



The iron-regulated surface determinant B (IsdB) protein from *Staphylococcus aureus* acts as a receptor for the host protein vitronectin

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Staphylococcus aureus is an important bacterial pathogen that can cause a wide spectrum of diseases in humans and other animals. *S. aureus* expresses a variety of virulence factors that promote infection with this pathogen. These include cell-surface proteins that mediate adherence of the bacterial cells to host extracellular matrix components, such as fibronectin and fibrinogen. Here, using immunoblotting, ELISA, and surface plasmon resonance analysis, we report that the iron-regulated surface determinant B (IsdB) protein, besides being involved in heme transport, plays a novel role as a receptor for the plasma and extracellular matrix protein vitronectin (Vn). Vn-binding activity was expressed by staphylococcal strains grown under iron starvation conditions when Isd proteins are expressed. Recombinant IsdB bound Vn dose dependently and specifically. Both near-iron transporter motifs NEAT₁ and NEAT₂ of IsdB individually bound Vn in a saturable manner, with K_D values in the range of 16–18 nM. Binding of Vn to IsdB was specifically blocked by heparin and reduced at high ionic strength. Furthermore, IsdB-expressing bacterial cells bound significantly higher amounts of Vn from human plasma than did an *isdB* mutant. Adherence to and invasion of epithelial and endothelial cells by IsdB-expressing *S. aureus* cells was promoted by Vn, and an $\alpha_v\beta_3$ integrin-blocking mAb or cilengitide inhibited adherence and invasion by staphylococci, suggesting that Vn acts as a bridge between IsdB and host $\alpha_v\beta_3$ integrin.

Staphylococcus aureus causes a wide range of opportunistic infections that range from superficial skin infections to life-threatening diseases, including endocarditis, pneumonia, and septicemia (1). Adherence of bacteria to host matrix components is the initial critical event in the pathogenesis of most infections. The extracellular matrix (ECM) essentially consists of macromolecules, such as collagens, proteoglycans, and glycoproteins, that serve as a substrate for the adhesion and migration of tissue cells. These processes involve integrins, a family of heterodimeric cell surface receptors that recognize specific ECM proteins (2, 3).

Bacteria, including *S. aureus*, also utilize the ECM as substrate for their adhesion through a family of cell wall-anchored

(CWA) adhesins called MSCRAMMs (microbial surface component recognizing adhesive matrix molecules) that specifically recognize host matrix components (4, 5).

Vitronectin (Vn) is a glycoprotein that is synthesized in the liver and secreted into plasma (6) and is also an important component of the ECM (7). Vn is found at a high concentration in plasma (200–700 $\mu\text{g/ml}$) (8, 9) and is also present in different human tissues (10). The N-terminal portion of mature Vn (43 aa residues) consists of a somatomedin B (SMB) domain followed by the classical integrin-binding motif, Arg-Gly-Asp (RGD). Members of the integrin family that engage in Vn binding include integrin $\alpha_3\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ (11). The next domain comprises four hemopexin-like domains with putative heme-binding motifs. In addition, Vn has three heparin-binding domains (HBD) spanning residues Vn82–137 (HBD-1), Vn175–219 (HBD-2), and Vn348–361 (HBD-3) (12–14) (Fig. S1A). Vn is present in the organism in different conformational states: as the native, folded monomer (65–75 kDa, the 65-kDa form being derived from the proteolytic cleavage in the C-terminal region of the protein) in plasma/serum and as a multimeric unfolded form in the ECM (6, 15, 16). Conformational change from the monomeric to the activated, multimeric state is promoted by exposure of Vn to agents, such as urea, or binding to physiological ligands, such as the thrombin-antithrombin complex and the membrane attack complex. Vn conformational activation reveals a number of cryptic sites, including the full exposure of the heparin-binding site at the C-terminal domain of the protein (17, 18) and cell-binding motif (RGD) (19, 20). Vn binds the terminal complement C5b-7 complex. It occupies the metastable membrane binding site and thereby inhibits membrane insertion of the complex (21). It also binds C9 and directly inhibits C9 polymerization (21, 22).

