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ORIGINAL ARTICLE

Clumping factor A, von Willebrand factor-binding protein and von Willebrand factor anchor *Staphylococcus aureus* to the vessel wall

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Essentials

- *Staphylococcus aureus* (*S. aureus*) binds to endothelium via von Willebrand factor (VWF).
- Secreted VWF-binding protein (vWbp) mediates *S. aureus* adhesion to VWF under shear stress.
- vWbp interacts with VWF and the Sortase A-dependent surface protein Clumping factor A (ClfA).
- VWF-vWbp-ClfA anchor *S. aureus* to vascular endothelium under shear stress.

Summary. *Objective:* When establishing endovascular infections, *Staphylococcus aureus (S. aureus)* overcomes shear forces of flowing blood by binding to von Willebrand factor (VWF). Staphylococcal VWF-binding protein (vWbp) interacts with VWF, but it is unknown how this secreted protein binds to the bacterial cell wall. We hypothesized that vWbp interacts with a staphylococcal surface protein, mediating the adhesion of *S. aureus* to VWF and vascular endothelium under shear stress. *Methods:* We studied the binding of *S. aureus* to vWbp, VWF and endothelial cells in a microparallel flow chamber using various mutants deficient in Sortase A (SrtA) and SrtA-dependent surface proteins, and *Lactococcus lactis* expressing single staphylococcal surface proteins. *In vivo* adhesion of bacteria was evaluated in the murine mesenteric circulation using real-time intravital vascular microscopy. *Results:* vWbp bridges the bacterial cell wall and VWF, allowing shear-resistant binding of *S. aureus* to inflamed or damaged endothelium. Absence of SrtA and Clumping factor A (ClfA) reduced adhesion of *S. aureus* to vWbp, VWF and activated endothelial cells. ADAMTS-13 and an anti-VWF A1 domain antibody, when combined, reduced *S. aureus* adhesion to activated endothelial cells by 90%. Selective overexpression of ClfA in the membrane of *Lactococcus lactis* enabled these bacteria to bind to VWF and activated endothelial cells but only in the presence of vWbp. Absence of ClfA abolished bacterial adhesion to the activated murine vessel wall. *Conclusions:* vWbp interacts with VWF and with the SrtA-dependent staphylococcal surface protein ClfA. The complex formed by VWF, secreted vWbp and bacterial ClfA anchors *S. aureus* to vascular endothelium under shear stress.

Keywords: endothelium; infection; shear stress; *Staphylococcus aureus*; von Willebrand factor.

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Introduction

Staphylococcus aureus (S. aureus) is the leading cause of life-threatening endovascular infections [1]. One of the most feared complications of invasive *S. aureus* disease is infective endocarditis. Compared with other pathogens, infective endocarditis caused by *S. aureus* has a higher mortality and is more frequently associated with severe complications [2,3].

Once *S. aureus* infects the heart valves, almost one in three patients will die, despite aggressive surgery and antibiotics [4]. The dramatic morbidity and mortality of

S. aureus endocarditis have remained unchanged over the past decades. This stresses the need for new therapeutic strategies to prevent and treat infective endocarditis.

To cause endocarditis, bacteria first need to adhere to the endothelium of the heart valve. However, binding to endothelial cells in flowing blood requires mechanisms to withstand shear stress. A better understanding of the initial binding of *S. aureus* to the valvular endocardium will allow the development of new strategies to prevent and treat endocarditis.

We and others showed that *S. aureus* adheres to the vessel wall under flow by binding to von Willebrand factor (VWF) [5,6]. VWF binds to sites of vascular damage and is exposed on the endothelial surface upon activation or injury [7]. VWF multimers are cleaved by ADAMTS-13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13) [8]. The binding of *S. aureus* to VWF is mediated by the von Willebrand factor-binding protein (vWbp); however, because vWbp is thought to be a secreted protein not anchoring to the cell wall, it remains unclear how vWbp mediates bacterial attachment.

S. aureus expresses a number of bacterial cell wall-anchored surface proteins that mediate bacterial adherence to host cells and to extracellular matrix components. Several of these *S. aureus* surface proteins, or MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), have been proposed to contribute to the pathogenesis of endovascular infections [9–11]. Many of those MSCRAMMs, which recognize fibronectin, fibrinogen, collagen or VWF, have a conserved C-terminal cell wall sorting signal with a Leu-Pro-X-Thr-Gly (LPXTG) motif [12]. Together, more than 20 members of this family of cell wall-anchored surface proteins have been identified in the *S. aureus* genome [13–15]. This sorting signal triggers the covalent anchoring of these proteins to the bacterial cell wall by Sortase A (SrtA), a transpeptidase [12]. Strains with a mutation in the *srtA* gene lack these cell wall-anchored proteins [14].

We hypothesized that vWbp bridges VWF with a cell wall-anchored surface protein of *S. aureus*. In this study we identify the bacterial binding partners for vWbp and unravel the mechanism of *S. aureus* binding to the vascular wall under shear stress, a crucial step in the early phases of the infectious process.

Materials and methods

Bacterial strains

S. aureus strains were stored in Brain Heart Infusion with 10% glycerol at −80 °C. Bacteria were grown overnight in tryptic soy broth at 37 °C. The reference strain used in this study is *S. aureus* Newman [16]. Isogenic single mutants of *S. aureus* Newman [17–19] are listed in Table 1. *Lactococcus lactis (L. lactis)* strains [11,20] were grown overnight at 37 °C in M17 medium (Fluka, Sigma-Aldrich, Darmstadt, Germany) supplemented with 0.5% glucose and 5 µg mL^{-1} erythromycin and stored in M17 medium supplemented with 10% glycerol at −80 °C. The strains used in this study are listed in Table 1. *Escherichia coli (E. coli)* strains DH5α and BL21 (DE3) were cultured on Luria agar or broth at 37 °C. Ampicillin (100 µg mL^{-1}) and erythromycin (10 µg mL^{-1}) were used for plasmid selection.

