha$ -defensins (Rooijakkers *et al.* 2005).

*S. aureus* invasion triggers a chemotactic response whereby anaphylotoxin C5a is released. Neutrophils recognize these as well as formylated peptides that are secreted from the bacteria (Foster, 2005; Rooijakkers *et al.* 2005) with the C5a receptor (C5aR) and the formyl peptide receptor (FPR) respectively. To prevent neutrophil chemotaxis, *S. aureus* secretes chemotaxis inhibitory protein

of staphylococci (CHIPS). CHIPS is an antagonist to both C5aR and FPR and in this way blocks ligand binding (Foster, 2005; Rooijackers *et al.* 2005). Neutrophil migration and adhesion is inhibited by extracellular adherence protein (Eap). Eap interacts with intercellular adhesion molecule 1 (ICAM-1) thereby inhibiting integrin mediated leukocyte adhesion (Chavakis *et al.* 2002).

*S. aureus* employs these and several other virulence factors to evade the immune attack. In addition, staphylocoagulases contribute to survival within the host blood system (DeDent *et al.* 2012). As mentioned, the blood coagulation system is also used to establish immune evasion. Some of the immune evasion virulence factors of *staphylococcus aureus* not only interact with specific sections of the immune response, but may also affect hemostasis. Protein A for instance, not only reduces antibody function, but also plays a role in platelet aggregation (O'Brien *et al.* 2002). A complete model of *S. aureus* virulence factors that influence the blood coagulation system is not fully comprehended.

### Coagulase and von Willebrand binding factor

The ability of *S. aureus* to clot blood has been shown to correspond with its virulence (McAdow *et al.* 2012). Coagulase (Coa) is the best known coagulation promoting virulence factor that is produced by *S. aureus*. Next to Coa, *S. aureus* also secretes von Willebrand binding protein (vWbp), which has a similar role in coagulation. Both of these proteins activate prothrombin without the usual proteolytic cleavage by factor Xa. The N-terminal ends of Coa and vWbp can associate with the prosite of prothrombin which results in an active site thereby promoting cleavage of fibrinogen to fibrin (Friedrich *et al.* 2003; Kroh *et al.* 2009). A model for this mechanism has been established by crystallization of the active staphylothrombin complex and differs from that which would normally have been formed as a result of prothrombin cleavage by factor Xa. Prothrombin activation at sites of platelet aggregation and thrombosis will result in fibrin deposition on damaged endothelium (Friedrich *et al.* 2003). Coa and vWbp are very important for *S. aureus* pathogenesis. They both contribute to staphylococcal survival and virulence. Immunohistochemical staining of abscesses show expression of Coa and vWbp suggesting a contribution of these factors to abscess formation (Cheng *et al.* 2010; McAdow *et al.* 2012). In fact, Cheng *et al.* showed that a Coa/vWbp double mutant was incapable to clot mouse blood. Next to this, the double mutant was unable to form abscesses or persist in infected tissue (Cheng *et al.* 2010).

### Platelet aggregation

*S. aureus* not only produces virulence factors that influence the coagulation cascade, it also has several different proteins that can bind to and in some cases stimulate platelets (Fitzgerald *et al.* 2006). Platelet interactions are considered to play a role in staphylococcal adhesion to damaged endothelium and to facilitate growth at the infected area (Hartleib *et al.* 2000). Indeed, adhesion to platelets has been shown to be a major determinant in virulence and represents an important step in *S. aureus* induced endocarditis (O'Seaghdha *et al.* 2006; Nguyen *et al.* 2000). *S. aureus* adheres to healthy endothelium and platelets through interactions between its adhesins and receptors either with or without the help of bridging proteins such as fibrinogen and fibronectin, but also to previously inflamed endothelial tissue, like atherosclerotic lesions, thereby furthering inflammation and inducing tissue destruction and vegetative growth (Heilmann *et al.* 2004; Que & Moreillon, 2011). Platelet interactions are mediated by several MSCRAMMs including protein A, clumping factors (Clfs) and fibronectin binding proteins (Fnbps) (Figure 7) (Martin *et al.* 2012).

