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Exploring *Staphylococcus aureus* pathways to disease for vaccine development

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Abstract

Staphylococcus aureus is a commensal of the human skin or nares and a pathogen that frequently causes skin and soft tissue infections as well as bacteremia and sepsis. Recent efforts in understanding the molecular mechanisms of pathogenesis revealed key virulence strategies of *S. aureus* in host tissues: bacterial scavenging of iron, induction of coagulation pathways to promote staphylococcal agglutination in the vasculature, and suppression of innate and adaptive immune responses. Advances in all three areas have been explored for opportunities in vaccine design in an effort to identify the critical protective antigens of *S. aureus*. Human clinical trials with specific subunit vaccines have failed, yet provide important insights for the design of future trials that must address the current epidemic of *S. aureus* infections with drug-resistant isolates (MRSA, methicillin-resistant *S. aureus*).

Keywords

MRSA; Iron; Coagulation; Immunmodulation

Introduction

Staphylococcus aureus colonizes the skin and nares of 30–50% of the human population. Nasal colonization typically persists for long periods of time and represents a predisposition to future disease, typically skin and soft tissue infections (SSTIs) but also bacteremia and sepsis. A key feature of *S. aureus* SSTIs is recurrence, which occurs in 20–30 % of all cases even following antibiotic and/or surgical therapy [1]. Following the initial discovery and use of antibiotics, concern and frequency of *S. aureus* disease decreased significantly. However, the golden age of antibiotic therapy was followed by *S. aureus* acquisition of a wide variety of antibiotic resistance genes that provided escape from the most commonly used therapeutics [2]. Of particular concern is the emergence of methicillin-resistant *S. aureus* (MRSA), from community origins (community-acquired or CA-MRSA) and acquisition of additional antibiotic resistance including vancomycin (VRSA), often the antibiotic of last resort for infections with CA-MRSA [3–5]. *S. aureus* infections currently account for ~4% of all hospital admissions in the United States with the related mortality in the US exceeding that of any other infectious disease [6]. In addition, *S. aureus* infections are the leading cause of respiratory, skin and soft tissue, and bloodstream infections [6]. Considering that *S. aureus* has evolved drug-resistance against every antibiotic licensed for the therapy of staphylococcal infections [7], it seems highly unlikely that a miracle drug or silver bullet will be discovered addressing these issues.

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Hygienic measures reduce the burden of staphylococcal infections. Although scientists have tried for decades to develop a vaccine that can protect against *S. aureus* infections, these efforts have not yet borne fruit and anti-staphylococcal vaccines are not available. An important obstacle in the development of vaccines is the clinical evidence for staphylococcal immune evasion. The very same individuals encounter recurrent infections with the same *S. aureus* strain, but are unable to mount protective immune responses [8]. The failure of a variety of subunit vaccines in late stage clinical trials highlights the formidable obstacles on the road towards a staphylococcal vaccine [7, 9–12]. Here we review recent work in three areas of *S. aureus* pathogenesis – iron scavenging, coagulation and immune evasion – and what this research has taught us about vaccine development.

I. Iron homeostasis

Iron in the host—Iron is an indispensable element for many organisms. In the human body iron is an essential component of hemoglobin, important for delivery and transport of oxygen through the blood to major organs and tissues. During cellular respiration, iron is important for energy generating redox reactions. The ability of iron to easily accept and donate electrons makes iron both essential and potentially toxic. Specifically, free, unregulated iron within the cell can catalyze the conversion of hydrogen peroxide into free radicals, having deleterious consequences. To prevent such harmful effects, the abundance and usage of iron in the body is tightly controlled, with free soluble iron concentrations kept at very low levels. As a result the majority of iron in the body is intracellular. 60–80% of the intracellular iron is located at the center of the porphyrin ring of heme [13, 14], a cofactor for hemoglobin in the blood or myoglobin in muscle tissue. Extracellular heme levels are controlled by the heme scavenging host protein hemopexin [15] while extracellular hemoglobin is bound by haptoglobin [16] and the complex removed by the reticuloendothelial system [17]. An additional 15–20% of iron is complexed with the storage molecule ferritin in non-erythrocyte cells [14]. The remaining extracellular iron is scavenged and tightly bound by transferrin in the plasma or lactoferrin in mucosal and similar secretions, aiding intercellular iron transport and preventing iron generated free radicals [18].

Iron homeostasis is regulated through the control of absorption and transport into cells. This occurs mainly through the effects of the small peptide hormone hepcidin which is made and released by the liver in response to iron levels in the body [19–22]. When iron levels are high, hepcidin levels increase and inhibit the uptake of transferrin iron from the plasma into iron storage cells (such as red blood cells) by binding to the Fe transporter ferriportin [23]. This results in the endocytosis and degradation of ferriportin and decreased iron absorption. Under low levels of iron, hepcidin levels are decreased and iron is more readily taken up by cells [23, 24]. Bacterial infection, one cause of inflammation in the host, also results in increased production of hepcidin [25, 26].

Host iron linked defense mechanisms—Much like in its human host, iron, required for cellular respiration, is also an essential element for *S. aureus*. As such, iron acquisition from the environment is vital to the survival of the pathogen. In addition to the usual tight control of free iron availability in the human body, the human host has a number of strategies, collectively referred to as the iron withholding defense system [27], to further limit iron availability to invading pathogens such as *S. aureus*. As mentioned, increased hepcidin levels during inflammation [28] function to suppress release of iron by macrophages and by duodenal enterocytes, concomitant with increased synthesis of macrophage ferritin [23, 29]. Additional iron withholding defenses are mediated by professional phagocytes called to the site of infection, including neutrophil release of apolactoferrin to bind iron [30], macrophage scavenging of hololactoferrin [31], release of

bacterial siderophore binding lipocalins [32, 33], and release of nitric oxide which interferes with the pathogen's iron metabolism [34]. Other described defenses include hepatic release of haptoglobin and hemopexin to bind and scavenge extracellular hemoglobin and heme [35] and B lymphocyte production of immunoglobulins to microbial cell surface receptors that mediate pathogen iron uptake [36]. These measures together limit free iron availability in human tissues to around 10^{-18} M or less [37], levels greatly below what is needed by the pathogen to continue to grow and cause disease.