Expression and purification of proteins

Recombinant His$_6$-vWbp (rvWbp) without the signal sequence and

lacking coagulase activity was cloned with plasmid pET15 and was purified from *E. coli* BL21 (DE3) using Ni-trilotriacetic acid (Ni-NTA) chromatography as previously described [21]. Recombinant vWbp-Strep was cloned with pET22b and was purified from *E. coli* BL21 (DE3) using Strep-Tactin affinity chromatography as previously described [22]. Recombinant His$_6$-ClfA$_{1\text{-}520}$ was (ClfA, Clumping factor A) cloned with plasmid pET15 and

Table 1 List of the bacteria used in this study, including abbreviations used in the text and the strain's origin and properties

Abbreviation	Original strain	Properties	References
WT	*S. aureus* Newman	*S. aureus* reference strain	[17]
vwb	*S. aureus* Newman	Deletion of *vwb* gene	[18,19]
coa/vwb	*S. aureus* Newman	Deletion of *coa* and *vwb* genes	[18,19]
srtA	*S. aureus* Newman	Deletion of *srtA* gene	[18,19]
spa	*S. aureus* Newman	Deletion of *spa* gene	[18,19]
clfA	*S. aureus* Newman	Deletion of *clfA* gene	[18,19]
clfB	*S. aureus* Newman	Deletion of *clfB* gene	[18,19]
fnbpA	*S. aureus* Newman	Deletion of *fnbpA* gene	[18,19]
fnbpB	*S. aureus* Newman	Deletion of *fnbpB* gene	[18,19]
srdcde	*S. aureus* Newman	Deletion of SdrCDE genes	[18,19]
sasB	*S. aureus* Newman	Deletion of *sasB* gene	[18,19]
sasC	*S. aureus* Newman	Deletion of *sasC* gene	[18,19]
L. lactis pIL253	*L. lactis* subsp. *cremoris* 1363	Empty vector expressing erythromycin resistance determinant	[11]
L. lactis FnBpA	*L. lactis* subsp. *cremoris* 1363	Insertion of staphylococcal *fnba* gene	[11]
L. lactis FnBpB	*L. lactis* subsp. *cremoris* 1363	Insertion of staphylococcal *fnbb* gene	[11]
L. lactis ClfA	*L. lactis* subsp. *cremoris* 1363	Insertion of staphylococcal *clfa* gene	[20]

purified from *E. coli* BL21 (DE3) using Ni-NTA chromatography as previously described [23]. Glutathione-S-transferase (GST)–VWF A1-domain proteins were produced as described before [24].

Cell wall and secreted protein extraction

S. aureus Newman (wild-type) and *coa/vwb* deletion mutant (Table 1) were grown overnight in Tryptic Soy Broth at 37 °C. Bacteria were washed, resuspended in 50 mL phosphate buffered saline (PBS) and incubated for 4 h at 37 °C. Where indicated, 20 μg mL^{-1} rvWbp was added to the *S. aureus coa/vwb* strain and incubated for 1 h at 37 °C. The bacterial pellet and supernatant were stored separately at –20 °C. To recover the cell wall proteins, the pellet was first washed in 0.05 M Tris buffer (pH 7.4) and resuspended in 0.05 M Tris buffer (pH 7.4) containing 0.002 M $MgCl_2$. Bacterial cells were disrupted with a homogenizer for 10 min and the cell suspension was placed at 75 °C for 10 min to inactivate cell-wall autolytic enzymes. Supernatants were recovered and centrifuged at high speed to recover the cell wall. Cell walls were washed with 5 mL 0.05 M Tris buffer pH 7.4 + 1 M NaCl and extracted with 2% Triton at room temperature for 30 min, followed by incubating the pellet in 0.05 M Tris buffer (pH 7.4) + 0.145 M NaCl + 50 μg mL^{-1} lysostaphin (AMBI Products, New York, NY, USA) at 37 °C for 2 h. To recover secreted proteins, supernatant was filtered (Millex Filter Unit 0.22 μm, Merck Millipore, Overijse, Belgium) and concentrated using centrifugal filters with a 50 kD threshold (Centrifugal Filer Units, Merck Millipore).

Western blot analysis

Cell wall and secreted proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes in a Trans-Blot Turbo apparatus (Bio-Rad, Nazareth, Belgium). Membranes were incubated overnight with a house-made polyclonal antibody against rvWbp (1:500). After adding horseradish peroxidase-conjugated secondary antibodies, immunoreactive bands were visualized by Enhanced chemiluminescence (Amersham Biosciences, Diegem, Belgium).

In vitro *perfusion experiments*

In vitro perfusion experiments were performed as previously described [6,25]. Glass coverslips (24 × 50 mm, VWR International, Leuven, Belgium) were coated with 50 μg mL^{-1} VWF (Haemate P, CSL Behring, Mechelen, Belgium), 200 μg mL^{-1} Horm collagen (Takeda, Linz, Austria), 30 μg mL^{-1} rvWbp or 50 μg mL^{-1} VWF A1-domain in a humidified container at room temperature for 4 h. The coverslips were mounted in a micro-parallel flow chamber [26] and perfused for 10 min with a high-accuracy Harvard pump (PHD 2000 Infusion, Harvard Apparatus, Holliston, MA, USA) generating flow rates of 1000 s^{-1}. Bacteria were labeled with 5(6)-carboxy-fluorescein N-hydroxysuccinimidyl ester (Sigma-Aldrich) (final concentration of 30 μg mL^{-1} for subsequent perfusion experiments) and diluted in PBS to an OD_{600} (optical density) of 0.65 or 1.2 (corresponding to approximately 3×10^8 and 6×10^9 colony forming units (CFU) mL^{-1}). Coated coverslips were perfused with labeled bacteria, with or without soluble VWF (60 μg mL^{-1}), rvWbp or bacterial supernatant added to the perfusate. Membrane-bound rvWbp was prepared by supplementing *vwb* (*S. aureus* strain lacking vWbp) with rvWbp and after an incubation period of 15 min, unbound rvWbp was removed by centrifugation. Bacterial supernatant was prepared by incubating washed bacteria for 4 h at 37 °C in PBS. Bacteria were then removed by centrifugation and filtering (Millex Filter Unit 0.22 μm, Merck Millipore). Live images were obtained using an inverted fluorescence microscope and video microscopy as reported [6]. Images were digitally stored and fluorescence was measured with ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA). The intensity of fluorescence is reported as relative bacterial adhesion.

Bacterial adhesion to endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords of healthy donors as described before [6,25]. HUVECs were seeded on 1% gelatin-coated (Sigma-Alderich) plastic coverslips (Sarstedt, Nümbrecht, Germany) and grown to confluence. The coverslips were mounted in a micro-parallel flow chamber and the HUVECs were activated with 0.1 mM Ca^{2+}-ionophore A23187 (Sigma-Aldrich) for 10 min and perfused for 10 min with fluorescently labeled bacteria (OD_{600} of 1.2). Where indicated, 20 μg mL^{-1} rvWbp, 2.5 μg mL^{-1} rADAMTS-13 and/or 10 μg mL^{-1} anti-VWF A1 domain antibody were added to the bacterial perfusate. Bacterial adhesion was recorded as described above.

