Adhesion of *Staphylococcus aureus* to the vessel wall under flow is mediated by von Willebrand factor-binding protein

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Key-points

• von Willebrand factor-binding protein (vWbp) mediates adhesion of S. aureus under flow to activated ECs and the subendothelium via VWF.
• vWbp activates prothrombin and triggers the formation of bacteria-fibrin-platelet aggregates, which enhance adhesion to vessels under flow.

Abstract

Adhesion of Staphylococcus aureus (S. aureus) to blood vessels under shear stress requires von Willebrand factor (VWF). Several bacterial factors have been proposed to interact with VWF, including von Willebrand factor-binding protein (vWbp), a secreted coagulase that activates the host’s prothrombin to generate fibrin.

We measured the adhesion of S. aureus Newman and a vWbp-deficient mutant (vwb) to VWF, collagen, and activated endothelial cells in a micro-parallel flow chamber. In vivo adhesion of S. aureus was evaluated in the mesenteric circulation of wildtype and VWF-deficient mice.

We found a shear-dependent increase in adhesion of S. aureus to the (sub)endothelium that was dependent on interactions between vWbp and the A1-domain of VWF. Adhesion was further enhanced by coagulase-mediated fibrin formation that clustered bacteria and recruited platelets into bacterial microthrombi. In vivo, deficiency of vWbp or VWF as well as inhibition of coagulase activity reduced S. aureus adhesion.

We conclude that vWbp contributes to vascular adhesion of S. aureus through two independent mechanisms: shear-mediated binding to VWF and activation of prothrombin to form S. aureus-fibrin-platelet aggregates.
Introduction

*Staphylococcus aureus* (*S. aureus*) is the most frequent cause of life-threatening bloodstream infections. *S. aureus* sepsis carries a poor prognosis, with a fatal outcome in about one out of five cases. A major determinant of this adverse outcome is its propensity to generate secondary sites of infection upon spreading through the bloodstream. In order to establish these metastatic infections and infective endocarditis in particular, circulating pathogens require a mechanism to adhere to the blood vessel wall and to overcome the shear stress of flowing blood.

Activation of endothelial cells (ECs) triggers the release of ultra-large von Willebrand factor (VWF) multimers that are temporarily retained on the EC surface. Furthermore, upon EC damage, circulating VWF binds to collagen fibers from the exposed subendothelial matrix. Vessel wall-bound VWF recruits platelets to sites of EC damage or activation through the shear-dependent interaction between the platelet glycoprotein GPIbα and the VWF A1-domain that is exposed after unfolding of VWF multimers by flowing blood. A recent study revealed that *S. aureus* may employ a similar VWF-dependent mechanism to adhere to ECs under shear stress. However, the bacterial factors involved in the *S. aureus*-VWF interactions remained to be elucidated.

A number of *S. aureus* proteins were shown to bind to VWF, including staphylococcal protein A (SpA) and the von Willebrand factor-binding protein (vWbp). However, the relevance of VWF as a ligand for vWbp was questioned in subsequent studies, and the role of vWbp-VWF interaction has not yet been studied in relevant disease models.

Nevertheless, vWbp has been shown to contribute to *S. aureus* pathophysiology by its ability to induce blood clotting. This coagulase activity, which differentiates *S. aureus* from less virulent coagulase-negative staphylococci, is the result of the conformational activation of prothrombin by two secreted proteins: staphylocoagulase (*Coa*) and vWbp. Staphylothrombin, the resulting complex of a bacterial coagulase and host prothrombin, converts fibrinogen into insoluble fibrin. Because of the direct activation of prothrombin, *S. aureus*-mediated activity is insensitive to most anticoagulant drugs, which has hampered the study of coagulase activity as a virulence factor. However, the generation of mutant strains that lack vWbp, Coa, or both, and the recent finding that the direct thrombin inhibitor dabigatran effectively inhibits *S. aureus*-mediated coagulation has allowed to demonstrate the contribution of this pathogen-induced clot formation to the virulence of *S. aureus* in local infections as well as in sepsis. Furthermore, staphylothrombin-generated fibrin was shown to promote interaction...
between *S. aureus* and platelets via the platelet fibrinogen receptor $\alpha_{\text{IIb}\beta_{3}}$, and contributes to the initiation and propagation of experimental infective endocarditis lesions in flowing blood. In this study, we have investigated the role of both vWbp functions, VWF binding and prothrombin activation, for the adhesion of *S. aureus* to activated ECs and to subendothelial matrix under shear stress. We further evaluated the role of platelets in *S. aureus*-vessel wall adhesion focusing on the role of the platelet fibrinogen receptor $\alpha_{\text{IIb}\beta_{3}}$, and present a model for the endovascular dissemination of *S. aureus* based on VWF-vWbp-*S. aureus* interactions, reinforced by coagulase activity and bacteria-fibrin-platelet interactions.
Materials and Methods

