***Staphylococcus aureus* Secretes Coagulase and von Willebrand Factor Binding Protein to Modify the Coagulation Cascade and Establish Host Infections**

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Key Words

Staphylococcus aureus infection • Coagulation • Host defense • Neutrophils • Proteinases • Sepsis

Abstract

Clinical isolates of *Staphylococcus aureus* secrete coagulases, polypeptides that bind to and activate prothrombin, thereby converting fibrinogen to fibrin and promoting the clotting of plasma or blood. Two staphylococcal products, the canonical coagulase (Coa) as well as the recently identified von Willebrand factor binding protein (vWbp), promote similar modifications of the coagulation cascade during host infection. Staphylococcal binding to fibrinogen or fibrin is an important attribute of disease pathogenesis, which leads to the formation of abscesses and bacterial persistence in host tissues and also enables the pathogen to cause lethal sepsis. Circumstantial evidence suggests that the product of coagulase activity, staphylococci captured within a fibrin meshwork, enable this pathogen to disseminate as thromboembolic lesions and to resist opsonophagocytic clearance by host immune cells. In addition, the coagulation products of staphylococci appear to display discrete differences when compared to those of thrombin-mediated coagulation, the latter representing a key innate defense mechanism against many

invading pathogens. Preclinical evidence suggests that inactivation or neutralization of coagulases may prevent the pathogenesis of staphylococcal infections, a strategy that could be used to combat the current epidemic of hospital-acquired infections with drug-resistant *S. aureus* isolates.

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Introduction

Coagulation is an ancient innate defense mechanism against microbial pathogens that traps and immobilizes invading bacteria in a clot [1–3]. However, as observed for many other host defense pathways, coagulation is also the target of bacterial immune evasive strategies [4]. *Staphylococcus aureus* is a commensal of the human skin and nostrils and frequently invades skin breaches to generate soft tissue infections. *S. aureus* also causes deadly invasive infections such as sepsis, endocarditis, osteomyelitis, pneumonia and toxemias of the gastrointestinal and reproductive tracts [5]. Invasive infections of *S. aureus* are epidemic in health care settings [6–8]. *S. aureus* is distinguished clinically from less pathogenic strains of staphylococci by the coagulase test [5]. Inoculation of calcium-chelated plasma or blood with *S. aureus* results in rapid

clotting. This phenomenon, first described in 1903 [4], has been studied with great interest for more than a century. The genes that produce this effect are important virulence factors during the pathogenesis of *S. aureus* infections, enabling the formation of abscesses for staphylococcal replication and the depletion of clotting factors from blood [9]. In this review, we describe the mechanisms whereby staphylococcal proteins coopt the coagulation cascade of their infected host and how these phenomena aid bacterial evasion of innate immune responses to promote staphylococcal diseases.

Physiological Coagulation/Fibrinolytic Cascade

Coagulation of blood or extracellular fluids is controlled by a cascade of serine proteases, which are activated following tissue injury to limit blood loss and are regulated closely to prevent systemic coagulation [10]. In the extrinsic coagulation cascade, tissue injury exposes tissue factor, which binds plasma factor VIIa (fVIIa) to form a complex that converts fX to fXa [11]. fXa and fVa form the prothrombinase complex that cleaves prothrombin to thrombin [10]. Both of these reactions require calcium and a phospholipid surface [10]. The degree of clotting is amplified by the intrinsic coagulation cascade and by positive feedback loops from activated coagulation factors [10]. Activated platelets further contribute to the amplitude of coagulation by localizing the prothrombinase complexes, calcium and phospholipids [10]. The coagulation cascade culminates in the conversion of fibrinogen to fibrin by thrombin [10].

Fibrinogen is a 340-kDa soluble glycoprotein found at high concentrations in blood and extracellular fluids. It is a dimer of trimers, composed of two A- α , two B- β and two γ -chains, linked together by 29 disulfide bonds [12]. The N-termini of the 6 polypeptides meet head-to-head in the central E domain, whereas their C-termini extend out to form symmetrical globular domains [13]. Thrombin initiates fibrin formation by cleaving fibrinopeptide A and fibrinopeptide B from the N-termini of the α - and β -chains [14]. Removal of these peptides initiates a structural rearrangement between adjacent polypeptides, resulting in elongation and lateral aggregation of fibrin into a polymer that constitutes the mesh network of a clot [15]. Fibrin aggregates are strengthened by fXIII cross-linking [16]. fXIII introduces ϵ -(γ -glutamyl)lysine cross-bridges between adjacent α - and γ -chains [16].