### Protein A

Protein A is one of the most abundant staphylococcal surface proteins (DeDent *et al.* 2012). As mentioned before, SpA plays a role in antibody-function reduction; however, it has also been shown to play a role in platelet aggregation (Engleberg *et al.* 2006, O'Brien *et al.* 2002). It was discovered that SpA directly binds to platelets and endothelial cells (ECs) via the gC1qR protein. Expression of the gC1qR protein on the surface of platelets and endothelial cells is upregulated by proinflammatory cytokines and binding to immobilized fibrinogen and fibronectin thus increasing *S. aureus* adhesion and platelet aggregation (Martin *et al.* 2012). Wildtype *S. aureus*, but not an *S. aureus* strain deficient of SpA, was able to bind to gC1qR, which indicates that SpA is the only *S. aureus* factor that binds gC1qR directly (Nguyen *et al.* 2000). Administration of an antibody against gC1qR reduced colonization and spreading of *S. aureus* in a rat model for infective endocarditis (Peerschke *et al.* 2006). However, *S. aureus* can still bind to platelets via fibrinogen and fibronectin bridges (Heilmann *et al.* 2004). Whether the interaction between SpA and vWf also contributes to platelet aggregation is not yet clear (McAdow *et al.* 2012).

### Clumping factor

Clumping factor A and B (ClfA and ClfB) are members of the serine-aspartate repeat protein family (O'Brien *et al.* 2002). They mediate attachment of *S. aureus* to fibrinogen (Fg) and thereby promote bacterial invasion into host tissues (Entenza *et al.* 2000, Gong *et al.* 2010). Clumping factors are divided in several different regions, as shown in **Figure 5**. The N-terminal secretory signal sequence is located in region S. Region A contains the Fg-binding domain of clumping factor and is connected to the cell wall-spanning domain (region W) by region R which contains repeats of serine and aspartic acid dipeptides (O'Brien *et al.* 2002, Riskey *et al.* 2007). At the C-terminal end of the factor, a LPXTG motif is situated, which is essential for correct localization in the bacterial cell wall (McDevitt *et al.* 1994). Via their Fg-binding domain, clumping factors can interact with platelets (**Figure 7**). Although it has been shown that ClfA can also bind to platelets independent of fibrinogen.



**Figure 5: Schematic overview of clumping factor A.** S: signal peptide; A non-repeat region containing the Fg-binding domain; R: repeat region; W: cell wall spanning domain; M: membrane spanning domain (McDevitt *et al.* 1994).

Both ClfA and ClfB have been shown to stimulate platelet activation and aggregation in a Fg-dependent manner, although ClfB aggregates platelets with a longer lag time, which is the time it takes to cause aggregation after the bacteria comes in contact with the platelet (Miajlovic *et al.* 2007). Differences in lag time may be caused by the affinity with which surface components bind to platelet receptors, or the density in which these surface components are present on the bacterial cell surface (Fitzgerald *et al.* 2005). The fact that the importance of ClfB in infection is limited was also shown in a rat model for infective endocarditis (Entenza *et al.* 2000). Although ClfB complementation increased infectivity, ClfB negative mutants were only slightly less infective. However, the individual contribution of ClfB in infectivity may be difficult to distinguish since there are additional fibrinogen-binding proteins, including ClfA and FbpA that can mask the role of ClfB *in vivo* (Entenza *et al.* 2000).

The same group also showed that ClfB was unable to make up for the strong decrease in fibrinogen binding capacities of a *S. aureus* strain lacking ClfA expression *in vitro* (Entenza *et al.* 2005).

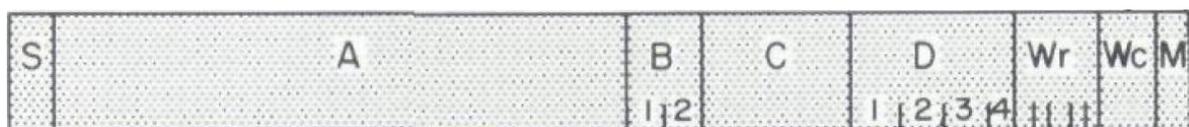
ClfA is the most important platelet aggregator during the stationary phase of *S. aureus*. O'Brien *et al.* demonstrated that ClfA is the primary factor involved in platelet activation. ClfA, ClfB and a Serine aspartate repeat protein (SdrE) were expressed on *L. lactis* which is a nonpathogenic bacterium often used to investigate the role of individual virulence factors. Compared to ClfB and SdrE, ClfA showed the shortest lag time in platelet aggregation (O'Brien *et al.* 2002).