Bacterial strains

The reference strain used in this study is *S. aureus* Newman, originally isolated from a case of osteomyelitis, with a well-described profile of coagulase activity. The isogenic single and double coagulase-deficient mutants *S. aureus* Newman *vwb*, *coa* and *coa/vwb* with complementing plasmid have been described. The double coagulase-deficient mutant has no detectable coagulase activity, i.e. is not able to activate host’s prothrombin and generate fibrin. Bacteria were fluorescently labeled with 5(6)-carboxy-fluorescein-N-hydroxysuccinimidyl ester (Sigma-Aldrich, Germany). Recombinant His6-vWbp without the signal sequence and lacking coagulase activity was cloned using plasmid pET15 and purified from *E. coli* BL21(DE3) using Ni-trilotriacetic acid (Ni-NTA) chromatography as previously described.

In vitro perfusion experiments

Glass coverslips (24x50 mm, VWR International, Belgium) were coated with 50 μg/mL VWF (Haemate P, CSL Behring, Belgium) or 200 μg/mL Horm collagen (Takeda, Austria) in a humidified container at room temperature (RT) for 4 hours. The coverslips were mounted in a micro-parallel flow chamber and perfused for 10 minutes using a high accuracy Harvard pump (PHD 2000 Infusion, Harvard Apparatus, USA) generating flow rates of 250 s⁻¹ to 2000 s⁻¹. Coverslips were perfused with labeled bacteria (OD₆₀₀ 0.65 or 1.2, corresponding to approximately 3x10⁸ and 6x10⁸ colony forming units (CFU)/mL) suspended in DMEM (Invitrogen, Belgium), PPP (platelet-poor plasma), VWF-deficient plasma (Hyphen, BioMed, France) with or without added VWF (60 μg/mL) and PRP (platelet-rich plasma), with or without dabigatran (500 nM, a gift of Joanne van Ryn, Boehringer Ingelheim, Germany), a reversible specific thrombin inhibitor and equally potent inhibitor of staphylothrombin, and/or eptifibatide (7.5 μg/mL, GlaxoSmithKline, UK), antagonizing fibrin(ogen) and VWF binding to the platelet α₁β₃-receptor. Where indicated, PPP and PRP were spiked with fluorescently labeled fibrinogen (F-13191, Alexa Fluor 488 labelled, Invitrogen, Belgium) to a final concentration of 37.5 μg/mL to assess fibrin formation during perfusion. Bacterial supernatant was prepared by incubating washed bacteria for 4 hours at 37°C in PBS. To investigate platelet adhesion, PRP was labeled with rhodamine-G (1 μg/mL). The neutralizing anti-VWF A1-domain murine monoclonal antibody 6D1 was added to DMEM, PPP or PRP at a final concentration of 10 μg/mL. An anti-tPA monoclonal IgG1 antibody was used as isotype control (10 μg/mL).
Live images were obtained using an inverted fluorescence microscope (Axio-observer DI, Carl-Zeiss NV, Belgium) and recorded using a black and white camera (Carl-Zeiss Axio-Cam MRm). Images were digitally stored and processed with the ImageJ analysis software (National Institutes of Health, Bethesda, USA) for fluorescent area quantification. Values are reported as Arbitrary Fluorescent Units (AFU) (1 AFU corresponds to the fluorescent area for one selected pixel).

Bacterial adhesion to ECs

Human umbilical vein endothelial cells (HUVECs) were seeded on coverslips and mounted in a micro-parallel flow chamber. The ECs were activated via perfusion with 0.1 mM Ca²⁺-ionophore A23187 (Sigma-Aldrich, Germany) for 10 minutes, followed by a perfusion for 10 minutes with fluorescently labeled bacteria (OD 600 1.2). Bacterial rolling and adhesion were monitored via video-microscopic imaging, as described above.