Fibrinolysis is the tightly regulated response that counters coagulation. Plasminogen is the circulating zy-

mogen that is converted to plasmin when activated [17]. Plasmin cleaves fibrin at specific sites, resulting in the sequential breakdown of the fibrin mesh into three major digestion products and many other, less abundant species [18]. Several proteins promote fibrinolysis by activating plasminogen (tissue-type plasminogen activator, urokinase plasminogen activator) [19]. Plasmin accumulates in the presence of the C-terminal lysines on fibrin that are exposed by plasmin cleavage, thereby potentiating further cleavage events [19]. Other proteins reduce fibrinolysis by sequestering plasmin or its activators into an inactive enzyme-inhibitor complex (α 2-antiplasmin, plasminogen activator inhibitor-1) [19]. To promote clot stabilization against lysis, antifibrinolytic proteins are incorporated into the growing fibrin clot by fXIII [19].

Fibrin morphology, strength and resistance to lysis are governed by many factors that are not yet completely understood. The concentration of both fibrinogen and thrombin plays a role in the kinetics of polymerization [20]. The rate of removal of fibrinopeptide A and, to a lesser extent, fibrinopeptide B, affects the rate of protofibril assembly, which in turn determines the structure of lateral aggregation and fibrin branching [20]. During clot formation, fXIIa increases the lag time of fibrin formation, density of fibrin fibers, and clot stiffness, independent of thrombin generation [21]. Further, fXIII affects the 3-dimensional structure of fibrin clots, as mutations that lead to more rapid activation of fXIII produce thin fibers [22] that are resistant to clot lysis [23]. Fibrin fiber morphology is influenced by other environmental determinants such as pH, calcium concentration and the presence of fibrinogen-binding proteins such as fibronectin and albumin [24]. Platelet aggregates promote the formation of dense fibrin networks [24]. These findings demonstrate that the specific environment in which a clot forms affect its 3-dimensional structure.

The structure of the fibrin network is the key determinant for its dissolution. As stated above, lytic enzymes and their regulators are incorporated into growing clots and affect the subsequent rate of lysis. Clots composed of thinner fibers with smaller pores are less permeable to lytic enzymes and more resistant to lysis [20]. Furthermore, the addition of polyphosphate, which is released from activated platelets, to fibrinogen and thrombin during clot formation results in clots that are less turbid and more resistant to lysis, independent of the rate of clot formation [25]. Thus, the unique conditions that promote hemostasis and the formation of a clot also affect its susceptibility to subsequent lysis and the eventual restoration of homeostasis.

Staphylococcal Coagulases

S. aureus secretes two proteins that promote coagulation, coagulase (Coa) and von Willebrand factor binding protein (vWbp). Both of these proteins activate prothrombin nonproteolytically [26, 27]. The N-terminal ends of Coa and vWbp each associate with the prosite of prothrombin, completing an active site that is otherwise only formed in thrombin. Of note, Coa- and vWbp-mediated activation does not involve fVa and fXa cleavage of prothrombin [26]. Coa is a protein of approximately 670 amino acids that varies in length among different strains [28]. The N-terminal 282 amino acids of Coa encompass the α -helical D1-D2 domains [26], which bind to the C-terminus of the β -chain of prothrombin [29]. The D1-D2 domains are followed by a 153-residue linker region, the function of which is still unknown [26]. The C-terminal end of Coa is comprised of 2–8 tandem repeats of a 27-residue peptide, designated the fibrinogen-binding domain that is found at the C-terminal end of another staphylococcal fibrinogen-binding protein [26, 30]. As a consequence of its interaction with fibrinogen and prothrombin, Coa mediates clotting of soluble fibrinogen, plasma or blood [9, 31, 32].

vWbp shares homology in the D1-D2 domains of Coa [33]. The remainder of vWbp is unique in sequence and includes a binding site for von Willebrand factor [33]. vWbp binds prothrombin and fibrinogen, the latter with a lower affinity than Coa [9, 27, 33]. Prior to the identification of the *coa* or *vwb* genes, studies on coagulases were based on purification methods that may or may not have isolated both proteins. We presume the two proteins display different activities in vivo. Nevertheless, to date, their differences are largely defined by their unique interactions with their binding partners, listed above.