Next to *in vitro* studies, several *in vivo* studies have shown that clumping factors influence *S. aureus* pathogenesis in various infection models (Moreillon *et al.* 1995, Entenza *et al.* 2000, Siboo *et al.* 2001, Stutzmann *et al.* 2001). Que *et al.* demonstrated that *L. lactis* expressing ClfA was able to adhere to fibrinogen and to induce experimental endocarditis. Individual expression of ClfA on *L. lactis* was sufficient to induce valve infection in rats to levels comparable to wild type *S. aureus* (Que *et al.* 2001). On the other hand, mice that expressed a version of fibrinogen lacking the ClfA binding motif were shown to exhibit increased survival compared to wild type mice (Flick *et al.* 2013). Together, this proves that ClfA is an important virulence factor in the pathogenesis of *S. aureus* infections.

### Fibronectin binding proteins

Fibronectin binding proteins (FnbpA and FnbpB) mediate *S. aureus* binding to platelets via fibronectin and to a lesser extent fibrinogen (Entenza *et al.* 2005). FnbpA and FnbpB can also activate endothelial cells, which results in monocyte adhesion, and trigger internalization into eukaryotic cells (Martin *et al.* 2012, Piroth *et al.* 2008).

FnbpA and FnbpB show significant similarity to the ClfA protein (McDevitt *et al.* 1994). They are composed of different regions, as shown in **Figure 6**. FnbpA also contains an N-terminal secretory signal sequence, located in Region S. Region A is similar to that of ClfA and consists of a Fg-binding domain. Interactions with fibronectin are mediated through several binding domains located in region B, C and D (Fitzgerald *et al.* 2006). FnbpA also contains a LPXTG motif, located at the C-terminal end of the protein.

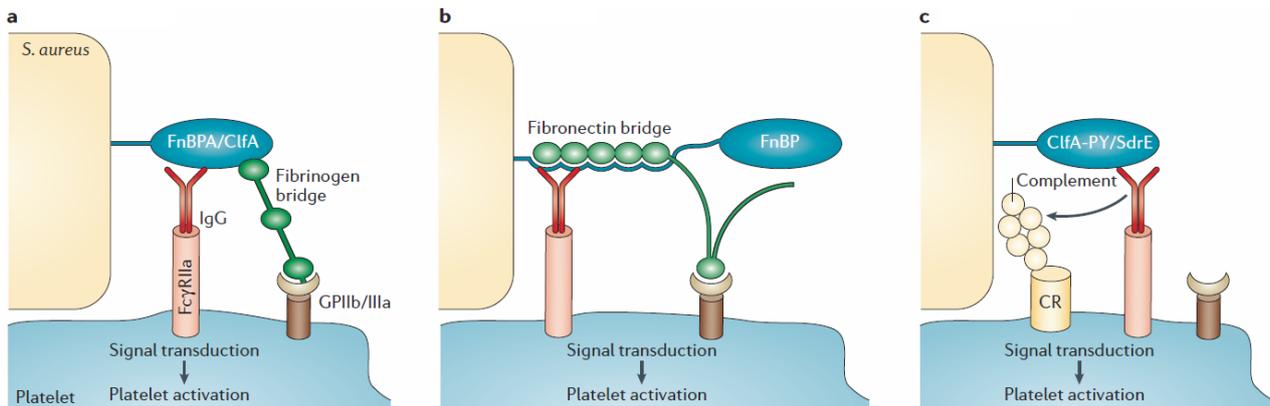


**Figure 6: Schematic overview of fibronectin-binding protein A.** S: signal peptide; A: Spacer region; B: repeat region; C: Spacer region; D: Repeat region; Wr: Repeat region in the cell wall spanning domain; Wc: non-repeat region in the cell wall spanning domain; M: membrane spanning domain (McDevitt *et al.* 1994).