Several bacterial species interact with host cell-bound multimeric Vn, facilitating adherence to epithelial cells and artificial surfaces (23). Simultaneous interaction of Vn with an integrin and bacterial surface proteins results in the formation of a bridge between bacteria and host cells. This leads to internalization of bacteria, as exemplified by *Streptococcus pneumoniae* (24) or *Pseudomonas aeruginosa* (25), resulting in downstream signaling events (24).

Staphylococci contain several Vn-binding proteins, including the autolysins AtlE and Aae from *S. epidermidis* and the homologous proteins AtlA and Aaa from *S. aureus* (26, 27). Also,

This article contains supporting information.

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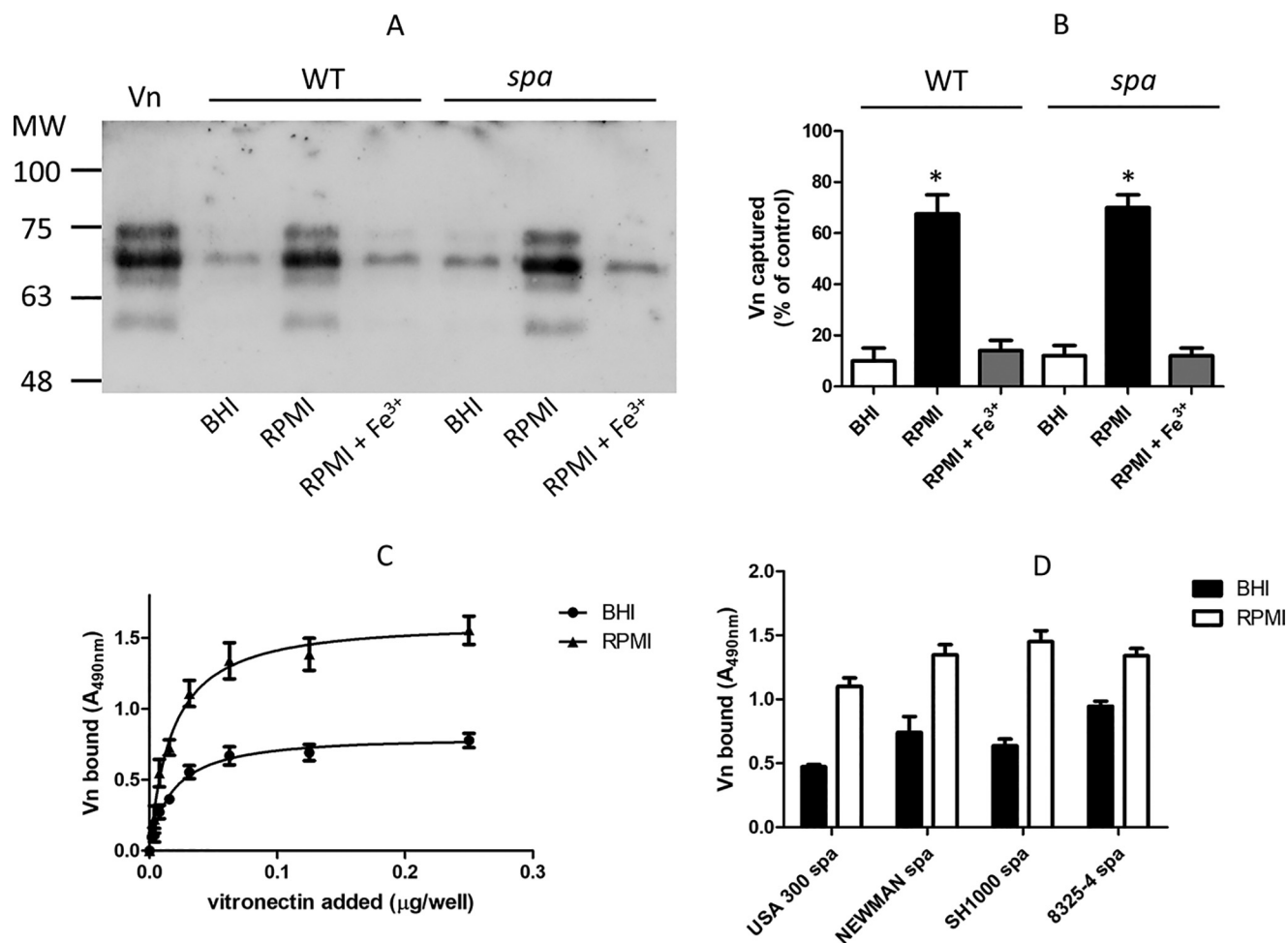