Pathogen obstacles and strategies for obtaining and regulating iron—In order to successfully replicate and divide, bacterial pathogens have developed numerous mechanisms for iron scavenging, uptake, and transport of limited iron which circumvent the iron withholding defenses of the host. *S. aureus* is not an exception, and has developed a variety of mechanisms to acquire iron from its human host. These mechanisms include systems for binding transferrin, lactoferrin, and/or heme containing proteins by specific bacterial receptors and synthesis and secretion of siderophores, which in turn bind back to specific receptors on the bacterial surface. In addition to systems for acquiring iron, much like the host, bacterial pathogens must also tightly regulate the factors that influence their own intracellular iron levels. *S. aureus* uses a variety of regulatory systems to monitor iron levels and levels in the surrounding environment. This can occur directly via iron dependent regulators, which in the presence of iron bind target sequences located in the promoter region of bacterial iron acquisition proteins repressing their expression. *S. aureus* possesses homologues from two such negative repressor metalloregulatory protein families, Fur (ferric uptake regulator) and DtxR (diphtheria toxin repressor). Two Fur family homologues, Fur and PerR together with the DtxR homologue, MntR (also called SirR or FeoA) [38–41] co-ordinate *S. aureus* metal ion homeostasis and influence the expression of iron responsive proteins. Fur is directly responsive to iron as a Fe(II)-dependent regulator while PerR and DtxR regulation is indirect, mediated through their Mn-dependent roles. Through these three regulators, S. *aureus* has been shown to coordinate the intracellular availability of free iron with the level of antioxidant proteins and manganese present in the cell [39]. The global importance of iron metabolism and acquisition is demonstrated by reduced virulence of *fur* mutant staphylococci in both murine skin [42] and pneumonia infection models [43].

A second iron sensing system recently described for *S. aureus* is involved in directly sensing and regulating intracellular levels of heme. The two component signal transduction system, HssRS (heme sensor system), directly controls expression of the heme regulated ABC transporter HrtAB, which is thought to function as an efflux pump, removing excessive heme, or a toxic byproduct, from the bacterial cell [44, 45]. In the absence of the response regulator HssR or the ATPase component of the efflux pump HrtA, the staphylococci exhibit increased sensitivity to hemin toxicity. *In vivo,* intravenous infection of mice with either mutant strain exhibited liver specific increases in bacterial load and abscesses and an up-regulation of secreted virulence factors with important immunomodulatory functions [46]. The authors speculate that the hypervirulence in the liver is due to this change in expression, perhaps resulting from membrane damage following buildup of heme or a toxic byproduct in the absence of effective export [47].

Staphylococcus aureus iron acquisition systems—In addition to systems that sense and regulate bacterial iron levels, staphylococci have evolved diverse mechanisms to acquire iron and overcome the host defenses aimed at ing iron from the pathogen. These systems can be broadly divided into siderophore-mediated uptake mechanisms and heme transport mechanisms. Siderophores are secreted molecules of low molecular weight that function as high affinity iron chelators. Siderophores are synthesized by specialized enzymes and can involve non-ribosomal peptide synthetases (NRPS) or NRPS-independent siderophore (NIS) synthesis [48]. The latter synthesize siderophores via the condensation of

dicarboxylic acids with diamines or amino alcohol substrates linked by amide or ester bonds [49]. The two *S. aureus* siderophores described to date employ the NIS system to synthesize staphyloferrin A (SA) and B (SB), which scavenge iron primarily from transferrin [50–52]. Following binding of the staphyloferrins to transferrin iron, specific receptors on the staphylococcal surface, HtsA (SA) and SirA (SB), bind the complex followed by permease import of the iron and/or siderophore-iron complex into the cell [53–55]. Mutation of *htsB* or *htsC* led to increased ratios of transferrin to heme-iron uptake, indicating that in addition to siderophore uptake, the Hts transporters can also function in heme-iron import. The same study also established a role for the Hts system in survival in host tissues, with mutations in *htsB* or *htsC* resulting in reduced staphylococcal load in organ tissues [56].

Two additional ABC transporters, Fhu and Sst, also contribute to staphylococcal iron acquisition and uptake. The FhuCBG ABC transporter and two lipoprotein receptors FhuD1 and D2 are important for import of Fe^{3+} hydroxamates [57, 58]. SstABCD has recently been shown to be required for growth promotion by catechol-type siderophores or chatecholamine liberated transferrin iron [59]. By possessing versatility in the class of siderophore transport systems available, *S. aureus* is better equipped to combat the limited iron availability within the host. The combined contribution of these seemingly redundant systems was experimentally confirmed as a triple mutant in *sir*, *hts*, and *sst* had significantly reduced bacterial cfu recovery from multiple organs in murine i.v. challenges compared to single or double mutants in these systems [59].

The *S. aureus* iron-regulated surface determinants (Isd) acquire iron from heme (Fe(III)-protoporphyrin IX). IsdB and IsdH are covalently attached to the cell wall peptidoglycan by the transpeptidase sortase A and displayed on the bacterial cell surface [60, 61]. IsdB and IsdH are receptors for host hemo-proteins, primarily hemoglobin or hemoglobin/haptoglobin complexes [62–64]. The sortase anchored proteins IsdA and IsdC facilitate the passage of heme from IsdB on the surface across the bacterial cell wall [60, 61, 65, 66]. Heme is then imported into the cell via the ABC transporters IsdDEF[67–69], where the iron is liberated by the mono-oxygenases IsdG and IsdI [70, 71]. Heme import across the plasma membrane may also be mediated by the ABC transporters Fhu or Hts [56].

Isd protein mediated protection and vaccine development—As discussed and detailed earlier (*vide supra*), a dichotomy exist for iron. While iron is required for respiratory processes, if uncontrolled cellular damage can result. This dual potential maintains that iron and the proteins involved in capturing, storing, and liberating it must be tightly regulated in most living organisms. In the case of bacterial pathogens, this ensures that specialized host-adapted iron acquisition systems like Isd are expressed only in iron-limiting environments as occurs during infection. Isd proteins are upregulated upon entry into the host, exposed to the immune system, and absent from non-invasive Coagulase-negative (CoNS) staphylococci. These proteins have been the focus of many *S. aureus* virulence and vaccine studies.