Fnbps are the most important platelet aggregators during the exponential growth phase of *S. aureus* and FnbpA and FnbpB are only expressed during this growth phase (Fitzgerald *et al.* 2012).

By expressing *S. aureus* FnbpA on *L. lactis*, it has been shown that FnbpA can stimulate platelet aggregation without the need for other *S. aureus* surface components (Fitzgerald *et al.* 2006). The same group also showed that FnbpA could mediate platelet aggregation in two different ways (**Figure 7**). Region BCD could mediate platelet aggregation via its fibronectin-binding domain, whereas region A could promote platelet aggregation through its fibrinogen binding domain. However, plasma factors are required for this, since neither domain could stimulate platelet activation in the presence

of only gel-filtered platelets and purified fibrinogen. Adding IgG to the platelets and fibrinogen resolved this (Fitzgerald *et al.* 2006).



**Figure 7: *S. aureus* platelet interactions.** **a.** Both ClfA and FnbpA can activate platelets rapidly via a fibrinogen bridge that binds to GPIIb/IIIa and a IgG specific for ClfA/FnbpA epitopes, which binds to FcγRIIa. **b.** Fnbp can activate platelets rapidly through a fibronectin bridge that binds GPIIb/IIIa and a specific IgG that engages FcγRIIa. **c.** antibody recognition of for example ClfA or SdrE can stimulate complement fixation. Complement recognition by a platelet complement receptor (CR) and IgG engaging FcγRIIa, results in slow activation of platelets . (Fitzgerald *et al.* 2012)

Fibrinogen and fibronectin binding are essential in *S. aureus* induced endocarditis. Fibrinogen binding promotes valve colonization, but is unable to promote invasion whereas fibronectin binding promotes invasion of endothelial cells and persistence. It seems these two binding properties cooperate to facilitate colonization and endothelial invasion (Que *et al.* 2005). Since Fnbp contains both a fibrinogen and a fibronectin binding domain, this virulence factor is able to promote colonization and invasion by itself (Piroth *et al.* 2008). Indeed, individual expression of FnbpA on *L. lactis* resulted in fibronectin adherence and infectivity in a model of experimental endocarditis that was similar to wild type *S. aureus* (Que *et al.* 2001).

## Other virulence factors involved in the hemostatic pathway

Next to Coa, vWbp, ClfA and FnbpA, *S. aureus* produces a myriad of factors that contribute to immune evasion, growth and surviving the hemostatic pathway. These proteins may also influence blood coagulation. Since there are interactions between different pathways and systems such as coagulation and complement, a complete model for staphylococcal blood pathogenesis is not fully established (DeDent *et al.* 2012).

### Staphylokinase

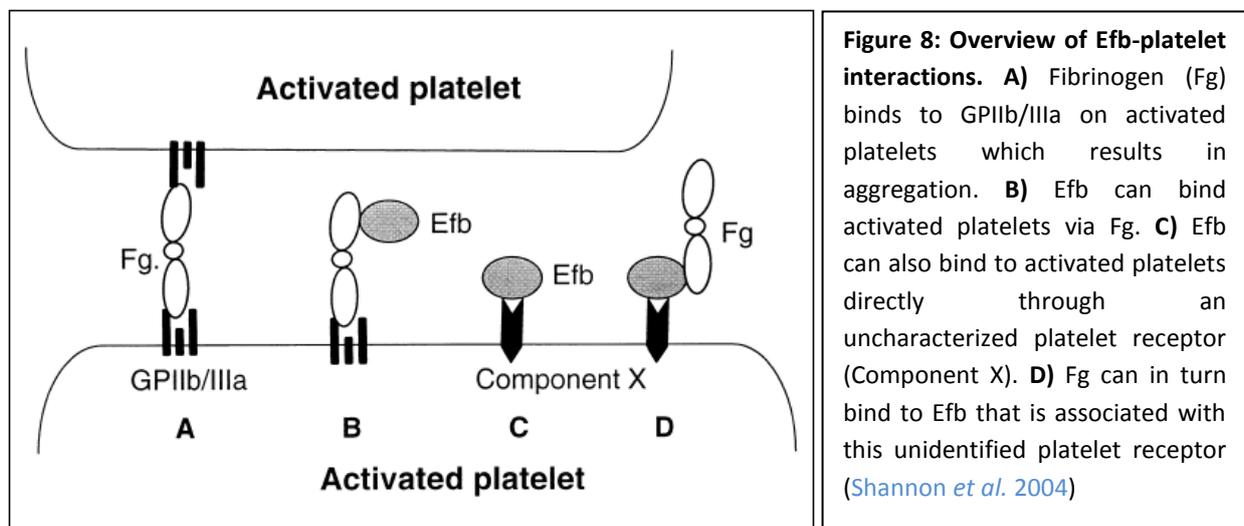
SAK is a 136 amino acid virulence factor that is produced by *S. aureus*. Its function in staphylococcal infection is assumed to be in its interaction with  $\alpha$ -defensins and plasminogen (Bokawera *et al.* 2006). Plasminogen binds to the bacterial cell surface by plasminogen receptors and is then converted into plasmin. This process requires fibrin as a co-factor which in turn protects against inactivation by  $\alpha$ 2-antiplasmin (Bergmann & Hammerschmidt 2007). Plasmin degrades fibrin clots, as well as extracellular matrix and connective tissue. It is hypothesized that active plasmin facilitates the degradation of either the layer of fibrin deposited by the host around the site of infection, or the release of bacteria from the reservoir formed by the bacterium itself, followed by ECM destruction,