Figure 1. Binding of purified Vn by *S. aureus* cells. A, *S. aureus* strain SH1000 and its *spa* mutant grown either in BHI or RPMI medium in the absence/presence of 1 mM FeCl₃ were incubated with purified Vn. Bacterium-bound proteins were released by extraction buffer and separated by SDS-PAGE under reducing conditions and analyzed by far Western blotting. The membrane was probed with sheep anti-human Vn, followed by HRP-conjugated rabbit anti-sheep IgG. Molecular masses of standard proteins are indicated on the left. B, densitometric analysis of Vn bound to *S. aureus* SH1000 and the *spa* mutant as reported in panel A. The band intensity was quantified relative to a sample of purified human Vn (8 µg). The reported data are the mean values ± S.D. from three independent experiments. C, binding of increasing concentrations of Vn to immobilized *S. aureus* SH1000 *spa* cells grown in RPMI or BHI medium is shown. Bound Vn was detected as described above. The data points are the means ± S.D. from three independent experiments, each performed in triplicate. A statistically significant difference is indicated (Student's *t* test; *, *p* < 0.05). D, binding of Vn to strains of *S. aureus spa*. Microtiter wells coated with *S. aureus spa* strains grown in RPMI or BHI were incubated with Vn. Bound Vn was detected by addition of sheep anti-Vn polyclonal IgG and rabbit HRP-conjugated anti-sheep IgG.

the multifunctional autolysin Atl from *Staphylococcus lugdunensis* interacts with Vn (28).

Atl autolysins have a similar modular organization (signal peptide, propeptide, amidase activity, three major repeats, R1 to R3, and glucosaminidase activity), share a high degree of sequence similarity, and are functionally interchangeable (29). R1-R2 repeats are critical for autolysin binding to Vn (30). Moreover, the major autolysin, Atl, mediates *S. aureus* internalization via direct interaction with host heat shock protein Hsc70 (31).

Studies on *S. aureus* adhesion to and invasion of host cells have been performed with bacteria grown in rich medium containing iron (4). In contrast, *in vivo S. aureus* has restricted access to iron, and the lack of available iron leads to the upregulation of a number of genes, among which are those that encode surface determinant (Isd) proteins (32). The Isd system contains nine proteins whose expression is coordinately upregulated under iron-depleted conditions (33–36). The primary role of Isd proteins is to capture heme from hemoglobin (Hb) and

transport it into the cell (32). These include IsdA, IsdB, IsdC, and IsdH, which are anchored to cell wall peptidoglycan by sortases and are exposed on the cell surface (37, 38). Each protein contains a structurally conserved near iron transporter (NEAT) motif(s) that binds Hb and heme. IsdA and IsdC contain one NEAT domain each, whereas IsdB and IsdH contain two and three NEAT domains, respectively. The NEAT domains adopt a beta sandwich fold that consists of two five-stranded antiparallel beta sheets (39).

Fig. S1B shows the organization and primary sequence comparisons between the seven known NEAT domains in *S. aureus*.

Sequence homologs of this class of proteins are found in a number of important human pathogens, such as *S. lugdunensis*, (40–42), *Listeria monocytogenes* (43), *Bacillus anthracis* (44), and *Streptococcus pyogenes* (45).

IsdA, IsdB, and IsdH of *S. aureus* are known to have other biological functions. IsdA interacts with an array of host proteins (36) and confers resistance to the innate defenses of the human

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skin (46). IsdH plays a role in the evasion of phagocytosis as a result of accelerated degradation of C3b (47). IsdB binds to platelets via direct interaction with the platelet integrin GPIIb/IIIa and also promotes *S. aureus* adherence to and internalization by nonphagocytic human cells (48). The objective of the current study was to investigate in more detail the binding of Vn to *S. aureus* cells. We show that cells expressing IsdB specifically bind to Vn and analyze the nature and the biological consequences of this interaction.