As the main heme transporter of *S. aureus*, the Isd pathway is one of the best characterized of the iron transport systems. Extensive biochemical and structural studies have been performed providing insight into the mechanism of hemoprotein binding, heme extraction and import into the cell, and extraction of iron from heme. In addition to the molecular and biochemical details, a vast amount of research has contributed to our understanding of the role of the Isd system in staphylococcal disease and use of the Isd surface components as protective antigens. Initial studies concerning the contribution of the Isd system to pathogenesis were directed at the role of the iron regulated transpeptidase sortase B and its single known substrate, the cell wall anchored surface protein IsdC. These studies established a role for *srtB* in later stages of infection, as a significant reduction in *srtB*

mutant staphylococci recovered from infected tissues was only observed after 9 days [61]. Additional studies examining individual Isd surface receptor mutations, *isdA*, *isdB*, *isdC*, or *isdH* in murine renal abscess models of infection further corroborated a role for the Isd surface molecules in persistence. Mutants in *isdA*, *isdB*, and *isdC*, but not *isdH*, resulted in significant reductions in cfu recovered from renal tissue as well as reduction of abscesses present within renal organs [56, 72, 73]. These same mutants were also investigated for their role in a murine sepsis model. Though isd mutation did not alter the lethal outcome of S. aureus infection the mutants displayed delay in time to death [72]. IsdA has also been shown to contribute to colonization, with a mutation in *isdA* leading to the inability of the mutant staphylococci to colonize the nasopharynx of cotton rats [74].

Initial vaccination studies in murine models of disease together with the identification of IsdB reactive antibodies in infected patients further suggested that the Isd system components, in particular the surface receptors IsdB, IsdH, and IsdA, warranted further examination for the development of a staphylococcal vaccine. Following examination of immune sera from healthy colonized and non-colonized individuals against a large number of staphylococcal proteins, IsdA and IsdH were identified. Reactive antibodies to both proteins were significantly higher in non-colonized compared to colonized individuals. These proteins were then used for vaccination in a cotton rat nasal *S. aureus* colonization model and provided protection in this model [74].

Two studies independently confirmed the protective capacity of IsdB in active vaccination. The studies used different approaches to identify protective staphylococcal vaccine antigens. One group screened a library of recombinant surface displaced polypeptides using sera from patients suffering from acute staphylococcal infections [75]. IsdB immunized mice challenged with a lethal dose of *S. aureus* resulted in significant survival and produced high level titres both in mice and rhesus macaques [11]. In the second study, mice were vaccinated with 19 sortase anchored surface proteins followed by challenge with a renal abscess model of infection. IsdA (and IsdB to a lesser extent) provided the most significant protection in the renal abscess model. A combination vaccine was designed which included the four most promising antigens including IsdA and IsdB, and tested protection in a lethal challenge model. The combination vaccine provided significant protection against multiple MRSA strains [76]. Merck and Co. developed IsdB for active vaccination and the resulting vaccine, V710, showed promising results in early clinical trials [77]. Unfortunately, the most recent Phase II/III trials were ended prematurely after early data analysis failed to show a statistically significant clinical benefit. The failure of V710 could result from, among other possibilities, significant differences between hosts such as variations in development of humoral immunity and/or disease pathogenesis. In addition, a single-valency vaccine may not be sufficient to curtail severe invasive disease and instead a multi-target vaccine or therapy may be required.

In addition to establishment of IsdB as an effective antigen for active vaccination, the studies described above (*vide supra*) also led to development and investigation of antibodies against the Isd surface receptors. Antibodies against IsdB and IsdA have also provided insight into the mechanism of protection afforded by the Isd surface proteins and important conformational epitopes. In addition to V710, Merck also developed murine monoclonal antibodies (MAbs) and assessed them for binding diversity, efficacy in opsonophagocytic killing assays (OPK), and protection in three murine staphylococcal infection models. One promising antibody, 2H2 (both IgG1 and IgG2b isotypes), resulted in greater than fifty percent killing in the OPK assay and was protective in two lethal challenge models as well as in a sublethal indwelling catheter model [78]. A fully human monoclonal was later identified by screening a single chain variable fragment (scFv) antibody library. The human MAb recognized a conformational epitope, exhibited OPK activity similar to that of the

mouse MAb 2H2, and displayed statistically significant protection in i.v. lethal challenge and central venous catheter (CVC) infection models in rats. In the CVC model, statistically significant protection was also achieved when administered therapeutically 1 hour post-challenge [79]. Studies with IsdA and IsdB rabbit polyclonal antibodies also demonstrated protection in murine renal abscess and lethal challenge models. Additionally, *in vitro* studies identified non-IsdB crosreacitve IsdA antibodies that interfered with IsdA heme-binding and non-IsdA crossreactive IsdB antibodies that significantly reduced hemoglobin binding to IsdB. Based on these in vitro assays, the authors speculate that protection mediated by IsdA and/or IsdB antibodies may in part be due to perturbation of Isd mediated heme acquisition by the pathogen [72]. Antibodies have also been used to develop clinical assays to assess protection in humans following vaccination via competitive ELISA, a strategy that could also be applied to other potential vaccine antigens [77, 80].

The importance of iron in the host in regards to *Staphylococcus aureus* infection is emphasized by iron use and storage genetic disorders which can serve to predispose patients to staphylococcal infections. These include hemochromatosis and thalassemia, both of which result in excessive absorption and storage of dietary iron [81]. In addition, host iron and iron binding proteins can also have secondary effects on inflammation and hemostatis. Hemoglobin liberated from red blood cells, lysed by bacterial hemolysins for example, can bind and inactivate nitric oxide (NO). This results in narrowed vessels and increased platelet aggregation, both of which contribute to thrombosis [82]. By reducing the available Fe^{2+} and limiting the generation of oxygen intermediates, the Isd proteins have also been linked to secondary effects resulting in resistance to phagocytic killing and H_2O_2 resistance within the phagosome [83, 84]. IsdB has also recently been shown to directly interact with the platelet receptor GPIIb/IIIa, slowing blood flow and contributing to platelet aggregation [85].

II. Blood Hemostasis

Host mechanisms for maintaining hemostasis—Most physiological processes of the human host, as already illustrated with iron homeostasis, are tightly regulated and interconnected. Blood hemostasis, the process of blood maintenance within vessels to ensure a fluid state in healthy endothelial tissue while mediating rapid initiation of the coagulation cascade and formation of a clot upon injury to the vasculature, most also be tightly controlled [82]. These events also influence one another. This is illustrated by extremely low levels of iron, as is the case with severe anemia, which affects the oxygenation state of the blood and can result in hypoxia in turn causeing an ischemic slowing of the blood flow [82]. On the reverse side, local iron excesses can result from blood hemorrhages and red blood cell lysis in damaged tissues (i.e. a bruise). Unrestricted and/or uncontrolled blood coagulation also trigger pathologies, for example thrombosis or embolism, while the inability to form an effective clot can lead to fatal blood loss, as is the case with hemophilia. Maintaining hemostasis involves three important components, the vascular wall (mainly the endothelium), platelets, and the coagulation cascade [82].