thereby promoting bacterial invasion and dissemination into deeper tissue (Bhattacharya *et al.* 2012, Sanderson-Smith *et al.* 2012).

The ability of human isolates of *S. aureus* to acquire a bound plasmin-like activity was dependent on the ability of the bacteria to secrete SAK and the presence of plasminogen (Christner & Boyle 1996). However, a correlation between the pathogenicity of *S. aureus* and whether or not it has the ability to secrete SAK has not been conclusively shown. Jin *et al.* showed that infection with staphylokinase deficient *S. aureus* strains is associated with a more pathogenic outcome compared to strains that do produce staphylokinase, whereas Piechowicz *et al.* did not find such a correlation (Jin *et al.* 2003, Piechowicz *et al.* 2010).

#### Extracellular fibrinogen-binding protein

Efb is a 15.8 kDa Fg-binding, secretable expanded repertoire adhesive molecule (SERAM). The C-terminal domain of Efb binds to C3, which is an immune evasion strategy (Ko *et al.* 2010). The N-terminal region contains a binding site for Fibrinogen and Efb was shown to bind to the A $\alpha$ -chain of Fg (Shannon & Flock 2004). Efb binds to fibrinogen and platelets and thereby inhibits platelet aggregation (Figure 8). Since *S. aureus* secretes many virulence factors that contribute to platelet aggregation, the function of Efb seems rather counterintuitive. However, platelets also contribute to tissue repair and wound healing and a compromised wound healing response is in fact a hallmark of *S. aureus* infections. This would suggest a potential role for Efb in the disturbance of wound healing (Shannon *et al.* 2005). Indeed, an Efb-negative *S. aureus* mutant had a significantly reduced ability to induce wound infection in a rat wound infection model (Palma *et al.* 1996, Shannon & Flock 2004).



Since Staphylokinase and Efb display anti-thrombotic properties, they have been considered as novel agents for the treatment or prevention of thrombosis (Lian *et al.* 2003, Shannon *et al.* 2005).

#### Extracellular matrix binding proteins

*S. aureus* produces several adhesion factors that interact with ECM to promote efficient colonization (Chavakis *et al.* 2007). Interactions with the extracellular matrix molecules such as collagen and fibrinogen are important for the activation of the blood coagulation system. These virulence factors may therefore be involved in blood clotting since they bind to extracellular matrix and plasma

proteins, although they have not been specifically linked to the coagulation cascade or platelet aggregation.

Extracellular adherence protein (Eap) is a SERAM that interacts with fibrinogen, fibronectin, prothrombin and other plasma proteins as well as ICAM-1. The effect of Eap binding to plasma proteins is not clear. One study showed that *S. aureus* lacking in Eap caused significantly less abscess formation in a mouse model of infection, compared to wildtype (Lee *et al.* 2002). However, this may be caused by its ability to contribute to immune evasion, rather than binding to plasma proteins. Indeed, another study did not find differences in abscess formation, besides this, Eap negative mutants did not show any differences in fibrinogen or fibronectin binding (Rivera *et al.* 2007).