Results

Vn binding by *S. aureus* is promoted by growth under iron-restricted conditions

In preliminary experiments, we tested the capture of Vn by *S. aureus* strain SH1000 grown to stationary phase in rich brain heart infusion (BHI) or iron-restricted Roswell Park Memorial Institute 1640 (RPMI) medium with or without FeCl₃. Bacteria grown in RPMI showed a higher ability to capture Vn than those grown in iron-rich medium or in RPMI supplemented with FeCl₃, suggesting that binding of strain SH1000 depends on proteins induced by iron starvation. Interestingly, a protein A-deficient (*spa*) mutant showed a Vn-binding profile overlapping that of the WT strain, suggesting that Vn binding to the bacterial surface is not related to protein A expression (Fig. 1, A and B).

To further analyze the interaction of Vn with *S. aureus*, SH1000 *spa* organisms grown in BHI and RPMI medium were immobilized onto microtiter wells and allowed to interact with increasing amounts of soluble Vn (Fig. 1C). Under both conditions, Vn bound to bacteria in a dose-dependent and saturable fashion. RPMI-grown bacteria captured significantly larger amounts of Vn, suggesting that *S. aureus* cells express a larger number of receptors on their surface or that novel receptors were induced when grown in iron starvation. To compare the Vn binding potential of different *S. aureus* strains, *spa* mutants of the *S. aureus* laboratory strains Newman, SH1000, and 8325-4 and the clinical strain USA300 grown in BHI or RPMI medium to the stationary phase were immobilized in microtiter wells and tested for binding to soluble Vn. All the strains grown in RPMI medium showed a higher ability to bind Vn when grown under iron starvation conditions than in iron-rich medium (Fig. 1D).

Identification of a Vn-binding protein

To identify the surface component(s) involved in Vn binding, SH1000 *spa* cells grown in BHI or RPMI medium were digested with lysostaphin and the released material subjected to SDS-PAGE under reducing conditions and far Western blotting. The nitrocellulose membrane was incubated with Vn, and the bound ligand was detected with anti-Vn IgG. A strong signal corresponding to a molecule of 75 kDa was noted in protein from cells grown in RPMI medium, whereas no significant signal was detected in material released from cells grown in BHI medium (Fig. 2, lanes 1 and 2).

To further investigate this issue, proteins were separated by SDS-PAGE under reducing conditions and visualized by staining with Coomassie brilliant blue (Fig. 2, lane 3). A candidate

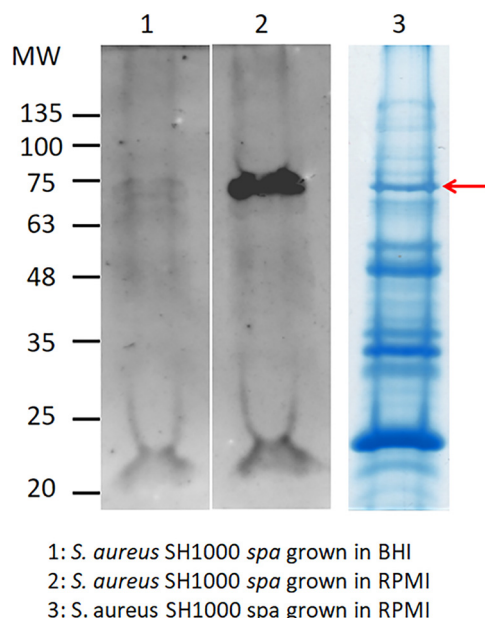


Figure 2. Far Western blotting of lysostaphin-released material from cells of SH1000 *spa*. Cells of SH1000 *spa* grown in BHI (lane 1) and RPMI (lane 2) were digested with lysostaphin and the released material subjected to far Western blotting. The nitrocellulose membrane was probed with Vn, followed by sheep anti-Vn and HRP-conjugated rabbit anti-sheep IgG. Molecular mass standards are indicated on the left. The lysostaphin-released material from cells of SH1000 *spa* grown in RPMI was also subjected to SDS-PAGE and stained with Coomassie blue (lane 3). The band corresponding to the 75-kDa bacterial component is marked by an arrow.

protein of 75 kDa was excised from the stained gel, digested with trypsin, and analyzed by MS. The database search unequivocally identified IsdB as the potential Vn-binding protein of *S. aureus* SH1000 (Fig. S2A).