In vivo *mesenteric perfusion model*

Six- to eight-week-old C57Bl/6 mice were anesthetized with ketamine/xylazine and their right jugular veins were catheterized (Portex intravenous cannula, 2F). The peritoneal cavity was opened via midline abdominal incision and the mesenteric microcirculation was visualized with an inverted microscope (Axio-observer D1, Carl-Zeiss NV, Zaventem, Belgium). To activate the endothelium and cause VWF release, 5 μL of the Ca^{2+}- ionophore A23187 (10 mM) was locally applied to the vascular bed. Subsequently, 100 μL of a suspension of fluorescently labeled bacteria (final concentration of 60 μg mL^{-1} carboxy-fluorescein and an OD_{600} of 1.8, corresponding to approximately 1×10^9 CFU mL^{-1}) was injected through the catheter. Where indicated, 20 μg mL^{-1} of rvWbp was added to the bacterial inoculum. Live time-lapse images (1 image per second, 40 images) were acquired with an inverted fluorescence microscope, captured via a black and white camera and developed using image software. The fluorescent signal in the blood vessel was quantified manually for each frame and reported as described above. Animal experiments were approved by the Ethical Committee of the University of Leuven (P110/2014). For immunofluorescence staining, the blood vessels were dissected and fixed with paraformaldehyde 4% overnight and imbedded in parafine. A polyclonal anti-VWF rabbit antibody (31 μg mL^{-1}) (Dako, Glostrup, Denmark) was used as primary antibody and a goat anti-rabbit Alexa Fluor-568 (20 μg mL^{-1}) (Invitrogen, Carlsbad, CA, USA) as secondary antibody. Endothelial cells were counterstained with 4',6-diamidino-2-phenylindole.

Surface plasmon resonance

Affinity and rates of association were measured on a BIAcore 3000 (GE Healthcare, Hillerod, Denmark). Buffers were sterile filtered and degassed. A nitrilotriacetic acid (NTA) chip (GE Healthcare, Diegem, Belgium) was used to capture histidine-tagged [27] ligands. The NTA chip was prepared for ligand capturing by injecting $NiCl_2$ (0.5 M) (GE Healthcare, Diegem, Belgium) followed by injection of 20 μL His-$ClfA_{1\text{-}520}$ (200 nM). His-$ClfA_{1\text{-}520}$ and vWbp-Strep were diluted in running buffer (HBS-P buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 0.005% [vol/vol] surfactant P20) (GE Healthcare, Diegem, Belgium), 50 μM ethylenediaminetetraacetic acid (EDTA) (GE Healthcare, Diegem, Belgium) and 1 mM imidazole). To study the His-$ClfA_{1\text{-}520}$–vWbp interaction, vWbp-Strep was injected at 6.25 nM, 12.5 nM and 25 nM for 180 s, followed by regeneration of the chip with 50 μM EDTA and 1 mM imidazole. All injections were performed at a flow rate of 10 μL min^{-1}. Kinetic coefficients, K_A and K_D, were determined using the BiaEvaluation software (GE Healthcare, Diegem, Belgium) and best fit was determined with a 1 : 1 binding model with drifting baseline and local R_{max}. All experiments were repeated in triplicate on at least three occasions.

Statistical analysis

All calculations were carried out with GraphPad Prism 5.0d (GraphPad Software, San Diego, CA USA). Groups were compared with 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

Secreted vWbp interacts with both VWF and the S. aureus *cell wall*

We previously described that the shear-dependent adhesion of *S. aureus* to endothelial cells and subendothelial matrix is mediated by complex formation between VWF and staphylococcal vWbp [6]. To promote bacterial adhesion, secreted vWbp has to be able to interact with the *S. aureus* cell wall.

Western blotting confirmed that vWbp is secreted by the *S. aureus* Newman wild-type strain (WT), but it was also found attached to the cell wall (Fig. 1A). An *S. aureus* mutant strain lacking vWbp lacked both secreted and cell wall-bound vWbp. Added exogenous rvWbp was able to bind to the cell wall of the mutant strain lacking vWbp.

Compared with WT, perfusion of *vwb* over VWF resulted in reduced adhesion. Normal adhesion could be restored by either exogenous rvWbp (Fig. 1B) or by adding supernatant of the WT strain (Fig. 1C), but not by adding supernatant of the *vwb* strain. Exogenous vWbp (rvWbp or vWbp present in WT supernatant) (Fig. 1B, C) led to a 2-fold increase in the adhesion of *vwb* to VWF under flow ($P = 0.021$ and $P = 0.048$, respectively), indicating that vWbp interacts with *S. aureus* regardless of its ability to secrete vWbp.

This increase in adhesion was seen both when *vwb* was pre-incubated with rvWbp and when rvWbp was pre-perfused over VWF (Fig. 1D) ($P = 0.0003$ and $P = 0.003$, respectively).

These findings show that vWbp is a secreted protein capable of both binding to VWF under flow and sticking to *S. aureus*, thereby promoting the adhesion of *S. aureus* to the vessel wall under flow via a ternary interaction.

vWbp binds to S. aureus *via an SrtA-dependent surface protein*

We hypothesized that *S. aureus* interacts with vWbp via a staphylococcal cell wall-anchored surface protein processed by SrtA.

In contrast to WT, the *srtA* strain (deficient in SrtA, which lacks all sortase A-dependent cell wall-anchored proteins) was not able to adhere to coated rvWbp (Fig. 2A) ($P < 0.0001$). Binding of *vwb* to the rvWbp coating was similar to that of the WT strain ($P = 0.56$).

Pre-perfusion of coated VWF with rvWbp increased the adhesion of *vwb* but had no effect on the adhesion of *srtA* (Fig. 2B) ($P = 0.10$), strengthening our interpretation that vWbp interacts with *S. aureus* through a sortase A-dependent cell wall-anchored protein.

vWbp forms a complex with VWF and ClfA, promoting bacterial adhesion to endothelial cells and subendothelial matrix

To identify which SrtA-dependent protein is crucial for vWbp binding, we screened a set of mutants deficient in one single SrtA-dependent cell wall-anchored surface protein for their adhesiveness to coated rvWbp under flow. When compared with the WT strain, two SrtA-dependent