Coagulases Are Virulence Factors for *S. aureus* Pathogenesis

The role of coagulases in disease has been a matter of considerable inquiry. Following the discovery that *S. aureus* isolates coagulate plasma or blood, microbiologists discovered the link between virulence, i.e. the association of a clinical staphylococcal isolate with human disease, and its ability to coagulate plasma [34, 35]. Injection of coagulase-negative staphylococci that had been incubated with purified coagulase into the bloodstream of mice resulted in increased mortality, even if the bacteria were washed prior to injection [36]. However, if coagulase was

injected prior to a challenge with coagulase-negative staphylococci, animal survival was unaffected [36]. These results suggested that the pathogenic role of coagulase relied on the proximity of staphylococci to the ensuing coagulation product.

Nevertheless, the results of experiments that have examined the role of coagulase during *S. aureus* infection have not been unequivocal. Chromosomal deletion of *coa* does not affect *S. aureus* virulence in mouse models of subcutaneous infection or mastitis [37], or in a rat model of infective endocarditis [38, 39]. Heterologous expression of *clfA*, which encodes the fibrinogen-binding clumping factor of *S. aureus*, but not of *coa* in *Streptococcus gordonii*, resulted in increased bacterial adherence to fibrin platelet thrombi and higher rates of infective endocarditis in rats when compared to *S. gordonii* without these genes [40]. In contrast, the analysis of various *S. aureus* isolates for coagulase activity and virulence following intravenous challenge of mice revealed a positive correlation between coagulase titer and bacterial load in the lung [41]. Moreover, in this study, the number of viable bacteria recovered from the lung 7 days after infection was significantly higher for the wild-type strain than for the *coa* deletion mutant [41]. Early work used chemical mutagenesis to isolate coagulase-negative variants of *S. aureus*; however, the mutational lesions were not characterized [42–44]. The field received a critical boost with the discovery that both *coa* and *vwb* encode secreted factors (coagulases) that clot blood [33]. The targeted deletion of either *coa* or *vwb* alone resulted in only a moderate decrease in virulence, whereas the *coa-vwb* double mutant displayed a dramatic reduction in the ability to cause abscesses or lethal sepsis in mice [9].

The activity of coagulase in vivo has been investigated by injecting rabbits with purified coagulase [45]. At a dose of 2–5 mg Coa, fibrinogen levels dropped and blood drawn from these rabbits failed to clot, indicating that the coagulation system was activated and fibrinogen stores were depleted [45]. Injection of 20 mg Coa caused rabbits to die within 20 minutes [45]. Necropsy revealed fibrin thrombi in the vasculature of the kidneys, adrenal glands and lung tissues [45]. Although such high doses of coagulase are likely nonphysiological, the experiment reveals the potency of coagulase to clot blood in vivo. Coagulases certainly facilitate the formation of abscesses, a hallmark of *S. aureus* infection [46]. Implantation of collodion bags, containing either live staphylococci or sterile staphylococcal extracts, into mice results in the formation of a fibrinous capsule with polymorphonuclear infiltrate surrounding the bag [47]. When the coagulase activity is re-

moved from the staphylococcal extracts, encapsulation is not visible [47]. Moreover, subcutaneous inoculation of mice with wild-type *S. aureus*, not the *coa* mutant, resulted in abscess formation [48]. As shown by immunohistochemical staining, abscesses formed during infection by *S. aureus* are surrounded by prothrombin as well as fibrin(ogen) and contain both Coa and vWbp [9]. Intravenous infection of mice with *S. aureus* lacking both coagulases results in an almost complete abolition of abscess formation in any organ system [9].

Coagulases serve an antiphagocytic role during infection [41, 49]. In one study, following intraperitoneal *S. aureus* infection, clumps of staphylococci surrounded by eosinophilic material were identified in peritoneal lavage fluid [50]. Although neutrophil recruitment does occur under these conditions, only very few of the recruited leukocytes can phagocytose staphylococci [50]. These results prompted the hypothesis that staphylococcal coagulation/clumping provides for staphylococcal escape from phagocytic clearance.