isdB gene expression is growth-phase dependent

Since *S. aureus* SH1000 *spa* cells grown to stationary phase in BHI medium bound Vn significantly less than when grown in RPMI medium (Fig. 1), we analyzed the expression of the *isdB* gene in cells grown both in BHI or RPMI medium to mid-exponential and stationary phases of growth by quantitative RT-PCR (qRT-PCR). Expression of *isdB* in cells grown to stationary phase in RPMI medium was about 5-fold higher than that in cells grown in BHI medium or cells grown to mid-exponential phase in either medium (Fig. S2B).

This finding was validated by comparing the expression levels of the IsdB protein by bacteria grown to mid-exponential and stationary phases in BHI and RPMI media by Western immunoblotting. While IsdB was virtually undetectable in material released from bacteria grown to both the mid-exponential and stationary phases in BHI medium, the protein was abundant in material released from bacteria grown to the stationary phase in RPMI medium (Fig. S2C).

Ectopic expression of *IsdB* by *L. lactis* and binding to Vn

To study IsdB in isolation from other *S. aureus* CWA proteins, a strain of *L. lactis* expressing IsdB from a gene cloned into the plasmid vector pNZ8037 was used. To validate the expression of IsdB from the bacterium, *L. lactis* pNZ8037::*isdB*

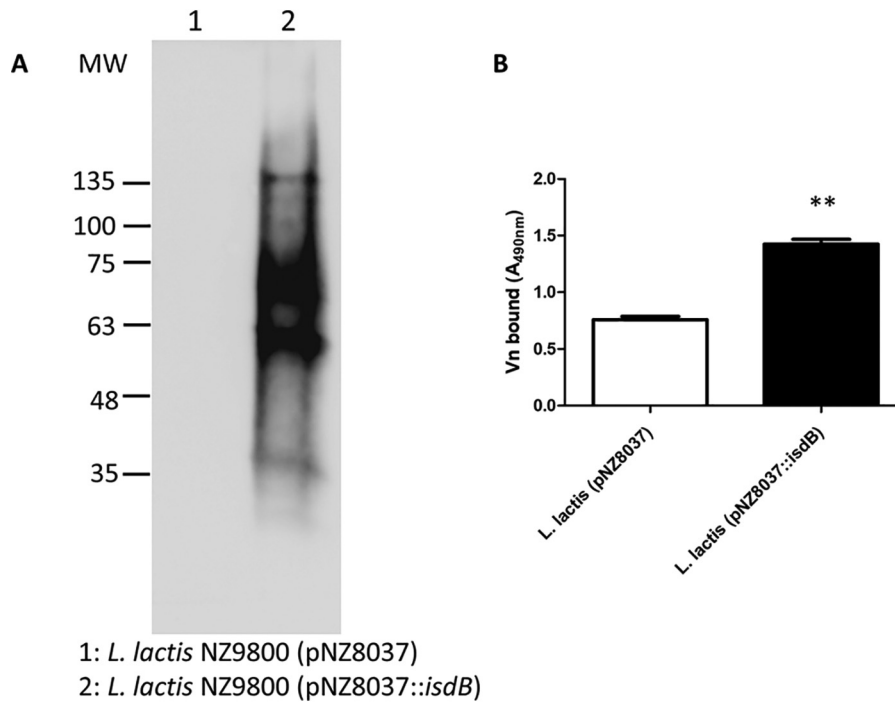


Figure 3. Interaction of *L. lactis*-expressing IsdB with Vn. *A*, cell wall proteins were released by mutanolysin/lysozyme treatment from *L. lactis* expressing IsdB (pNZ8037::isdB) and control cells carrying the empty vector. The protein components in the mixture were separated by SDS-PAGE and subjected to far Western blotting. The membrane was probed with Vn, followed by sheep anti-Vn and HRP-conjugated rabbit anti-sheep IgG. The figure is representative of three independent experiments. Molecular mass standards are indicated on the left. *B*, interaction of Vn with *L. lactis*-expressing IsdB and cells carrying the empty vector assessed by ELISA. Bacteria were immobilized in microtiter plates and incubated with Vn, followed by addition to the wells of sheep anti-Vn polyclonal IgG and HRP-conjugated rabbit anti-sheep antibody. The data points are the means \pm S.D. from three independent experiments, each performed in triplicate. Statistically significant differences are indicated (Student's *t* test; **, $p < 0.01$).