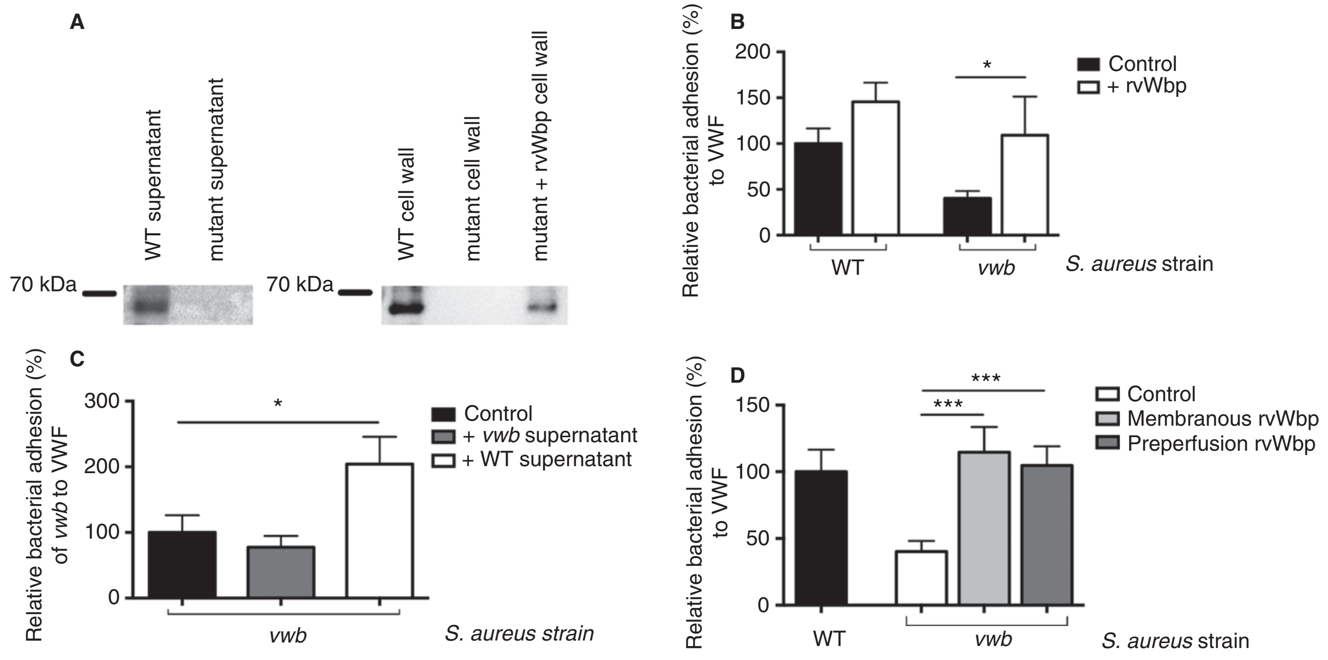


Fig. 1. vWbp is a secreted staphylococcal protein that binds to *S. aureus*. (A) Western blot analysis on secreted proteins and cell wall proteins of *S. aureus* WT and mutant lacking vWbp. Where indicated, 30 μg mL^{-1} rvWbp was added to the mutant. (B) Micro-parallel plate flow chamber perfusion over coated VWF (50 μg mL^{-1}) with fluorescently labeled WT and *vwb* strains at a shear rate of 1000 s^{-1} ($n \geq 8$). Where indicated, 15 μg mL^{-1} rvWbp was added to the bacterial perfusate. All results are expressed as mean ± SEM. *$P < 0.05$. (C) Micro-parallel plate flow chamber perfusion over coated VWF (50 μg mL^{-1}) with fluorescently labeled *vwb* strain at a shear rate of 1000 s^{-1} ($n \geq 8$). Where indicated, supernatant of the WT strain (containing secreted vWbp) or *vwb* supernatant (lacking secreted vWbp) was added to the bacterial perfusate. All results are expressed as mean ± SEM. *$P < 0.05$. (D) Micro-parallel plate flow chamber perfusion over coated VWF (50 μg mL^{-1}) with fluorescently labeled WT or *vwb* strains at a shear rate of 1000 s^{-1} ($n \geq 8$). Where indicated, prior to perfusion, *vwb* was supplemented with 30 μg mL^{-1} rvWbp and after an incubation period of 15 minutes, unbound rvWbp was removed. Where indicated, VWF was pre-perfused with 30 μg mL^{-1} rvWbp. All results are expressed as mean ± SEM. ***$P < 0.001$. vWbp, von Willebrand factor-binding protein; WT, wild type; rvWbp, recombinant His$_6$-vWbp; VWF, von Willebrand factor.

cell wall-anchored surface protein deletion mutants showed decreased adhesion to rvWbp (i.e. the mutant lacking Clumping factor A [*clfA*] and the mutant lacking staphylococcal protein A) [28] (Fig. 3A) ($P = 0.0001$ and $P = 0.0092$, respectively).

We further assessed the adhesion profile of *srtA*, *clfA* and *spa* to VWF (Fig. 3B). Compared with the WT strain, absence of SrtA and ClfA reduced adhesion by more than 75% ($P = 0.0066$ and $P = 0.0066$, respectively), whereas absence of SpA did not significantly reduce bacterial adhesion ($P = 0.48$).

Similarly, when compared with WT, *srtA* and *clfA* were unable to bind to collagen, the main component of the subendothelial matrix, regardless of the presence of VWF in the perfusate (Figure SI) ($P = 0.0009$ and $P = 0.0069$, respectively). The adhesion of *spa* to collagen in the presence of VWF was similar to that of WT ($P = 0.49$), suggesting that ClfA is the main bacterial surface binding partner for vWbp mediating *S. aureus* adhesion to VWF under flow.

Exogenous rvWbp increased the adhesion of *vwb* to VWF under flow; however, different concentrations of rvWbp did not affect the adhesion of *clfA* to VWF (Figure S2).

To validate whether this mechanism is capable of explaining bacterial adhesion to the endothelium, we examined the adhesion of WT, *vwb*, *srtA*, *clfA* and *spa* to resting and stimulated endothelial cells under flow.

Activation of endothelial cells facilitated the adhesion of the WT strain (Fig. 3C,D). In contrast, in the absence of vWbp, SrtA or ClfA, *S. aureus* was no longer able to bind to the VWF-strings and bacterial adhesion was low even to stimulated endothelial cells (Fig. 3C) ($P = 0.0087$, $P = 0.0053$ and $P = 0.0077$, respectively). However, addition of rvWbp increased the adhesion of *vwb* comparable to WT. Cleavage of VWF multimers by rADAMTS-13 decreased the adhesion of the WT strain by 60% ($P = 0.0085$) and combining rADAMTS-13 with an anti-VWF A1 domain antibody further decreased the WT adhesion to 10% ($P = 0.0007$) (Fig. 3E).