Staphylococcal Aggregation with Platelets

Platelets play a key role in innate defenses against staphylococci by releasing platelet microbicidal proteins that kill many *S. aureus* isolates [51]. Staphylococci have also been reported to adhere to platelets, in particular during infective endocarditis or endovascular infections [52]. ClfA and ClfB, two fibrinogen-binding proteins of *S. aureus* [53], mediate platelet aggregation in the presence of fibrinogen [54]. Fibrin, not fibrinogen, is the relevant form during ClfA/ClfB-mediated aggregation between staphylococci and platelets [55]. *S. aureus*-platelet aggregates form when platelets are activated by thrombin, not by ADP, and this is independent of the GPIIb/IIIa fibrinogen receptor [55]. Ancrod, a viper venom enzyme that cleaves the α -chain of fibrinogen to generate soluble fibrin fragments, promotes neither platelet activation nor the association between platelets and staphylococci. Nevertheless, the simultaneous activation of platelets with ADP and treatment of fibrinogen with ancrod leads to the association of staphylococci with platelets [55]. This interaction is dependent on *clfA*, suggesting that the clumping receptor of *S. aureus* may interact with fibrin, not fibrinogen, to promote platelet aggregation [55]. The role of coagulases during platelet aggregation has not yet been investigated. Nevertheless, we imagine a model whereby staphylococcal coagulation provides a fibrin matrix for the aggregation between staphylococci and platelets.

Shortly after the discovery that *S. aureus* clots blood, Much [56] reported that staphylococci, once immersed in calcium-chelated plasma, rapidly agglutinate into visible clumps. Staphylococcal agglutination in plasma requires fibrinogen [56] as well as prothrombin [57], and most strains expressing coagulase are capable of agglutination (clumping) [58, 59]. More recently, staphylococci have been suggested to clump in the presence of soluble fibrinogen alone [60], an activity that is absolutely dependent on the structural gene for clumping factor (*clfA*), but not on coagulase (*coa*) [31, 61, 62]. Nevertheless, *clfA* as well as *coa/vwb* are required for staphylococcal agglutination in plasma [63], which, as demonstrated by scanning electron microscopy, generates a mesh of fibrin cables with which staphylococci associate [63]. These results are consistent with earlier reports that fibrin cables are visible between staphylococci clumped in fibrinogen [64], which may harbor small amounts of contaminating prothrombin. Histopathology of heart tissue from septic mice infected with wild-type *S. aureus* revealed aggregates of staphylococci surrounded by fibrin(ogen) and prothrombin that appear to represent the pathological product of staphylococcal agglutination in vivo [63]. These lesions are absent in mice lacking the three agglutination factors, *coa*, *vwb*, and *clfA*. We presume that *S. aureus* generates such thromboembolic lesions in order to disseminate into and penetrate all organ systems of an infected host.

S. aureus strains express protein A [65], a surface polypeptide that binds to the Fc γ portion of immunoglobulins [66] as well as the Fab portion of the V_H3 type IgM B cell receptors [67]. Protein A also interacts with von Willebrand factor [68] via its Fc γ binding site [69]. It is not yet clear whether the binding of protein A to von Willebrand factor contributes to the aggregation of staphylococci with platelets [68].

Coagulation in Immunity and Pathogenesis

The negatively charged surface of bacteria activates the contact-dependent intrinsic coagulation cascade resulting in thrombin formation [10]. The transglutaminase, represented by fXIII in mammals, is the most conserved factor of the coagulation cascade [1]. Both *S. aureus* and *Escherichia coli* become cross-linked by the transglutaminase in the hemolymph of *Drosophila melanogaster* and in the fibrin matrix of human plasma [1]. The capacity to cross-link bacteria in clots reduces the burden of infection in insects [1]. Similarly, in a subcuta-

neous mouse model of *Streptococcus pyogenes* infection, the transglutaminase acts to reduce the bacterial burden [2]. These experiments document that the coagulation cascade functions as a key component of the innate immune defense against bacterial infection.