and the isogenic strain carrying the empty vector were treated with mutanolysin and lysozyme, and the released material subjected to far Western blotting. A Vn binding protein of 75 kDa was detected (along with an approximately 60-kDa protein, which may be a breakdown product), whereas the material released from *L. lactis* (pNZ8037) lacked reactivity (Fig. 3A). Moreover, the lactococci were immobilized onto microtiter wells, and binding of soluble Vn was examined by ELISA. Significantly higher binding of Vn to *L. lactis* (pNZ8037::isdB) was observed compared to that of *L. lactis* harboring the empty vector (Fig. 3B).

Specificity of Vn binding to IsdB

To investigate the specificity of Vn binding to IsdB, recombinant IsdB NEAT₁-NEAT₂ protein was immobilized onto microtiter wells and tested for binding to extracellular matrix proteins, including fibrinogen, fibronectin, collagen, and Vn. Only Vn bound to the surface-coated IsdB, whereas no binding of the other proteins was observed (Fig. 4A).

Localization of the binding sites within IsdB

To localize the Vn-binding region within the IsdB protein, the NEAT₁ and NEAT₂ domains were expressed in *E. coli* and employed in binding studies. First, the binding of soluble Vn to immobilized recombinant NEAT₁ and NEAT₂ was determined by ELISA. Vn bound dose dependently and saturably to both NEAT₁ and NEAT₂ fragments and with a binding profile

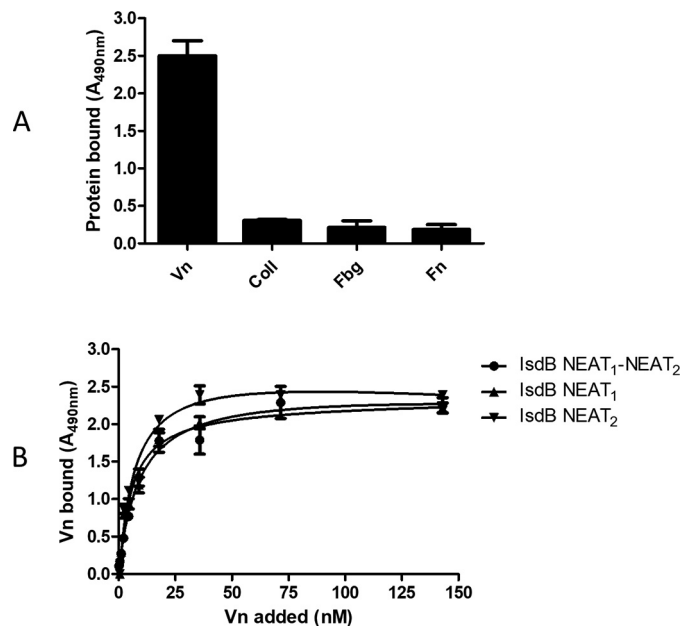


Figure 4. Characterization of Vn-binding activity of IsdB. *A*, specificity of recombinant IsdB interaction with extracellular matrix proteins. Microtiter wells were coated with IsdB NEAT₁-NEAT₂ and then incubated with the indicated extracellular matrix proteins Vn, collagen type I, fibrinogen, and fibronectin. Each bound protein was detected by the addition of ligand-specific polyclonal antibody and HRP-conjugated secondary IgG. The data points are the means \pm S.D. from three independent experiments, each performed in triplicate. *B*, Vn binding to IsdB NEAT₁-NEAT₂ and its derivatives, NEAT₁ and NEAT₂. Recombinant IsdB NEAT₁-NEAT₂ and its derivatives, NEAT₁ and NEAT₂, were immobilized onto microtiter wells and incubated with increasing amounts of Vn. Bound Vn was detected by the addition of sheep anti-Vn polyclonal IgG and HRP-conjugated rabbit anti-sheep. The data points are the means \pm S.D. from three independent experiments, each performed in triplicate.