In vivo mesenteric perfusion model

The mouse mesenteric perfusion model was used to study real-time bacterial adhesion to the vascular bed in wild type Vwf+/+ and homozygous Vwf−/− C57Bl/6 mice. Six- to eight-week old mice were anesthetized using ketamine/xylazine intraperitoneally and the right jugular vein was catheterized (Portex intravenous cannula, 2F). The peritoneal cavity was opened via midline abdominal incision to visualize the mesenteric arteriolar and venular circulation under an inverted microscope (Axio-observer D1). Following topical application of 5µL of the Ca²⁺-ionophore A23187 (10 mM) to trigger ECs activation and VWF-release, 300 µL of a suspension of labeled bacteria (OD600 1.8, corresponding to approximately 1x10⁹ CFU/mL) was injected through the jugular catheter. Where indicated, bacterial inoculation was preceded by a bolus of 50 µL of dabigatran (10 µM). Time-lapse images were acquired via online video-microscopy for analysis. The fluorescent signal in the blood vessel was quantified manually for each frame and reported as AFU. Animal experiments were approved by the Ethical Committee of the University of Leuven.

Statistical analysis

All calculations were done using GraphPad Prism 5.0d (GraphPad Software, San Diego California USA). Groups were compared using the one-way ANOVA or a two-tailed Student’s t-test. All values are reported as mean ± standard error of the mean (SEM). A P-value of < 0.05 was considered significant (* P < 0.05; ** P < 0.01; *** P < 0.001).
Results

vWbp-VWF interactions mediate *S. aureus* adhesion under flow

First, we assessed the adhesion of *S. aureus* Newman (WT) to surface-bound VWF under static and flow conditions. Adhesion of *S. aureus* to VWF increased with increasing shear rates (Fig 1A, Fig S1A-B). In contrast, adhesion of the vWbp-deficient mutant strain (*vwb*) remained low at all shear rates, and further decreased with increasing shear (Fig 1A).

Reconstitution of vWbp expression restored the adhesive phenotype, confirming that vWbp is required for the shear-dependent increase in adhesion of *S. aureus* to VWF (Fig S2A). Furthermore, the addition of exogenous vWbp increased the adhesion of *vwb* to VWF under flow (Fig S3A-C). Preperfusion of the coated VWF with recombinant His<sub>6</sub>-vWbp decreased the subsequent adhesion of the WT strain, suggesting competition between bound His<sub>6</sub>-vWbp and subsequently perfused bacterial vWbp. In contrast, the *vwb* strain showed a mildly increased adhesion when VWF was preperfused with the His<sub>6</sub>-vWbp (Fig S3D). Both WT and *vwb* were found to bind under flow to coated His<sub>6</sub>-vWbp with comparable efficacies (data not shown), showing that deletion of *vwb* had no effect on the binding of exogenous vWbp to *S. aureus*.

Second, we studied whether VWF-vWbp interaction contributes to the adhesion of *S. aureus* to collagen, the main subendothelial component. The addition of VWF significantly increased the adhesion of WT (Fig 1B, Fig S1C), but had only a limited effect the binding of *vwb*. At 1000 s<sup>-1</sup>, a large difference in adhesion was observed between the WT and the *vwb* strains (35,195±6959 AFU vs. 5847±1644 AFU, n=12, *P*<0.001) (Fig 1B). Again, addition of exogenous vWbp from WT supernatant or in recombinant form increased the adhesion of *vwb* (Fig S3A) and WT (Fig S3B), and the decreased adhesion of the *coal/vwb* strain was rescued by a plasmid carrying the *coal* and *vwb* gene (Fig S2B).

We further compared the adhesion of the WT and *vwb* strains to collagen in VWF-deficient plasma, before and after spiking with VWF. When suspended in VWF-deficient plasma, the WT and *vwb* strains bound comparably to collagen, but VWF repletion only increased the adhesion of the WT strain significantly (Fig 1C).
"S. aureus" coagulase activity enhances bacterial adhesion to collagen

Activation of coagulation through coagulase activity is a hallmark of "S. aureus." Indeed, in the presence of plasma, we noted a time-dependent formation of microscopic aggregates that facilitated bacterial adhesion to collagen when perfused under flow. To study the interaction between coagulase activity and vWbp-VWF interactions on flow-dependent adhesion, we compared adhesion of "S. aureus" WT, which possesses 2 coagulases; vwb, which is deficient in vWbp but retains Coa-mediated coagulase-activity; and the coa/vwb strain, which has no detectable coagulase activity.