Bacterial surfaces also activate the complement cascade through the lectin pathway. Activation of mannan-binding lectin-associated serine protease 2 (MASP-2), which converts prothrombin to thrombin at a slower rate than the prothrombinase complex, results in the deposition of fibrin on the target surface [3]. If bacterial surfaces activate coagulation, resulting in their entrapment, why do staphylococci secrete two molecules (Coa and vWbp) that perform a similar reaction? If the fibrin polymerization products of thrombin and staphylocoagulases (Coa-prothrombin and vWbp-prothrombin) were identical, it would seem difficult to appreciate the advantage for *S. aureus* pathogenesis. The Coa-prothrombin complex cleaves fibrinopeptides at the same site as thrombin [70]. However, the biochemical and physiological attributes of the fibrin formed by staphylocoagulases are thought to be distinct from those generated by thrombin. Staphylocoagulase cleaves fibrinogen much more slowly than thrombin [71–74] and dissolution of the staphylococcal clot is more rapid than that of a physiological clot [71]. Thromboelastography reveals that clots produced by staphylocoagulase are weaker than thrombin-generated clots [75]. These data support a hypothesis whereby staphylococcal coagulases produce a fibrin clot that is mechanically and morphologically distinct from the clot generated by endogenous thrombin activation.

Thrombin has many substrates for proteolytic cleavage; however, only some of the thrombin substrates have been investigated for staphylocoagulases. For example, Coa binding to prothrombin masks the substrate recognition sites for thrombin-mediated activation of fV and fVIII [74]. While thrombin activates fXIII, it is not yet clear whether fXIII-mediated cross-linking occurs during staphylococcal coagulation; some reports observed that fXIII is not activated by staphylocoagulase [71], while others reported fXIII activation [73]. Additional thrombin activities include the stimulation of platelet aggregation (interaction with the receptor GpIB, glycoprotein V and protease-activated receptor and anticoagulant activities (activation of protein C and sequestration of antithrombin) as well as the reduction of plasmin activity through thrombin-activated fibrinolysis inhibitor [76]. Recently, thrombin was demonstrated to activate the complement cascade through the conversion of C5 to C5a

[77]. Future work will need to examine whether staphylocoagulases have retained some of the many functions of thrombin.

Concluding Comments

Healthcare systems in many countries combat the current epidemic of staphylococcal infections. *S. aureus* is an extremely versatile pathogen with many immune evasion strategies, toxins and virulence factors [5]. Two of its secreted molecules, Coa and vWbp, coopt the mammalian coagulation cascade for pathogenesis. By nonenzymatically activating prothrombin, coagulases trigger the polymerization of fibrin. Fibrin deposition and cross-linking of bacteria typically represent innate immune defenses that reduce the burden of infection. Not so for *S. aureus*, where the secretion of Coa and vWbp are key virulence strategies that promote the pathogenesis of abscess formation and persistent infection as well as staphylococcal sepsis and endocarditis. It seems plausible that staphylocoagulase-generated fibrin networks display unique structural, biochemical and physiological attributes that enable staphylococci to form a pseudocapsule and escape opsonophagocytic clearance in host tissues. Moreover, staphylococcal coagulation generates thromboembolic events that promote bacterial dissemination into all organ systems. Future work will need to examine the biophysical attributes of staphylococcal agglutination, i.e. the clot formed by staphylocoagulase to which *S. aureus* cells adhere. It seems plausible that staphylococcal clots provide not only protection from host defenses but also allow for access to essential nutrients. The fibrin meshwork may function as a scaffold to position bacterial virulence factors as well as host cells in an ordered fashion and in the vicinity of staphylococcal abscess communities.

We presume the structure and dimensions of staphylococcal clots are dynamic: the clots need to be dissolved as the pathogen aims to disseminate deeper into host tissues yet the coagulase-assembled network must resist dissolution by host enzymes as a means to clear the invading microbes. Of note, staphylococci secrete another nonenzymatic coagulation factor: staphylokinase, which acts on plasmin and plasminogen associated with fibrin to lyse fibrin clots [78, 79]. The activity of staphylokinase-plasmin(ogen) has been reported to reduce the mortality and morbidity of *S. aureus* infections [80]. What role staphylokinase plays during infection, and how its activity relates to the coagulases, is still to be investigated.

Considering their key role in several disease processes, the two *S. aureus* coagulases, Coa and vWbp, represent an important target for either the prevention or therapy of *S. aureus* infection [9, 63, 81]. Antibodies that neutralize the ability of Coa and vWbp to associate with prothrombin can protect experimental animals from intravenous challenge with *S. aureus* and small molecules that block coagulase activity can reduce the severity of staphylococcal sepsis in a mouse model of infection [63]. Thus, future studies of staphylococcal coagulases will likely generate important biological insights and may help address the public health crisis precipitated by the current epidemic of *S. aureus* infections.

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