Upon injury and or inflammation of endothelia, inhibitory signals to prevent coagulation are removed and arteriolar vasoconstriction is signaled via reflex neurogenic mechanisms and release of endothelin [86]. Platelets are activated and recruited to the site of injury. Activation, involving secretory granule release, is primarily due to injury mediated exposure to ECM components like collagen and the glycoprotein vWF [82]. vWF mediates adhesion by bridging the platelets and the underlying endothelium through binding the platelet glycoprotein GpIb [87]. Additionally the platelet glycoproteins GpIIb and GpIIIa bind soluble fibrinogen [88, 89]. During these initial stages, a reversible hemostatic plug is formed at the site of trauma. Direct signals from the site of trauma trigger the coagulation

cascase, series of serine zymogens that culminate in thrombin activation by the prothrombinase complex, which consists of factor Xa and factor Va [90]. In addition to injury, inflammatory cytokines and/or bacterial factors (such as endotoxin) can activate the coagulation cascade [91–93]. Initiation occurs via endothelial surface exposure of tissue factor (factor III/thromboplastin), the major activator of the extrinsic clotting cascade [94]. Polyanion contact dependent activation of the coagulation cascade intrinsic pathway can also occur, and this is mediated via the serum protease Factor XII [95].

Concomitant with platelet activation and recruitment, tissue factor and factor VIIa generate thrombin, which in turn cleaves soluble fibrinogen circulating in the blood to insoluble fibrin cables [95, 96]. Location of this reaction in the vicinity of the fibrinogen bound platelets stabilizes the clot and mediates platelet contraction, resulting in an irreversible mass or secondary hemostatic plug [82]. Speaking to its central role in blood clot formation, thrombin also serves a pro-inflammatory signaling role. Thrombin fibrinogen cleavage products result in chemotactic signaling of neutrophils to the site of infection while thrombin directly mediates adhesion of neutrophils and monocytes to the clot [97–99]. Lastly, a permanent plug to prevent further vascular leakage is formed following covalent crosslinks of the fibrin cables by Factor XIIIa [100]. The balance then swings back in the reverse direction to favor the anticoagulant mechanisms and assure that a clot only forms at the site of injury.

In order to maintain a hemostatic balance, Factor X conversion of prothrombin to thrombin and initiation of the coagulation cascade is normally inhibited within healthy vessels by endothelial cell secretion of heparan sulfate proteoglycans, thrombomodulin, and tissue factor pathway inhibitor [82]. Clot formation is further inhibited by nitric oxide and prostacyclin which prevent the aggregation of platelets [101]. In the absence of injury, endothelial cells may also cause the degradation of ADP within dense granules of platelets via adenosine diphosphatase secretion, further preventing platelet aggregation [102]. Under normal conditions, active fibrinolysis is also in effect and the protease tissue-type plasminogen activator (t-PA) activates plasmin to cleave fibrin clots [103]. In addition to preventing clot formation under steady state, many of these signaling cascades and mechanisms also function to terminate and resolve coagulation following formation of a stable clot.

Staphylococcus aureus, appropriating host hemostatic mechanisms—During *S. aureus* invasive disease, either at the site of primary infection or as the bacteria disseminate to other tissues and seed abscess formation, staphylococcal cells will come into contact with the host's circulatory system [8]. *S. aureus* has been clinically distinguished from other staphylococcal species primarily based on bacterial molecular interactions with the host's blood system. Of these clinical tests is the distinction between coagulase positive *S. aureus* from other CoNS. This clinical designation, referred to as the coagulase test, is historically based on *S. aureus* dependent coagulation of blood [104]. A related *S. aureus* dependent phenomenon, also exploited as a diagnostic tool, monitors the aggregation or agglutination of staphylococci exposed to calcium-chelated plasma on a glass slide [105, 106]. The slide agglutination test has further been reduced to the clumping or precipitation of bacteria in the presence of soluble fibrinogen [107]. These observations, mediated by molecular interactions between *S. aureus* and its host, have been studied for over 100 years. The major bacterial effectors of these observations have been known for some time, namely the staphylocoagulase Coa, and more recently identified, vWbp, which mediate host prothrombin and fibrinogen interactions [108, 109] and ClfA which mediates the clumping phenotype and binds to soluble fibrinogen [110, 111]. A handful of new studies have provided additional insight into these multi-component molecular interactions. Specifically, multi-gene mutation virulence studies and the identification of additional bacterial mediators

have significantly furthered our understanding of *S. aureus* pathogenesis in blood hemostasis.

Early *S. aureus* studies on the bacterial dependent coagulation of blood/plasma were based primarily on the biochemical separation and isolation of this activity [112–114]. With the advancement of genetic and recombinant DNA technologies, the gene encoding staphylococcal coagulase (*coa*) was identified [115–118]. Later studies identified a second staphylococcal gene product that also possesses coagulase activity, von Willebrand factor binding protein (vWbp), first isolated based on its interactions with vWF [119, 120]. Both *S. aureus* coagulases bind to human prothrombin resulting in the zymogen dependent cleavage of the Aα and Bβ chains of fibrinogen to fibrin [108, 109]. It is this reaction that mediates the early observations of staphylococcal agglutination and coagulation.

A model delineating the mechanism for the coagulation and rapid conversion of fibrinogen to fibrin by the staphylococcal coagulases has been elegantly established following crystallization of the active staphylothrombin complex [108]. The N-terminal Ile1-Val2 of Coa insert into the Ile16 cleft of prothrombin while the remainder of the N-terminal D1D2 domain (present in both Coa and vWbp) binds and stabilizes the complex. Coagulase binding to prothrombin induces a conformational change resulting in a functionally active site [121]. Interestingly, while binding of the D2 region blocks the α– Thrombin fibrinogen recognition site, the Coa-Prothrombin complex is still able to bind fibrinogen with high affinity [108, 121]. In addition, coagulase induced formation of an active site in prothrombin in the absence of cleavage to thrombin and blockage of exosite1 also blocks the action of thrombin inhibitors such as heparins, vitamin K antagonists and the bivalent thrombin inhibitors such as hirudin [115]. In this same manner, the staphylococcal coagulases are also able to circumvent the regulatory processes of the coagulation cascade [122]. Univalent direct thrombin inhibitors (DTIs) such as argatroban, melagatran, and dabigatran are exceptions and are effective in inhibiting the Coa Prothrombin complex as they only target and bind the active site [123, 124]. Differences in zymogen activation [109, 125] and possessing very distinct C-termini, suggests that the role of the two coagulases is not simply redundant and that each provides a unique contribution to establishment of disease.