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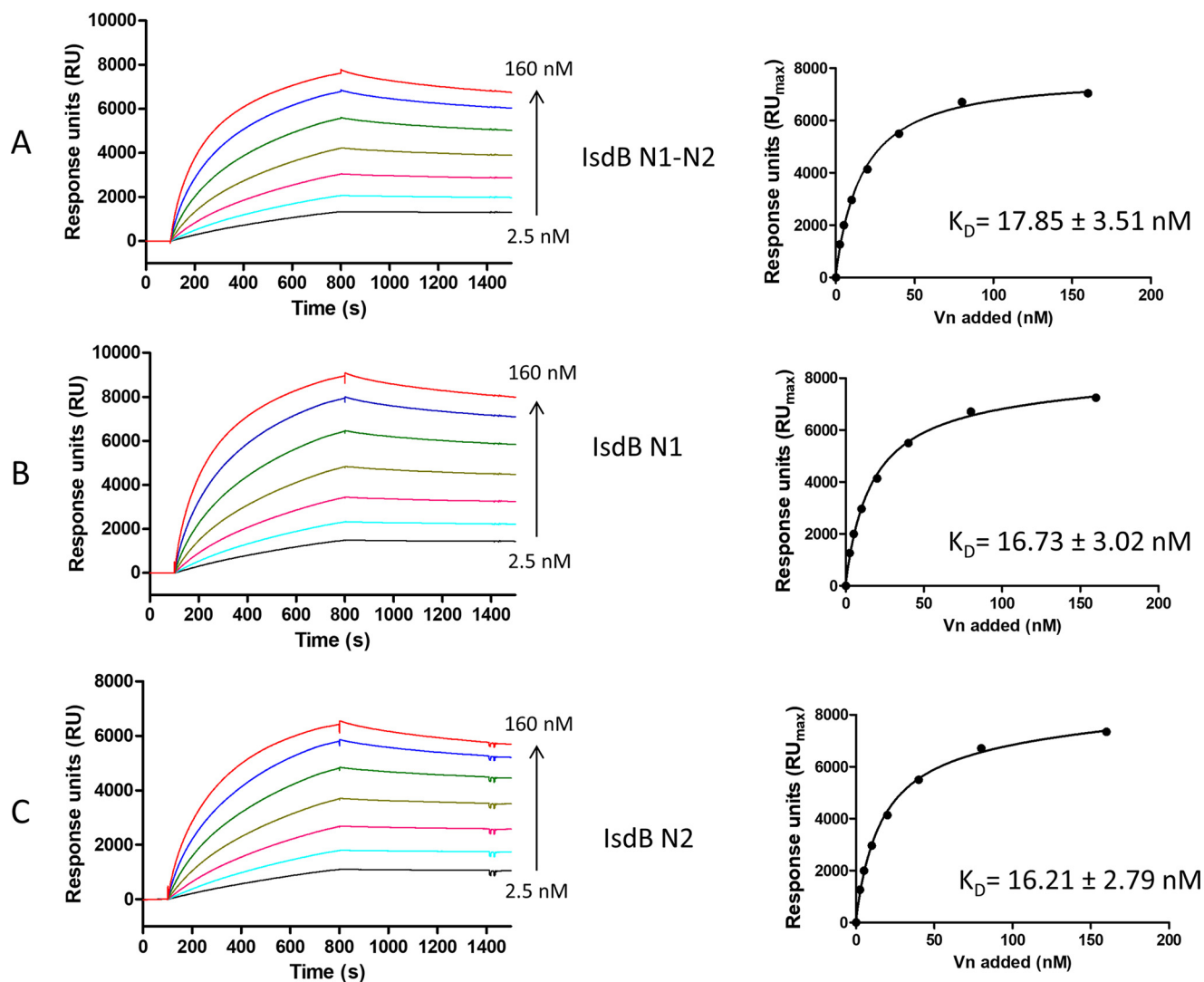


Figure 5. Analysis of the interaction between IsdB NEAT₁-NEAT₂ and its derivatives, NEAT₁ and NEAT₂, with Vn by SPR. Twofold linear dilution series (2.5–160 nM) of Vn were injected over the IsdB NEAT₁-NEAT₂ or its derivatives, NEAT₁ and NEAT₂, immobilized on the surface of a CM5 sensor chip. The sensorgrams obtained were normalized versus the response obtained when Vn was flowed over uncoated chips. The affinity was calculated from curve fitting to a plot of the response unit values at the steady state (RU_{max}) against increasing concentrations of Vn (left). Shown is one representative of three experiments.

resembling that exhibited by intact full-length IsdB NEAT₁-NEAT₂ (Fig. 4B).