Preincubation of the WT strain in plasma led to a ~7-fold increase in adhesion for the WT strain (1891±314 AFU vs. 13,130±1867 AFU, n≥5, P<0.01) (Fig 2A) (Video 1-2), which could be prevented by the (staphylo)thrombin inhibitor dabigatran. This increased adhesion of the WT strain was paralleled by a deposition of fibrin fibers (Fig 2B). In contrast, preincubation of the coagulase-deficient coa/vwb strain had only a minor effect on bacterial adhesion (Fig 2A), and no fibrin formation could be detected (Fig 2B).

Interestingly, absence of vWbp (vwb strain), which abolished shear-mediated adhesion in the absence of plasma (Fig 1C), had only a limited effect on adhesion if coagulase-activity was present (Fig 2A). Although fibrin deposition of vwb was reduced by approximately 80% compared to WT, fibrin fibers could still be detected (Fig 2B). When absence of vWbp was combined with pharmacological inhibition of coagulase (dabigatran), bacterial adhesion to collagen was completely abrogated (Fig 2A).

The finding that coagulase activity could promote flow-dependent adhesion of "S. aureus" independent from vWbp was confirmed by studying adhesion of the coa strain in which the vWbp-prothrombin complex is the only source of staphylothrombin (Fig S4).

"S. aureus"-platelet interactions contribute to vessel wall adhesion

Platelets interact with "S. aureus" via fibrin(ogen) bridges and they also display typical shear-dependent adhesion to VWF. To study the influence of platelet-VWF, platelet-fibrin and platelet-"S. aureus" interactions on the VWF-dependent adhesion of "S. aureus" to collagen, perfusion experiments were carried out using both platelet-poor plasma and platelet-rich plasma, spiked with fluorescently labeled bacteria or platelets.

The presence of platelets increased the adhesion of the WT strain to collagen (13,130±1867 AFU vs. 39,504±6431 AFU, n≥11, P<0.01) (Fig 3A) in control conditions. The increased adhesion of "S. aureus" in the presence of platelets was no longer found when coagulase
activity was prevented by dabigatran (Fig 3A). Interestingly, when the platelet fibrin(ogen) receptor $\alpha_{\text{IIb}}\beta_3$ was inhibited by eptifibatide, addition of platelets decreased S. aureus WT adhesion to collagen (Fig 3A). Finally, the combined inhibition of the coagulase activity of S. aureus and the $\alpha_{\text{IIb}}\beta_3$ platelet integrin almost completely abolished bacterial adhesion (39,504±6431 AFU vs. 739±258 AFU, $n \geq 4$, $P < 0.01$) (Fig 3A).

To explain this, we hypothesized that S. aureus and platelets individually compete for VWF-mediated binding to collagen. However, clustering of bacteria to platelets, which requires coagulase-mediated fibrin formation and subsequent $\alpha_{\text{IIb}}\beta_3$-mediated fibrin bridging, increases the binding capacity. To test this hypothesis, we performed scanning electron microscopy imaging of the coverslips following perfusion, which showed fibrin bridging of bacteria and platelets (Fig 3C-D). Hence, inhibition or absence of one of these players mitigated the increased bacterial adhesion.

Platelets had less effect on the adhesion of the $\text{vwb}$ strain compared to the WT strain, and both eptifibatide and dabigatran further reduced the adhesion of the $\text{vwb}$ strain in the presence of platelets (Fig 3A). The elimination of both vWbp-VWF interactions and staphylothrombin-mediated fibrin formation in the $\text{coa/vwb}$ strain resulted in a limited adhesion, irrespective of the presence of platelets (Fig 3A).

The changes in bacterial adhesion were paralleled by changes in platelet deposition on the collagen surface. Absent vWbp reduced platelet adhesion to collagen 2-fold. Adhesion of platelets to collagen was further decreased upon perfusion of $\text{coa/vwb}$ (Fig 3B), underpinning the role of both fibrin generation and vWbp-VWF interactions in the generation of S. aureus-platelet microthrombi.

vWbp binds to VWF via the VWF A1-domain

Neutralization of the VWF A1-domain by a neutralizing anti-A1 mAb reduced bacterial adhesion of S. aureus to collagen (Fig 4A-B), demonstrating the importance of the A1-domain of VWF for the shear-dependent adhesion of S. aureus to VWF. The impact of the anti-A1 domain was larger in the presence of platelets, which also bind to the VWF A1-domain via the GP1b$\alpha$ receptor.