Contribution of staphylocoagulases to virulence and protection—The contribution of staphylococcal coagulation toward *S. aureus* pathogenesis had been a matter of debate. Conflicting reports on the importance of the staphylocoagulase in a variety of disease systems had left the role of coagulase during infection unresolved. However, clinical observations that infections caused by coagulase positive strains such as *S. aureus* are much more invasive and severe compared to CoNS such as *S. epidermidis* together with early experimental evidence that injection of CoNS with coagulase material enhances virulence support the inclusion of coagulase as a *S. aureus* virulence factor [113]. Initial studies of mutants isolated from chemical or transposon mutagenesis strategies found decreased virulence in experimental endocarditis, i.v. and i.p. LD_{50} infections, and murine mastitis [112, 126–129]. However the mode of mutagenesis and mutant selection may have also led to additional mutations, for example in important virulence regulatory genes. Generation of an isogenic *coa* mutant did not display any defect in skin abscess, endocarditis, or mastitis murine infection models [117, 130], while an independently generated isogenic *coa* mutant did exhibit a virulence defect in a hematogenous pulmonary infection model [131]. Based on the finding that staphylococcal vWbp also possesses coagulase activity, more recent work generated mutant strains in *S. aureus* Newman by allelic replacement of each *coa* and *vWb* genes or both in combination. The single mutants had significant defects in renal abscess formation, lethal bacteremia, as well as survival in blood. Importantly however, these phenotypes were greatly enhanced for the double mutant [132].

Studies aimed at further dissecting the formation of staphylococcal vegetations during acute endocarditis established that prothrombin bound to staphylocoagulase was found at the leading edge of the vegetations. Further, the survival of animals infected with the double coagulase mutant significantly improved and detection of staphylococci by positron emission tomography (PET) imaging revealed significantly reduced bacteria within vegetations [133]. The related biochemical reaction of agglutination employed citrate treated plasma and coagulase positive staphylococci, resulting in bacterial aggregation [105]. Distinct from the later reduction of this reaction, the fibrinogen clumping reaction, which employs only purified, soluble fibrinogen and bacteria, the agglutination reaction was proposed to require both fibrinogen and prothrombin [106, 107]. Mediating the fibrinogen clumping reaction, N2 and N3 of the A-domain of *S. aureus* ClfA, a staphylococcal sortase anchored surface protein, bind to soluble fibrinogen through the C-terminal ends of the γ chains [107, 111, 134] resulting in precipitation of staphylococci [107, 110, 135, 136] The N2 and N3 of a second staphylococcal clumping factor, ClfB which also binds to cytokeratin, bind to a short region of fibrinogen located within the C terminus of the α-chain [137]. The involvement of clumping factor as well as the coagulases in the agglutination reaction, more closely mimicking what may occur within the host, was recently confirmed. In addition, staphylococcal agglutination mediated by Coa, vWbp, and Clumping factor A (ClfA) in blood was shown to contribute to thromboembolic lesions in heart tissues and fatal outcome in a lethal i.v. infection model of bacteremia, as a triple *coa*, *vWbp* and *clfA* mutant resulted in complete survival [138].

Development of staphylocoagulase based therapies against S. aureus disease—The combined research investigating mutations in *coa*, *vWbp*, and *clfA* individually and in combination have contributed a great deal to furthering our understanding of the molecular events involved in staphylococcal disease and in the development of therapies aimed at prevention. As vaccines provide the greatest promise for treatment, investigation of antibodies raised against these factors has also provided significant insight into designing effective therapies. Specifically, active immunization and passive immunization with polyclonal antibodies raised against Coa and vWbp provide significant protection in *S. aureus* renal abscess and lethal models of infections, mimicking the virulence of the genetic deletions such that the combination of both antibodies provides increased protection compared to either alone [132]. In addition to providing significant protection in animal models, the antibodies were able to significantly extend the clotting time of human and mouse blood in vitro, again with the combination of both antibodies more significant than each alone. The antibodies were also able to block the association of the coagulases with fibrinogen (αvWbp) and prothrombin (αvWbp as well as αCoa) and inhibit the conversion of fibrinogen to fibrin [132].

Recent findings have shown that a new class of direct thrombin inhibitors including Pradaxa (dabigatran etexilate) are able to inhibit staphylocoagulase activity [124]. This finding together with the role of the coagulases and ClfA agglutination in murine lethal bacteremia were the basis of a successful combination therapy for treatment of murine acute *S. aureus* disease involving dabigatran and polyclonal ClfA antibodies [138]. Of interest, previous isolation of a monoclonal antibody to ClfA that interferes with fibrinogen γ–chain binding [139, 140] provided promising results in preclinical studies, however, no significant differences were detected between treatment and placebo groups in phase II end point analysis [141]. ClfA interactions with other host proteins, including complement regulatory factor I which mediates escape from phagocytic killing [142] or incomplete inhibition by the monoclonal if the gamma chain is not accessible following conversion to fibrin [143, 144], could help to explain the failure of Tefibazumab in phase II trials.

In addition to the specific contributions to survival and adaptation within the host blood system mediated by the staphylocoagulases and ClfA, *S. aureus* encodes an arsenal of additional gene products that aid in its ability to escape, grow, and survive the hemostatic pathway. These proteins, many of which also interact with fibrinogen, complement, and host mediators of inflammation, may also influence hemostatsis. A complete model for staphylococcal blood pathogenesis is thus not fully appreciated.

III. Immunmodulation

Upon breach of the epithelial surface barrier defenses, either as a result of trauma or other disease pathology, staphylococci gains access to deep tissues and/or the blood stream. There they encounter the host's innate immune system, mainly composed of professional phagocytes (macrophages, dendritic cells, neutrophils and eosinophils) and antimicrobial proteins (cytokines, complement proteins and antimicrobial peptides). Immediate activation of complement proteins through recognition of foreign particles including pathogens is amplified by secretion of pro-inflammatory cytokines and recruitment of phagocytic cells. Neutrophils are the primary host defense against staphylococcal infection (reviewed in [145]). This is exemplified by the recurrence of *S. aureus* infection in patients suffering from agranulocytosis or chronic granulomatous disease (CGD), genetic disorders that result in deficiencies in NADPH activation and failed neutrophil respiratory bursts [146]. *S. aureus* possesses a variety of molecules and mechanisms to combat and diminish neutrophil defenses including secretion of proteins that inhibit the deposition and activation of complement, generation of molecules that prevent or deter neutrophil recruitment to the sites of infection, and molecules with lytic properties that target neutrophils and other phagocytic cells [145, 147–150].