These data identify ClfA as a crucial factor in the VWF-mediated binding of *S. aureus* to endothelial cells in flow conditions by acting as a bacterial binding partner for vWbp.

vWbp forms a complex with the VWF A1-domain and ClfA

Presence of isolated VWF A1-domain on the coverslip was sufficient to trigger adhesion of the WT strain, but not of *vwb*, *srtA* and *clfA*, suggesting that vWbp binds to

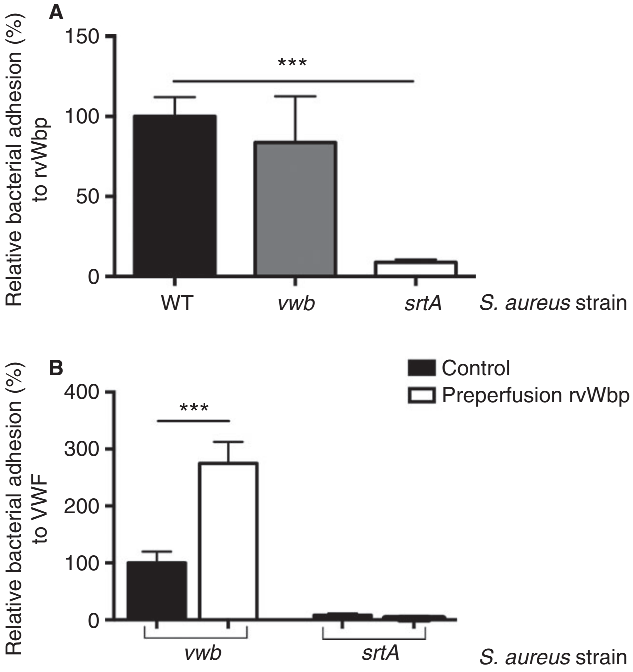


Fig. 2. vWbp binds to *S. aureus* via an SrtA-dependent surface protein. (A) Micro-parallel plate flow chamber perfusion over coated rvWbp (30 μg mL^{-1}) with fluorescently labeled WT, *vwb* and *srtA* strains at a shear rate of 1000 s^{-1} ($n \geq 8$). All results are expressed as mean ± SEM. ***$P < 0.001$. (B) Micro-parallel plate flow chamber perfusion over coated VWF (50 μg mL^{-1}) with fluorescently labeled *S. aureus vwb* and *srtA* strains at a shear rate of 1000 s^{-1} ($n \geq 8$). Where indicated, pre-perfusion with rvWbp (30 μg mL^{-1}) was performed. All results are expressed as mean ± SEM. ***$P < 0.001$. vWbp, von Willebrand factor-binding protein; SrtA, Sortase A; rvWbp, recombinant His_6-vWbp; WT, wild type; VWF, von Willebrand factor.

the A1-portion of VWF (Fig. 4A) ($P = 0.0072$, $P = 0.0013$ and $P = 0.0045$, respectively). This finding further provides an explanation for the previously recognized role of the VWF A1-domain in *S. aureus* binding to VWF [6].

To extend the submolecular localization of vWbp binding to the VWF A1-domain to interactions with ClfA, we measured the association of His-$ClfA_{1\text{-}520}$ with vWbp-Strep by surface plasmon resonance (Fig. 4B). Using a range of concentrations, we calculated the dissociation constant (K_D) to be around 1 nM for the interaction between soluble vWbp-Strep and immobilized His-$ClfA_{1\text{-}520}$, representative of a moderately high affinity (Fig. 4B).

These findings confirmed that vWbp interacts with the VWF A1-domain and with the *S. aureus* surface protein ClfA simultaneously.

L. lactis *expressing staphylococcal ClfA binds to vWbp and to VWF in the presence of vWbp*

We independently verified our findings using *Lactococcus lactis* (*L. lactis*) bacteria expressing single staphylococcal surface molecules on their cell walls. The control *L. lactis* pIL253 strain showed only minimal adhesion to rvWbp under flow. Expression of ClfA in *L. lactis* sufficed to allow adhesion to rvWbp under flow (Fig. 5A) ($P = 0.0018$). *L. lactis* expressing FnBPA (Fibronectin binding protein A) or FnBPB (Fibronectin binding protein B), two well-described staphylococcal SrtA-dependent surface proteins, was unable to adhere to rvWbp. No adhesion of the *L. lactis* strains was observed to coated VWF A1-domain. However, when rvWbp was present, *L. lactis* expressing ClfA showed a significantly increased adhesion to the VWF A1-domain under flow (Fig. 5B) ($P = 0.0354$). rvWbp had no effect on the adhesion of *L. lactis* pIL253 or the lactococci expressing FnBPA or FnBPB (data not shown). Next, we examined the adhesion of *L. lactis* to endothelial cells under flow. *L. lactis* expressing ClfA was not able to adhere to activated endothelial cells. Adding rvWbp to the perfusate remarkably increased adhesion of this strain to activated endothelial cells under flow (Fig. 5C) ($P = 0.0013$).

Together, these data show that expression of ClfA is sufficient to bind to the VWF A1-domain, but only in the presence of vWbp.

Adhesion of S. aureus *to the vessel wall* in vivo *is mediated by the VWF-vWbp-ClfA complex*

We confirmed these findings using an *in vivo* intravital mesenteric perfusion model. The WT strain was able to roll over and adhere to the activated murine vessel wall (Fig. 6A/B, Video S1) at the site of locally stimulated VWF release. Similar to our previous observations, the absence of ClfA or vWbp mitigated bacterial adhesion to the vessel wall compared with the WT strain ($P = 0.0033$ and $P = 0.0050$, respectively). However, supplementing *vwb* with rvWbp restored its adhesion to the vessel wall ($P = 0.0005$) (Video S2 and S3).

We conclude that vWbp interacts both with sheared VWF and with the staphylococcal surface protein ClfA. The ternary complex formed by endothelial VWF, secreted vWbp and bacterial ClfA mediates adhesion of *S. aureus* to the vascular endothelium with a high efficiency.

Discussion

Despite improvement in medical supportive care and the implementation of a more aggressive surgical approach, *S. aureus* infective endocarditis continues to have a very high mortality [29]. Furthermore, antibiotic resistance spreads at an alarming pace, urging new ways to prevent and treat this severe disease. The inability to improve outcome once infective endocarditis has been diagnosed underlines the importance of intervening at the early stages of the disease, preferably to prevent infective endocarditis from developing in the first place.