vWbp enhanced bacterial adhesion to endothelial cells

To investigate the adhesion of S. aureus to ECs under flow, HUVECs were perfused with fluorescently labeled bacteria at 1000 $\text{1/d}$ with or without EC activation, which leads to the release
Specific VWF staining on activated ECs confirmed release of multimeric VWF (not shown). EC activation and VWF release increased adhesion of the WT strain, which formed typical ‘string’ patterns of fluorescently labeled bacterial clusters, aligned in the direction of the shear force (Fig 5B), suggesting the binding of bacteria along a linear-stretched VWF molecule. Compared to WT, \(vwb\) adhered 3-fold less (982±207 AFU vs. 305±150 AFU, \(n \geq 5\), \(P < 0.05\)) (Fig 5A). The VWF A1-domain antibody, but not an isotype control antibody, significantly decreased the adhesion of the WT strain to activated ECs (Fig 5A).

\textit{In vivo} bacterial adhesion in splanchnic veins is mediated by vWbp-VWF interactions

Finally, we validated our findings in an \textit{in vivo} model. Real-time video-microscopy of the murine splanchnic veins allowed the \textit{in vivo} visualization of circulating fluorescently labeled \textit{S. aureus}, injected via a catheter in the jugular vein. After pharmacological activation of the endothelium by the Ca\(^{2+}\)-ionophore, we observed rapid local accumulation of individual bacteria and aggregates of bacteria to the vessel wall of wild type mice (Fig 6B) (Video 3-4). Inoculation of the \(vwb\) strain resulted in reduced vessel wall adhesion of bacteria (64.5±28.1 AFU vs. 143±31.2 AFU, \(n \geq 10\), \(P < 0.01\)) (Fig 6A) (Video 5). Inhibition of coagulase activity by dabigatran reduced the formation and adhesion of aggregates of the WT strain, leading to a large reduction in total bacterial adhesion, but did not prevent the adhesion of individual bacteria (Fig 6A). When the \(vwb\) strain was inoculated along with dabigatran treatment, the combined genetic deletion of vWbp and inhibition of coagulase activity resulted in the strongest reduction in adhesion (WT vs. \(vwb\) +dabigatran: 143±31.2 AFU vs. 10.2±4.01 AFU, \(n \geq 7\), \(P < 0.01\) and WT vs. coa/\(vwb\): 143±31.2 AFU vs. 21.6±7.89 AFU, \(n \geq 10\), \(P < 0.01\)).

Almost no adhesion of bacteria was observed on the activated vessel wall of VWF-deficient mice as compared to adhesion in wild type mice (\(Vwf^{-/-}\)mice vs. \(Vwf^{+/+}\)mice: 143±31.2 AFU vs. 14.4±9.01 AFU, \(n \geq 13\), \(P < 0.001\)) (Fig 6A) (Video 6). No further reduction was found when we compared the adhesion of the WT strain with the \(vwb\) or the coa/\(vwb\) strains to the activated vessel wall of VWF-deficient mice, with or without the inhibition of staphylothrombin.

In conclusion, the absence of both Coa and vWbp or the absence of VWF completely abolished the ability of \textit{S. aureus} to adhere to the activated vessel wall.
Discussion

In this study, we identify vWbp as a key protein involved in the early steps of vascular infections by *S. aureus* through a unique synergism between its shear-dependent interaction with VWF and its coagulase activity. vWbp mediates bacterial binding to collagen and to ECs under shear via vWbp-VWF interactions. Bacterial adhesion is further enhanced by vWbp- and Coa-mediated fibrin formation and by *S. aureus*-fibrin-platelet interactions. *S. aureus* adhesion to activated mesenteric endothelium required both VWF and vWbp, whereas the coagulase activity of Coa and vWbp increased the local infective burden through the formation of *S. aureus*-containing microthrombi.

The ability for bacteria to adhere to the vessel wall is a crucial first step to initiate metastatic infections and infective endocarditis\(^{20,21}\). Activation or inflammation of ECs will induce rapid release of highly reactive VWF multimers on the vessel wall. Temporarily retained VWF multimers are a ligand for circulating platelets but also for *S. aureus*, as recently reported\(^{6,22}\). However, the mechanisms by which *S. aureus* adheres to VWF under flow conditions remained uncertain.

The ability of *S. aureus* to bind to VWF had previously been reported to be mediated by the staphylococcal protein A (SpA)\(^{23}\). SpA binding to VWF has been documented in static conditions and at low shear stress\(^{24,25}\), however, in high flow, the recruitment of *S. aureus* to ECs appeared to be SpA-independent\(^{6}\). In 2002, vWbp was described as a novel secreted VWF-binding molecule with coagulase activity, present in all tested strains of *S. aureus*\(^{27}\). However, other studies could not confirm VWF as an important ligand for vWbp when assessed in plasma in static conditions\(^{9}\).