Prevention of phagocytic killing and promotion of intracellular survival— Several bacterial factors also mediate interactions with phagocytes, aiding survival within the phagocyte. *S. aureus* is capable of secreting cytolytic toxins such as alpha-hemolysin, leukocidins and PVL, which as well as killing the phagocyte and liberating key nutrients such as heme bound iron for extracellular staphylococci, may provide intracellular staphylococci an escape from phagolysosomes. Recent identification and characterization of phenol soluble modulins (PSMs), which are short amphiphathic and alpha helical peptides generated by most *S. aureus* strains, implicated an additional mode of bacterial neutrophil killing. In addition to secreted factors to interfere with phagocytic cells, *S. aureus* also retains surface associated virulence factors that inhibit phagocytic uptake. Two sortase anchored proteins, ClfA and SpA, inhibit phagocytosis, partly due to their interactions with fibrinogen and the Fc portion of immunoglobulin, respectively.

A third recently characterized staphylococcal surface protein, AdsA, harbors two 5′-nucleotidase enzymatic domains that convert AMP to adenosine. Early experiments had provided that this protein was important in promoting staphylococcal survival in the phagolysosome of neutrophils. Following intravenous staphylococcal infection of mice, a significant Increase in extracellular adenosine was generated. Further confirming the role in generation of increased adenosine levels, a mutant strain expressing non-catalytic AdsA failed to produce a similar increase [151], While the exact details of this process are currently being examined, it is clear that the staphylococcal cell has adapted to hijack an immunosuppressive host signal for promotion of its own survival. Other staphylococcal strategies, including an additional potential role for adenosine, to prevent generation of a productive adaptive immune response will be discussed later in this review (*vide infra*). Staphylococci also elaborate a polysaccharide capsule and the polysaccharide intercellular adhesin (PIA, Poly-N-Acetyl-Glucosamine) both of which are thought to possess anti-opsonic properties [152, 153]. However, as mutant *S. aureus* incapable of expression of CPs

and PIA did not exhibit decreased survival in naïve mouse blood, questions remain regarding the anti-opsonic role of these molecules (ref?).

S. aureus targets the intersection of the innate and adaptive immune response—Development of a pathogen specific adaptive immune response is a complex process whereby proper engagement of innate immune components and the proper cross-talk between T- and B-cells with phagocytic cells are essential [154–157]. While it is well established that the complement system is activated upon interaction with *S. aureus* in the blood stream, it was not fully appreciated until recently that the proper engagement and presentation of complement factors is critical to elicit a neutralizing immune response [158–161]. Not surprisingly, *S. aureus* produces an arsenal of proteins specifically aimed at targeting this important intersection between the innate and adaptive immune responses [162]. Three different pathways of complement activation (classical, alternative and lectin) converge at the formation of C3 convertases (C4bC2a for classical and lectin pathways and C3bBb for the alternative pathway). The C3 convertases enzymatically cleave C3 into its active products C3a and C3b. Release of the chemoattractant C3a reveals a reactive thioester in C3b that is necessary for covalent attachment and deposition onto the surface of foreign particles, including staphylococcal cells [163]. C3b deposition results in phagocytosis via complement receptor-containing cells. The secreted staphylococcal complement inhibitor (SCIN) protein interacts directly with both C3 convertases and stabilizes them, resulting in inhibition of further C3b deposition [148]. Staphylococci also interfere directly with the products of the C5 convertases. *S. aureus* superantigen-like protein 7 (SSL7) strongly inhibits C5a neutrophil recruitment through IgA dependent binding to C5, preventing its cleavage [164]. The staphylococcal secreted chemotaxis inhibitory protein (CHIPs) interacts directly with the C5a Receptor (C5aR) on neutrophils blocking C5a chemoattractant signaling [150].

S. aureus strains also express two immunoglobulin binding molecules, SpA and Sbi. These two proteins share significant amino acid homology in their Ig binding domains [165]. The interaction and sequestration of anti-staphylococcal immunoglobulins mediated by these proteins may severely limit host immunoglobulin mediated immune clearance of the pathogen. In contrast to SpA which is composed of four to five Ig binding domains (*vide infra*)[166], Sbi contains two Ig binding domains at the N-terminus followed by two domains that interact with complement protein C3 via C3d [165, 167]. C3d binds to antigens enhancing their uptake by dendritic cells and B cells[168]. The interaction with C3d is also mediated by two additional staphylococcal surface associated proteins, Efb and Ehp [169, 170]. Interestingly, mutational analysis revealed that the C3d residues involved in the interaction with complement receptor 2 (CR2) on B cells also participate in the interaction with the staphylococcal proteins [171]. Of note, CR2 can be found on the surface of follicular dendritic cells, B cells and T cells [168]. It is believed that synergistic B cell activation occurs through the simultaneous C3d-CR2 ligation and antigen specific immunoglobulin interactions [168].

Although complement activation is a necessary step to detect and eliminate invading pathogens, excessive complement activation can lead to increased, uncontrolled inflammation. In order to prevent uncontrolled activation, the host expresses complement factors H and I that downregulate complement fixation. Many pathogens have evolved to take advantage of these proteins by producing virulence factors that bind complement factors H and I. *S. aureus* is not an exception. The surface protein ClfA has been shown to capture and activate Factor I, enhancing the cleavage of nascent C3b into iC3b, ultimately reducing phagocytosis of bacteria by human neutrophils [147]. It was also suggested that Sbi binds Factor H and C3 simultaneously to form a tripartite complex, consuming a significant level of C3 in serum [167]. In order to promote its own survival, *S. aureus* has adapted to its

human host in part by limiting complement mediated defenses which are essential for convergence of the innate and adaptive immune responses [162].