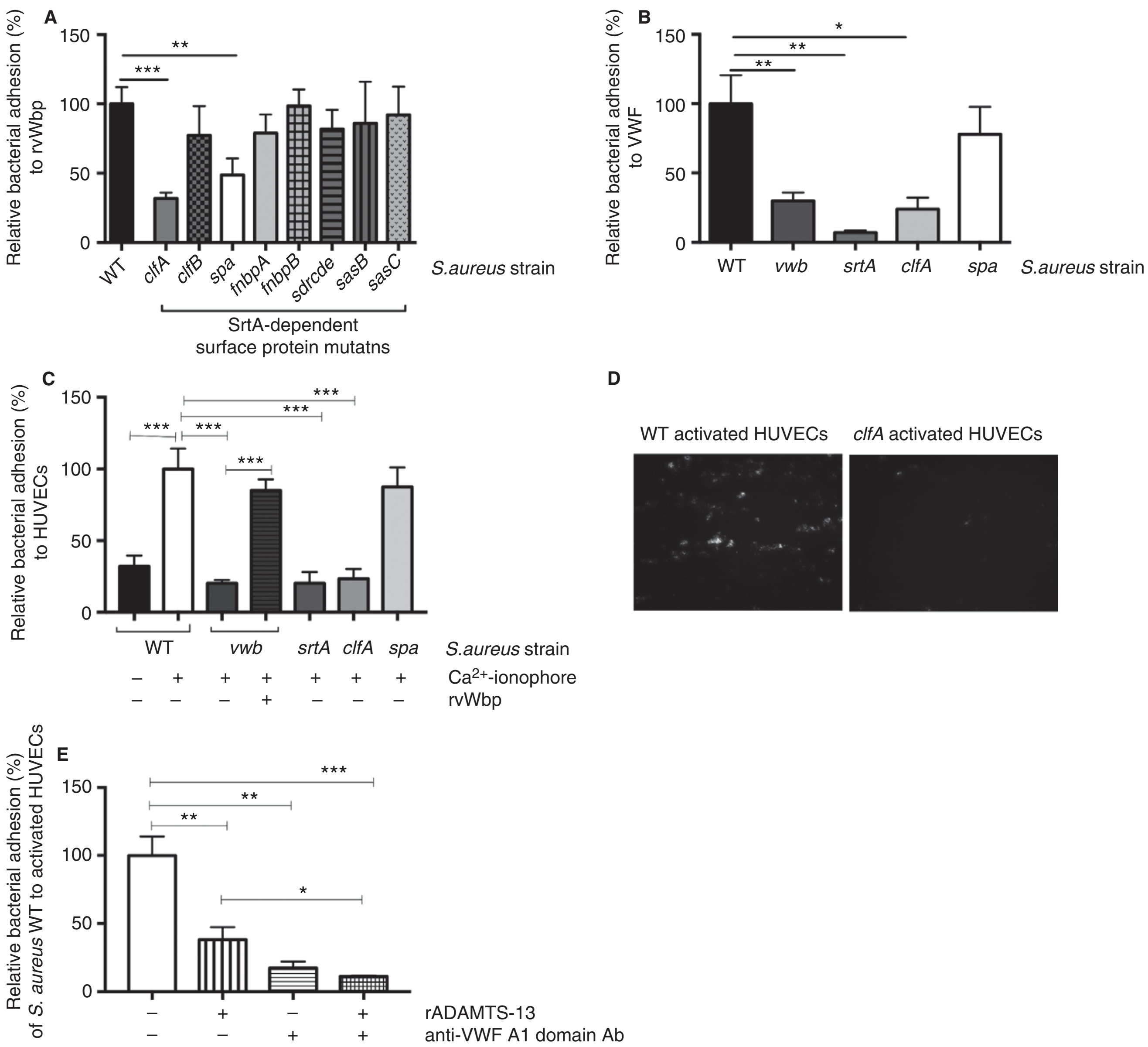
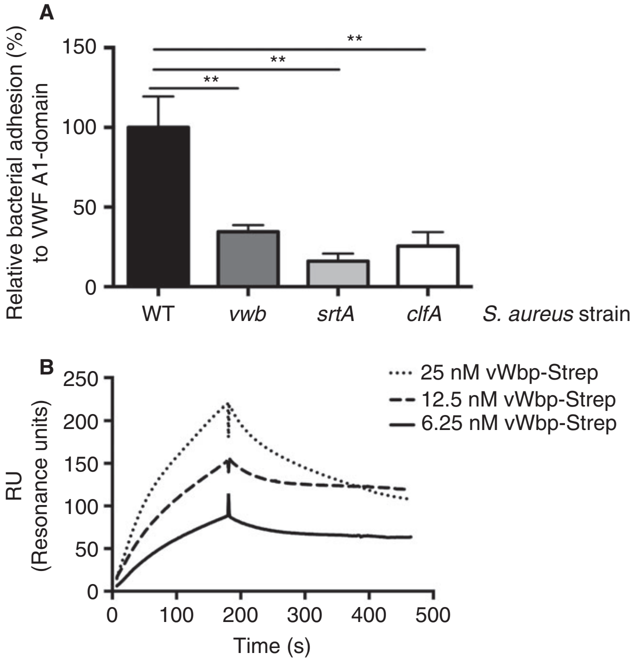


Fig. 3. vWbp forms a complex with VWF and ClfA to promote bacterial adhesion to VWF and to endothelial cells. (A) Micro-parallel plate flow chamber perfusion over coated rvWbp (30 μg mL^{-1}) with fluorescently labeled WT, *vwb*, *srtA*, *clfA*, *clfB*, *spa*, *fnbpA*, *fnbpB*, *sdrcde*, *sasB* and *sasC* strains at a shear rate of 1000 s^{-1} ($n \geq 8$). All results are expressed as mean ± SEM. $^{**}P < 0.01$ $^{***}P < 0.001$. (B) Micro-parallel plate flow chamber perfusion over coated VWF (50 μg mL^{-1}) with fluorescently labeled WT, *vwb*, *srtA*, *clfA* and *spa* strains at a shear rate of 1000 s^{-1} ($n \geq 8$). All results are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. (C) Micro-parallel plate flow chamber perfusion over HUVECs with fluorescently labeled WT, *vwb*, *srtA, clfA* and *spa* strains at a shear rate of 1000 s^{-1}. Where indicated, HUVECs were activated by a 5-min perfusion with a Ca^{2+}-ionophore. Where indicated, 20 μg mL^{-1} rvWbp was added to the perfusate ($n \geq 6$). All results are expressed as mean ± SEM. $^{***}P < 0.001$. (D) Representative image of WT bacteria (left) and *clfa* bacteria [36] adhering to activated endothelial cells under flow. White bar is 100 micron. (E) Micro-parallel plate flow chamber perfusion over HUVECs with fluorescently labeled WT strain at a shear rate of 1000 s^{-1}. HUVECs were activated by a 5-min perfusion with a Ca^{2+}-ionophore. Where indicated, 2.5 μg mL^{-1} rADAMTS-13 and/or 10 μg mL^{-1} anti-VWF A1 domain antibody were added to the perfusate ($n \geq 6$). All results are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. vWbp, von Willebrand factor-binding protein; VWF, von Willebrand factor; ClfA, Clumping factor A; rvWbp, recombinant His_6-vWbp; WT, wild type; HUVECs, human umbilical vein endothelial cells.

Patients with *S. aureus* bacteremia are at high risk of developing infective endocarditis [30]. Infection of cardiac valves requires the binding of *S. aureus* to the endothelium under the high shear stress of flowing blood. Identifying virulence factors that mediate this initial binding can help to develop strategies to prevent infective endocarditis.