Collagen binding adhesin (Cna) is an MSCRAMM that mediates *S. aureus* adhesion to collagen which contributes to invasion of tissues. Cna has been shown to contribute to the development of septic arthritis, osteomyelitis and endocarditis (Chavakis *et al.* 2007, Therrien *et al.* 2007).

Many other adhesion factors, such as Extracellular matrix protein-binding protein and several *S. aureus* surface proteins (Sas) interact with the extracellular matrix. However, their function, beyond their contribution to colonization, is mostly unknown (Chavakis *et al.* 2007).

## Conclusions

The blood coagulation system is important for the prevention of bleeding after vessel injury, but it is also used by the immune system as a defense against infections. Some microorganisms, however, use the coagulation system to their advantage. *S. aureus* produces many virulence factors that influence the hemostatic pathway. The interaction between *S. aureus* and plasma proteins fibrinogen, fibronectin and prothrombin leads to the formation of abscesses. Best known for their role in coagulation are Coa and vWbp which activate prothrombin to thrombin. This results in fibrin deposition and eventually the formation of abscesses. Although virulence factors other than Coa and vWbp do not have the capacity to induce the coagulation system on their own (Cheng et al. 2010), they do influence certain parts of the system, like platelet aggregation. FnbpA and ClfA are the most important *S. aureus* virulence factors that influence platelet aggregation. Both have been shown to bind to platelets with or without the help of fibrinogen bridges. Two other virulence factors, SAK and Efb seem to have a conflicting role in *S. aureus* pathogenesis, since they inhibit clot formation. However, these have also shown to conduce to *S. aureus*' pathogenesis by affecting invasion of host tissue by destruction of the extracellular matrix and wound healing respectable.

The abundance of mechanisms for platelet aggregation and activation shows the importance of this interaction in infection. However, this redundancy has made the identification of the role of individual virulence factors difficult. In the case of fibronectin binding abilities of *S. aureus* for example, not only FnbpA, but also FnbpB, Clf and possibly others are able to mediate this. Differences in *in vitro* and *in vivo* studies can be observed because of this. The redundancy of factors could complement the function of the inactivated adhesions during the disease process (Que et al. 2001). The use of *L. lactis*, which is not only poorly pathogenic, but also doesn't contribute to infective endocarditis, has been shown to be a useful tool. By transferring single MSCRAMMs to *L. lactis* it is possible to look at individual contributions to colonization.

Redundancy of certain factors may also be a pitfall with regards to the creation of a vaccine. Interference with one of the MSCRAMMs like for example ClfA may not work, since its function can be substituted by one of the other fibrinogen binding proteins that *S. aureus* produces. Indeed, many virulence factors have been individually tested as monovalent vaccines and these failed in human trials (Maira-Litrán et al. 2012). Because of this, polyvalent vaccines became an interesting new possibility. Since Clf plays an important role in the early stage of *S. aureus* infection, and studies have shown that antibodies against ClfA were opsonic and protective, this virulence factor poses a very suitable candidate for the production of a polyvalent vaccine (Maira-Litrán et al. 2012; Schennings et al. 2012). Immunization with Fnbp inhibited fibronectin binding and resulted in opsonic antibodies and protection against endocarditis in a rat model (Rennermalm et al. 2001; Brennan et al. 1999). Protein A, on the other hand, may not be a useful candidate, since SpA reduces the availability and function of antibodies (DeDent et al. 2012). In fact, it has been suggested that previous infection does not ensure protective immunity because of the immunoglobulin binding properties of SpA. Indeed, immunization of mice with SpA that was mutated in the immunoglobulin binding domains resulted in raised antibodies and protection against MRSA (Kim et al. 2010)

Since Coa, vWbp, ClfA and FnbpA have been shown to be important in *S. aureus* pathogenesis, these virulence factors should be further investigated with regards to a polyvalent vaccine against *S. aureus* (Flick et al. 2013).

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