Our results indicate that vWbp interactions with VWF are enhanced in a flow field. VWF circulates in a compact globular form, but is progressively unfolded in a shear field\(^{26}\) or when bound to collagen\(^{27,28}\), thereby exposing the VWF A1, A2 and A3-domain. The A1-domain is the ligand-binding site for platelet GPIbα, the major VWF receptor on platelets\(^{29}\), whereas the A3-domain is required for VWF binding to collagen fibers. We determined that not only binding of circulating platelets, but also of circulating *S. aureus*, could largely be blocked by an A1 neutralizing antibody. Hence, *S. aureus* has developed an adhesion mechanism very similar to that used by platelets.
vWbp also interacts with prothrombin to mediate the conversion of soluble fibrinogen into fibrin. *S. aureus* staphylothrombin activity constitutes an important determinant of bacterial virulence in local abscess formation, sepsis mortality, as well as device-related infections. Comparing adhesion of the single and double mutants *coa, vwb* and *coa/vwb* respectively revealed a unique synergistic action in vessel wall adhesion between coagulation activation and adhesion to VWF. Fibrin clusters bacteria and these aggregates interact with immobilized VWF more effectively than single bacteria. Cluster formation was abolished upon staphylothrombin inhibition by dabigatran or when using the double *coa/vwb* mutant, in line with our earlier findings.

Platelets rapidly bind to collagen under high shear stress, with VWF acting as a bridging molecule. Our results show that, rather than reducing the adhesion of bacteria by competing for VWF binding, the presence of platelets increased the adhesion of *S. aureus* to collagen under flow conditions. This increased adhesion in the presence of platelets required both coagulase activity and platelet-fibrin binding through αIβ3. Fibrin bridges between bacteria and platelets, may facilitate bacterial recruitment and the formation of bacteria-platelet aggregates. We have previously shown that even when staphylothrombin, in contrast to thrombin, does not directly activate the platelet thrombin receptor, *S. aureus*-mediated fibrin generation indirectly facilitates bacteria-platelet interactions via common interactions with fibrin.

The presence of platelets also increased the adhesion of the *vwb* mutant. Since this effect was lost in the presence of dabigatran or in the double *coa/vwb* mutant strain, these findings confirm that the role of platelets in adhesion requires coagulase activity and fibrin formation, even when direct bacterial receptors on platelets have been described. The bacteria-platelet interactions via fibrin may allow bacteria to dock more firmly onto VWF. Platelets will likewise interact with VWF multimers, and use GPIbα-VWF interaction to dock to collagen, further stabilizing adhesion of the conglomerate between bacteria and platelets. When αIβ3-fibrinogen-mediated platelet-platelet and αIβ3-fibrin-mediated platelet-bacteria interactions are prevented by eptifibatide, single platelets compete for VWF-binding with vWbp and reduce *S. aureus* adhesion to collagen.

Interactions of bacteria with VWF represent an efficient strategy to focus circulating pathogens to breaches in the protective endothelial cell lining of the vasculature. Inflammation-triggered exposure of VWF allowed the recruitment of *S. aureus* to the mesenteric vasculature. Adhesion was abrogated in *VWF−/−* mice. The role of staphylothrombin in vivo was also apparent from the formation of large vegetations, a process abrogated by dabigatran. The supplemental
videos further illustrate how large bacterial conglomerates embolize in the face of increasing shear forces, potentially giving rise to metastatic infectious foci.

Previous studies using \textit{S. aureus} \textit{coa} mutants failed to provide insight into the role of Coa in the initial infection of heart valves \textsuperscript{30}. Because vWbp had not been discovered at the time of these investigations, the molecular contribution of this secreted factor could not have been appreciated. Here, we show that vWbp is a key molecule for bacterial binding to the vasculature and that it represents an interesting therapeutic target to contain intravascular \textit{S. aureus} infections.