In our previous work we showed that *S. aureus* exploits the VWF-mediated binding that localizes platelets to sites of vascular damage or inflammation [6]. Although we



Ligand immobilized to chip	Protein ligand	k_a (1/Ms)	k_d (1/s)	K_A (1/M)	K_D (M)
His-ClfA$_{1\text{-}520}$	vWbp-Sterp	$5.93 \times 10^5 \pm 1.60$	$6.58 \times 10^{-4} \pm 2.30$	9.01×10^8	1.11×10^{-9}

Fig. 4. vWbp forms a complex with the VWF A1-domain and ClfA. (A) Micro-parallel plate flow chamber perfusion over coated VWF A1-domain (50 μg mL^{-1}) with fluorescently labeled WT, *vwb*, *srtA*, *clfA* and *spa* at a shear rate of 1000 s^{-1} ($n > 8$). All results are expressed as mean ± SEM. $^{**}P < 0.05$. (B) Protein–protein interaction study with surface plasmon resonance. 200 nM His-ClfA$_{1\text{-}520}$ was captured with an NTA chip and perfused with different concentrations of recombinant vWbp-Strep. All injections were performed with a flow rate of 10 μL min^{-1}. k_a = association rate, k_d = dissociation rate, K_A = association constant, K_D = dissociation constant. vWbp, von Willebrand factor-binding protein; VWF, von Willebrand factor; ClfA, Clumping factor A; WT, wild type; NTA, nitrilotriacetic acid.

identified the bacterial protein vWbp as a crucial factor, the precise mechanisms remained unclear, because vWbp is a secreted protein that lacks a cell-wall anchoring sequence.

In this study, we unravel how vWbp, a secreted protein, can bind to the bacterial cell wall, thus allowing shear-resistant binding of *S. aureus* to the inflamed or damaged endothelium and to the subendothelial matrix. We identified ClfA, an SrtA-mediated surface protein, as the bacterial surface binding partner for vWbp. Both vWbp and ClfA were shown to be crucial factors in the initial adhesion of *S. aureus* to the vascular endothelium.

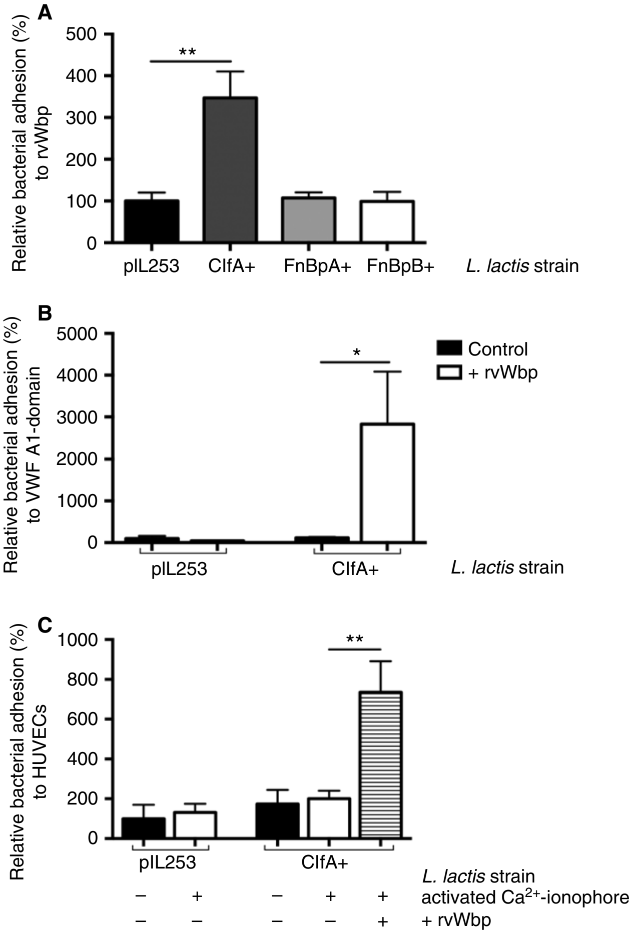


Fig. 5. *L. lactis* expressing staphylococcal ClfA binds to vWbp. (A) Micro-parallel plate flow chamber perfusion over coated rvWbp (30 μg mL^{-1}) with fluorescently labeled *L. lactis* pIL253, *L. lactis* ClfA+, *L. lactis* FnBpA+ and *L. lactis* FnBpB+ strains at a shear rate of 1000 s^{-1} ($n = 8$). All results are expressed as mean ± SEM. $^{**}P < 0.01$. (B) Micro-parallel plate flow chamber perfusion over coated VWF A1-domain (50 μg mL^{-1}) with fluorescently labeled *L. lactis* pIL253 and *L. lactis* ClfA+ strains at a shear rate of 1000 s^{-1} ($n \geq 9$). Where indicated, rvWbp (20 μg mL^{-1}) was added. All results are expressed as mean ± SEM. $^{*}P < 0.05$. (C) Micro-parallel flow chamber perfusion over HUVECs with fluorescently labeled *L. lactis* pIL253 and *L. lactis* ClfA+, strains at a shear rate of 1000 s^{-1}. Where indicated, HUVECs were activated by a 5-min perfusion with a Ca^{2+}-ionophore. Where indicated, rvWbp (50 μg mL^{-1}) was added ($n \geq 8$). All results are expressed as mean ± SEM. $^{**}P < 0.01$. ClfA, Clumping factor A; vWbp, von Willebrand factor-binding protein; rvWbp, recombinant His$_6$-vWbp; VWF, von Willebrand factor; HUVECs, human umbilical vein endothelial cells.

S. aureus has many surface proteins that enable its binding to host proteins, endothelial cells or to subendothelial matrix. These surface proteins or MSCRAMMs (e.g. ClfA, FnBPA/B and SpA) are covalently bound to the cell wall by a transpeptidase, SrtA. Absence of SrtA leads to the defective anchoring of about 20 staphylococcal surface proteins [13–15]. Our findings indicate that the *srtA* gene and the subsequent correct anchoring of staphylococcal surface proteins are vital for *S. aureus* binding to the vascular endothelium via VWF. However, the adhesive contribution of these proteins in a flow field remains uncertain. Whereas the surface proteins ClfA and SpA can bind to vWbp, only ClfA contributed to the adhesion of *S. aureus* to the vascular endothelium via VWF in flow. ClfA is known to adhere to endothelial