Preventing development of productive host adaptive immunity—As discussed earlier in this review (*vide supra*), AdsA supported delayed killing of *S. aureus* in blood neutrophils [151]. Though the exact mechanisms and strategies for survival of the staphylococci inside a professional phagocyte have not been determined, they would necessitate *S. aureus* circumventing and/or modulating a variety of bactericidal processes including reactive oxygen species (ROS) and hydrolytic enzymes. If one considers that *S. aureus* may possess mechanisms to evade the defense and killing strategies of professional phagocytes by altering phagocyte function, such strategies would also have downstream effects on the development of a proper adaptive immune response. In addition, as part of the anti-inflammatory signaling properties of adenosine, levels of MHC-II on macrophages and dendritic cells are reduced together with production of IL-12, a pivotal stimulus for Th1-type immune responses [172–174]. These signaling effects may also add to staphylococcal survival by interfering with the adaptive immune response, for example the generation of ineffective T-cell effector mechanisms.

As mentioned (*vide supra*), most human clinical isolates express two immunoglobulin binding proteins, protein A and Sbi. Protein A, which is one of the most abundant staphylococcal cell wall anchored proteins, harbors four to five immunoglobulin binding domains [166]. Individual Ig binding domains fold into triple helix bundles connected by short linkers [175]. The five successive Ig domains are followed by region X [176]. Protein A is well known for its binding to the Fcγ portion of most Ig subclasses [177]. Protein A specific interactions have more recently been expanded to include interactions with the F(ab)2 of VH3 type B cell receptors, von Willebrand factor, gC1qR/p33, and tumor necrosis factor receptor 1 (TNFR1) [178–181]. Protein A crosslinking to $F(ab)_2$ B cell receptors results in B cell superantigen activity, apoptosis and deletion of V_H3 type supra-clonal B cells. During staphylococcal infection, protein A is anchored to the peptidoglycan of staphylococci by the transpeptidase sortase [182]. Additional protein A is also released into the surrounding environment during peptidoglycan turnover resulting from normal cell growth and division. Both forms of protein A may interact with VH3+ BCR clones, which account for up to 50% of the total B cell population in humans [178, 183]. In mice, B-1 and marginal zone B cells, both innate like B cells which secrete natural antibodies, were the most susceptible B cells to soluble protein A treatment [184]. B-2 cells, follicular B cells which generate antigen specific antibodies, required more persistent superantigen activity [184]. Once depleted by protein A, the B cell subsets were not readily replenished, creating a deficit in the ability to elicit anti-staphylococcal immunity [185].

Staphylococcal immune evasion and vaccine design considerations—Despite being one of the most abundant staphylococcal surface proteins for potential interaction with the host immune surveillance system, wild-type Protein A makes an unattractive candidate for a *S. aureus* vaccine due to its toxicity and B cell superanitgen activity. Encouragingly however, Infection of mice with a Protein A knock out strain elicited protective immunity against secondary infection with wild-type *S. aureus*. This may be due to the resulting increased antibody responses [186, 187]. Subsequent generation of a non-toxigenic variant of Protein A with mutations in each one of five Ig binding domains (SpA_{KKAA}) eliminated Fcg and F(ab)V(H)3 binding and prevented B cell apoptosis. Vaccination of mice with this antigen resulted in generation of high titre antibodies that promoted *S. aureus* clearance by opsonophagocytosis and protection of mice against the current US epidemic MRSA strain, LAC. Vaccination with the SpA_{KKAA} also resulted in increased IgG titres to a variety of *S. aureus* antigens compared to adjuvant alone [186]. It is tempting to speculate that while also providing a new potential vaccine antigen, that SpA_{KKAA} could also be thought of in an

adjuvant capacity, administered in early childhood to help promote development of productive anti-staphylococcal immunity from natural infection.

Synthesis—*S. aureus* iron scavenging, coagulation and escape from innate and adaptive immune responses are key virulence strategies in the pathogenesis of skin and soft tissue infections, bacteremia or sepsis. Are these genetic determinants of staphylococcal disease also protective antigens that enable the development of efficacious vaccines? Research reviewed herein proposes several key virulence genes and presumed protective antigens but also describes the multi-factorial nature whereby staphylococci cause abscess lesions or sepsis. Thus, active or passive immunization strategies that focus on single antigens are more likely to fail than those that disrupt multiple pathogenetic processes. We therefore propose the development of combination vaccines that incorporate multiple protective determinants to raise immune responses that block staphylococcal iron uptake, interfere with coagulation and disrupt the immune evasion strategies of *S. aureus*.

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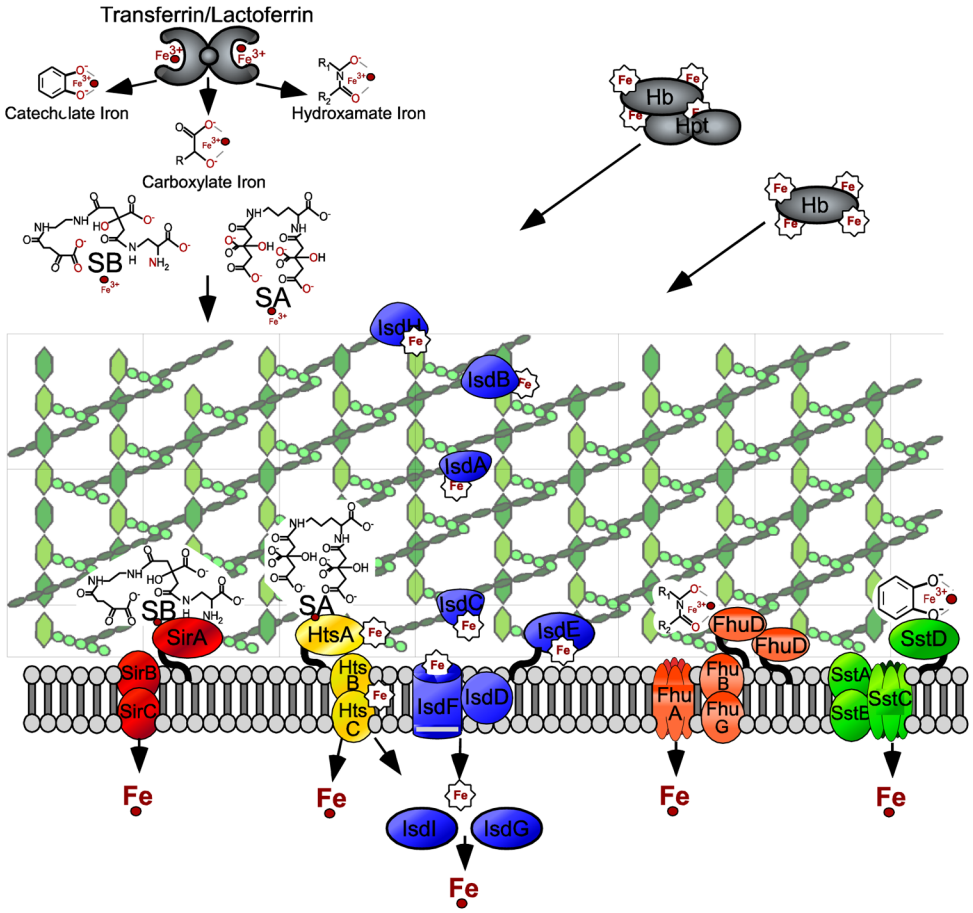