In conclusion, our work identifies vWbp as a key protein in \textit{S. aureus}-vessel wall interactions, through unique bimodal adhesive properties and by combining adhesive and procoagulant activities. Combined inhibition of coagulase activity and VWF binding completely abolished \textit{S. aureus} adhesion to the vessel wall, despite the redundancy of \textit{S. aureus} mechanisms including two coagulases and potentially other VWF-binding proteins. To explain the propensity of \textit{S. aureus} to adhere to the endothelium under shear stress, we have shown synergistic interactions between \textit{S. aureus}, platelets, fibrin(ogen) and adhesive bacteria-vessel wall interactions. Disruption of such interactions, may lead to novel therapeutic strategies reducing the high mortality of \textit{S. aureus} bloodstream infections, including infective endocarditis.
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Authorship

P.V., M.H., and R.H. designed the research, analyzed the data and wrote the manuscript; J.C. and T.V. designed and performed the research, analyzed the data and wrote the manuscript; K.V., M.P., L.L. and C.V. performed experiments and helped interpret data; D.M. and O.S designed the research, contributed vital new reagents, and contributed to writing the manuscript.

Conflict of interest disclosure

P.V. has received honoraria from Boehringer-Ingelheim for lectures and advisory committees. The other authors declare no conflict of interests.
References


**Figure Legends**

**Figure 1. vWbp mediates shear-dependent adhesion of *S. aureus* to VWF.**

(A) Micro-parallel flow chamber perfusion over coated VWF (50 μg/mL) with fluorescently labeled WT and vwb strains at shear rates of 500 s\(^{-1}\) and 1000 s\(^{-1}\) in medium (n\(\geq\)6).
(B) Perfusion over coated collagen with WT and vwb strains at 500 s\(^{-1}\) or 1000 s\(^{-1}\). VWF (60 μg/mL) was present in the medium where indicated (n\(\geq\)4). (C) Perfusion over coated collagen with WT or vwb strains in the presence of VWF-deficient plasma at 1000 s\(^{-1}\). VWF (60 μg/mL) was added where indicated (n\(\geq\)4). All results are expressed as mean ± SEM. * P < 0.05, ** P < 0.01, *** P < 0.001.

**Figure 2. Coagulase activity increases *S. aureus* adhesion to collagen under shear stress.** (A) Micro-parallel flow chamber perfusion over coated collagen with fluorescently labeled WT, vwb and coa/vwb strains in plasma with or without preincubation (37°C for 15 minutes) at 1000 s\(^{-1}\). The ability of bacteria to generate coagulase-mediated fibrin during the preincubation phase in plasma increases subsequent bacterial adhesion. Where indicated, dabigatran (500 nm) was added to the plasma (preincubation 37°C for 15 minutes) (n\(\geq\)5). (B) Perfusion over coated collagen with fluorescently labeled fibrinogen and WT, vwb and coa/vwb strains in the presence of plasma and platelet-rich plasma (preincubation 37°C for 15 minutes) (n\(\geq\)4). All results are expressed as mean ± SEM. * P < 0.05, ** P < 0.01, *** P < 0.001.

**Figure 3. Platelets increase *S. aureus* adhesion under shear stress.**

(A) Micro-parallel flow chamber perfusions over coated collagen with fluorescently labeled WT, vwb and coa/vwb strains in the presence of plasma and platelet-rich plasma (preincubation 37°C for 15 minutes) at 1000 s\(^{-1}\). Addition of dabigatran (500 nM) or eptifibatide (7.5 μg/mL) where indicated (n\(\geq\)5). (B) Perfusion over coated collagen with WT, vwb and coa/vwb strains in platelet-rich plasma at 1000 s\(^{-1}\). Platelets were labeled with with rhodamine-G (preincubation 37°C for 15 minutes). Adhesion of platelets was lower when perfused with a strain lacking vWbp compared to WT, and was further reduced when perfused together with a mutant strain lacking both coagulases (vwb/coa) (n\(\geq\)4). All results are expressed as mean ± SEM. * P < 0.05, ** P < 0.01. (C) SEM image (x20,000) of WT perfusion in PRP over collagen at 1000 s\(^{-1}\), illustrating the interactions between bacteria, fibrin and platelets. Bar represents 1 μm. (D) SEM image (x5000) of WT perfusion in PRP over collagen at 1000 s\(^{-1}\). Bar represents 2 μm. White full arrows indicate bacteria and white dotted arrows indicate platelets.
Figure 4. Blocking A1-domain of VWF inhibits *S. aureus* binding to collagen under shear stress. (A) Perfusion over coated collagen with fluorescently labeled WT strain and 60 μg/mL VWF in medium at a shear rate of 1000 s⁻¹. Anti-A1 VWF domain antibody 6D1 (final concentration 10 μg/mL) or anti-tPa monoclonal IgG1 antibody (10 μg/mL) were added where indicated (n≥5). (B) Perfusion over coated collagen at 1000 s⁻¹ with WT strain in PPP and PRP (PPP or PRP preincubation of 15 minutes at 37°C). 6D1 (final concentration 10 μg/mL) was added where indicated (n≥4). All results are expressed as mean ± SEM. * P < 0.05, ** P < 0.01.