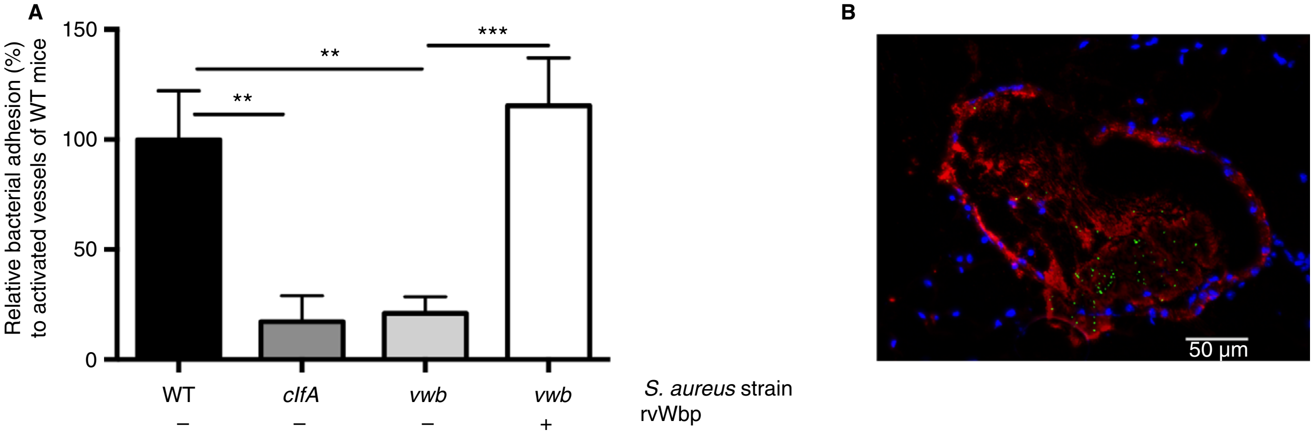


Fig. 6. Adhesion of *S. aureus* to the vessel wall *in vivo* is mediated by the ternary complex VWF-vWbp-ClfA. (A) *In vivo* venous mesenteric perfusion model with WT mice. A total of 5 µL of the Ca^{2+} -ionophore A23187 (10 mM) was applied to the region of the visualized vascular bed to trigger endothelial cell activation and VWF release. A suspension of fluorescent-labeled WT, *clfA* and *vwb* strains was injected through the jugular catheter. Where indicated, 20 µg mL^{-1} rvWbp was added to the bacterial perfusate ($n \geq 14$). All results are expressed as mean ± SEM. **$P < 0.01$, ***$P < 0.001$. (B) Fluorescence image (× 630) of *S. aureus* (green) adhering to activated murine vessel wall with immuno-staining for VWF (red) and 4',6-diamidino-2-phenylindole-staining of the cell nucleus (blue). White bar is 50 µm. VWF, von Willebrand factor; vWbp, von Willebrand factor-binding protein; ClfA, Clumping factor A; WT, wild type. [Color figure can be viewed at wileyonlinelibrary.com]

cells via fibrinogen and fibronectin, but it has never been associated with VWF binding. SpA binding to VWF has been reported in static conditions; however, in flow, the recruitment of *S. aureus* to endothelial cells is SpA independent [5,6].

VWF circulates in a compact globular form, but is progressively unfolded in a flow field or when bound to collagen, thereby exposing VWF A1, A2 and A3-domain [7]. It has been described that bacteria can bind to endothelial cells via VWF [5,6,31]. Similar to our findings, Pappelbaum *et al.* showed that ADAMTS-13 decreased VWF-mediated *S. aureus* adhesion to endothelial cells by 50% [5]. We have previously shown that binding of *S. aureus* to VWF can be blocked by an A1 neutralizing antibody, an antibody that also blocks the binding of platelets to VWF [6]. We now show that *S. aureus* binds directly to the VWF A1-domain and does so via vWbp and ClfA.

L. lactis, non-pathogenic bacteria, process their surface proteins in a similar way to *S. aureus* via a LPXTG motif. *L. lactis* expressing single staphylococcal surface molecules on their cell walls are therefore widely used to study the adhesive properties of a single surface protein. In 2013, Veloso *et al.* demonstrated the relevance of these lactococci expressing single surface proteins using a low-grade bacteremia model [11,20,32]. Using an *L. lactis* strain expressing ClfA, we confirmed that the simple presence of ClfA was sufficient to confer adhesion to the VWF A1-domain, but only in the presence of vWbp.

S. aureus exploits a variety of mechanisms to interact with and bind to the host's tissue. Therefore, targeting a single virulence factor may be insufficient to block clinically relevant bacterial adhesion. We confirmed the pathophysiological relevance of this adhesion mechanism by studying *in vivo* adhesion of *S. aureus* to the murine mesenteric circulation. Indeed, *S. aureus* binding to activated endothelium *in vivo* was also VWF, vWbp and ClfA dependent. Adding vWbp restored the adhesive phenotype of the vWbp mutant strain, again highlighting the importance of this protein in bacterial adhesion to the vessel wall. As absence of either ClfA or vWbp prevented bacterial adhesion *in vivo*, it is tempting to speculate about the therapeutic potential of a strategy targeting these factors in patients with *S. aureus* bacteremia [33–35]. Interestingly, it was previously shown that a vaccine strategy against ClfA reduced the incidence of experimental infective endocarditis in bacteremic mice [34]. Similarly, vaccinating against ClfA also reduced binding of *S. aureus* to an aortic patch in mice [29].

In summary, our work identifies ClfA as a novel bacterial binding partner for staphylococcal vWbp. Together, these two proteins promote the adhesion of *S. aureus* to vascular endothelium. Further unraveling of the interactions between VWF, secreted vWbp and bacterial ClfA may lead to novel preventive or therapeutic strategies that reduce the high mortality of *S. aureus* infective endocarditis.

Addendum

T. Vanassche, P. Verhamme, M. F. Hoylaerts, and R. Heying designed the research, analyzed the data, and wrote the manuscript; J. Claes designed and performed the research, analyzed the data, and wrote the manuscript; L. Liesenborghs, M. Peetermans, and T. R. Veloso performed experiments and helped interpret data; D. Missiakas, O. Schneewind, S. Mancini, and J. M. Entenza designed the research, contributed vital new agents, and contributed to writing the manuscript.

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Disclosure of Conflict of Interests

M. Peetermans reports grants from Research Foundation Flanders (FWO-Vlaanderen, grant number 11I0113N) during the conduct of the study and non-financial support from Pfizer outside the submitted work. P. Verhamme reports grants and personal fees from Boehringer-Ingelheim, Bayer, and Daiichi Sankyo, as well as personal fees from Pfizer, outside the submitted work. The other authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Micro-parallel flow chamber perfusion over coated collagen (200 μg mL^{-1}) with fluorescently labeled wild-type (WT), *vwb*, *srtA, clfA* and *spa* strains at a shear rate of 1000 s^{-1} ($n \geq 6$). Where indicated, 60 μg mL^{-1} von Willebrand factor (VWF) was added to the perfusate. All results are expressed as mean ± SEM.

Fig. S2. Micro-parallel flow chamber perfusion over coated von Willebrand factor (VWF) (50 μg mL^{-1}) with fluorescently labeled *vwb* and *clfA* strains at a shear rate of 1000 s^{-1} ($n \geq 6$).

Video S1. Real-time adhesion of *S. aureus* Newman to activated vessel wall of wild-type (WT) mice.

Video S2. Real-time adhesion of *vwb* to activated vessel wall of wild-type (WT) mice.

Video S3. Real-time adhesion of *vwb* supplemented with 20 μg mL^{-1} rvWbp to activated vessel wall of wild-type (WT) mice.

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