Fig 1. Iron Acquisition and Transport in *Staphylococcus aureus*

Five Iron import systems have been described to date in *Staphylococcus aureus*. The Isd system imports heme iron (Fe(III)-protoporphyrin IX). IsdB and IsdH are hemoprotein receptors, capturing hemoglobin (Hb) and hemoglobin-haptoglobin (Hpt) complexes, respectively. Following extraction from the hemoprotein, the heme moiety likely passes through the cell wall by increased binding interactions with IsdA, IsdC, and the lipoprotein IsdE, respectively. The heme is then imported into the cell by the IsdDEF and/or possibly the Hts or Fhu ABC transporters. The heme is then degraded and free iron liberated by the heme oxygenases IsdI and IsdG. Siderophore mediated transport systems include the Hts (multi-iron source import?), Sir, Sst, and Fhu (multi-iron source import?). These transporters acquire iron primarily from transferrin and/or lactoferrin and may demonstrate preferences for siderophore type (carboxylate, catecholate, or hydroxamate). HtsA and SirA are the receptors for the staphylococcal synthesized carboxylate-type siderophores Staphyloferrin A (SA) and Staphyloferrin B (SB), respectively. The Sst ABC transporter have been linked to catechol-type siderophore and chatecholamine iron sources and the Fhu transporter is important for hydroxamate-type iron sources. This diverse set of systems for acquiring iron from different sources in the host provides *S. aureus* with versatility and a better chance for survival in the iron limited environment within the host.

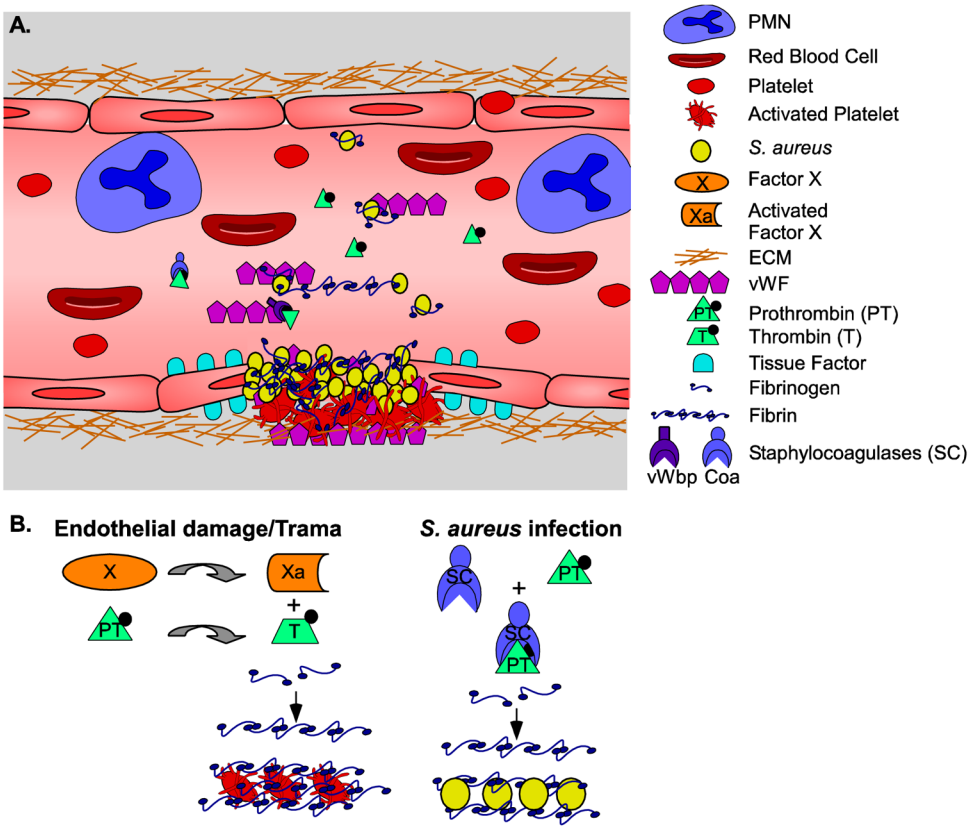

Fig 2. Staphylocoagulase mediated coagulation

A. The *S. aureus* genome encodes for two gene products able to bind Prothrombin (PT) and mediate coagulation. In addition to PT, the staphylocoagulases (SC) Coa and the more recently discovered vWbp, mediate additional interactions to aid blood coagulation and clot formation. Normal clot formation is initiated by trama or damage to the endothelium, followed by release of tissue factor and exposure of ECM factors such as collagen and vWF. vWF bridges platelets and the underlying tissue, aiding in platelet activation, which bind soluble fibrinogen. *S. aureus* vWbp and Protein A both bind vWF and these interactions together with fibrinogen binding proteins such as ClfA, mediate accumulation of the products necessary to form fibrin cables and further reinforcement of the clot. The generation of thrombin serves additional pro-inflammatory signaling roles including neutrophil (PMNs) recruitment and covalent crosslinking of fibrin cables by Factor XIII helping to form a permanent clot and activation of fibrinolysis. These actions may be avoided or limited during staphylococcal coagulation. B. A mechanism and model for staphylococcal coagulase mediated conversion of fibrinogen to fibrin was established following crystallization of the active (Coa Prothrombin)$_2$ complex. In this model, the N-terminal Ile1-Val2 of Coa (SC) inserts into the Ile16 cleft of prothrombin (PT). The D1D2 domain makes additional stabilization contacts with PT. A functional active site is formed following SC induced conformational changes and the complex binds fibrinogen with high affinity. By not requiring PT cleavage by Factor X (X-inactive, Xa-active) to thrombin (T) together with SC D2 blockage of the α-Thrombin recognition site for fibrinogen (●) on PT, the regulatory processes of the coagulation cascade are avoided