Figure 5. vWbp mediates bacterial adhesion to activated HUVECs under flow conditions. (A) Micro-parallel flow chamber perfusions. ECs were activated with the Ca²⁺-ionophore A23187 (0.1 mM) followed by a 10 minutes perfusion of fluorescently labeled WT and vwb strains at a shear rate of 1000 s⁻¹ in DMEM. Where indicated, the anti-A1 VWF domain antibody 6D1 (final concentration 10 μg/mL) was present. No difference was observed in the presence or absence of the anti-tPa monoclonal IgG1 antibody. All results are expressed as mean ± SEM. * P < 0.05, ** P < 0.01, n≥5. (B) Image of micro-parallel flow chamber perfusion over activated HUVECs with WT at a shear rate of 1000 s⁻¹. *S. aureus* forms strings on VWF over a distance of 200 micron.

Figure 6. Bacterial adhesion to activated endothelium *in vivo* is VWF and vWbp-mediated. (A-B) *In vivo* venous mesenteric perfusion model with C57Bl/6-Vwf⁺/+ and C57Bl/6-Vwf⁻/⁻ mice. Five μL of the Ca²⁺-ionophore A23187 (10 mM) were applied to the region of the visualized vascular bed to trigger ECs activation and VWF-release. A suspension of carboxy-fluorescein-labeled WT, vwb or coa/vwb strains was injected through the jugular catheter. Where indicated, bacterial inoculation was preceded by a bolus of 50μL of dabigatran (10μM). All results are expressed as mean ± SEM. ** P < 0.01, *** P < 0.001, n≥7. (B) Image of *in vivo* venous mesenteric perfusion model with C57Bl/6-Vwf⁺/+ mice.
A. Adhesion of *S. aureus* to VWF is shear-dependent and vWbp mediated

- **Bacterial adhesion (arbitrary fluorescent units)**
- **WT**
- **vwb**

<table>
<thead>
<tr>
<th>Shear Rate</th>
<th>WT</th>
<th>vwb</th>
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<tr>
<td>500 s⁻¹</td>
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<td></td>
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<tr>
<td>1000 s⁻¹</td>
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B. Shear-dependent adhesion of *S. aureus* to collagen is VWF mediated

- **Bacterial adhesion (arbitrary fluorescent units)**
- **WT**
- **vwb**

<table>
<thead>
<tr>
<th>Condition</th>
<th>WT</th>
<th>vwb</th>
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</thead>
<tbody>
<tr>
<td>no VWF</td>
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</tr>
<tr>
<td>+ VWF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500 s⁻¹</td>
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<tr>
<td>1000 s⁻¹</td>
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</table>

C. VWF-deficient plasma decreases *S. aureus* Newman adhesion to collagen

- **Bacterial adhesion (arbitrary fluorescent units)**
- **VWF-deficient plasma + VWF**
- **VWF-deficient plasma**

<table>
<thead>
<tr>
<th>WT</th>
<th>vwb</th>
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</table>
A  Increased bacterial adhesion to collagen of preincubated bacteria with plasma is reduced by dabigatran

B  Coagulase mediated fibrin accumulation during bacterial adhesion under shear stress
Figure 3

A. Platelet interactions increase S. aureus adhesion to collagen under shear stress

B. vWbp contributes to platelet adhesion under shear stress

C.

D.
A

Blocking VWF A1-domain inhibits *S. aureus* Newman binding to collagen in the presence of VWF under shear stress

![Graph A]

B

Blocking VWF A1-domain inhibits *S. aureus* Newman binding to collagen under shear stress in plasma and PRP

![Graph B]

Figure 4
A. *S. aureus* adhesion to activated endothelial cells under shear stress is vWbp mediated

B. Figure 5
A

The initial bacterial adhesion to activated endothelium \textit{in vivo} is VWF and vWbp mediated

![Bar graph showing bacterial adhesion to different genotypes](image)

- **WT**
- **vwb**
- **coa/vwb**

**B**

![Image of bacterial adhesion](image)

Figure 6
Adhesion of *Staphylococcus aureus* to the vessel wall under flow is mediated by von Willebrand factor-binding protein

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