IsdB NEAT₁-NEAT₂ and single NEAT₁ and NEAT₂ domains bind to Vn with high affinity

Surface plasmon resonance (SPR) experiments were performed to compare the binding of Vn to those of immobilized IsdB NEAT₁-NEAT₂ and single NEAT₁ and NEAT₂ fragments. Vn displayed a high binding activity, as indicated by the high response values and the slow dissociation of the IsdB-Vn complexes upon removal of the ligand. The best fit of the data points was obtained with the Langmuir isotherm equation describing a one-site binding model. From this analysis, we obtained dissociation constant (K_D) values of 17.85 ± 3.51 nM, 16.73 ± 3.02 nM, and 16.21 ± 2.79 nM for IsdB NEAT₁-NEAT₂/Vn, NEAT₁/Vn, and NEAT₂/Vn, respectively (Fig. 5). All these findings show that full-length IsdB contains two separate bind-

ing sites that interact with nearly identical high affinities for Vn.

Binding of Vn to IsdB is blocked by heparin and dependent on ionic strength

To investigate whether IsdB competes with glycosaminoglycans for binding to Vn, the effect of heparin, heparan sulfate, and chondroitin sulfate on Vn binding to immobilized IsdB NEAT₁-NEAT₂ was studied. In contrast to heparan sulfate and chondroitin sulfate, heparin dose dependently inhibited the IsdB-Vn interaction, suggesting that IsdB interacts with the heparin-binding domain(s) of the protein (Fig. S3A). To determine if ionic forces play a role in the interaction of IsdB with Vn, the effect of sodium chloride on Vn binding to IsdB was assessed. Addition of NaCl significantly reduced binding of Vn to immobilized IsdB, and at a concentration of 500 mM, NaCl reduced Vn binding to IsdB by almost 80% (Fig. S3B).

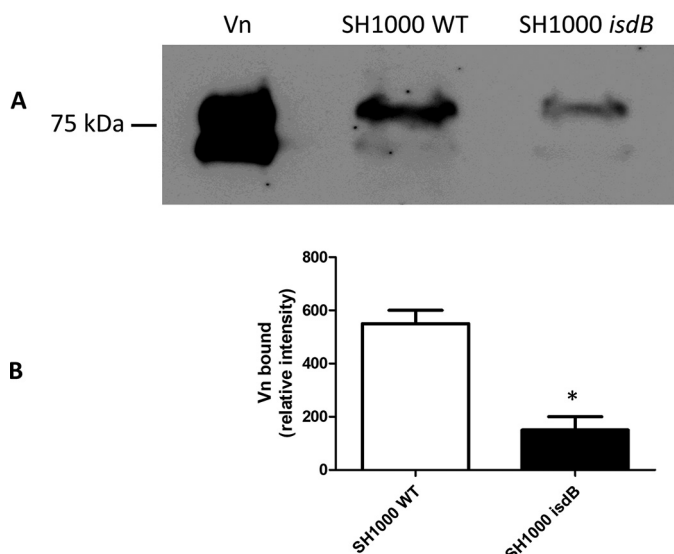


Figure 6. Capture of plasma vitronectin by *S. aureus* strain SH1000. A, *S. aureus* SH1000 and its isogenic *isdB* mutant were mixed with 1 ml of human plasma for 60 min. Proteins bound to the cell surface were released by extraction buffer, separated by SDS-PAGE under nonreducing conditions, and analyzed by Western blotting. The membrane was probed with sheep anti-Vn polyclonal IgG and HRP-conjugated rabbit anti-sheep and developed with the ECL Western blotting detection kit. The pure human Vn sample is shown on the left. B, densitometric analysis of Vn bound to and released from *S. aureus* SH1000 and its isogenic *isdB* mutant. The reported data are the mean values \pm S.D. from three independent experiments. Statistically significant differences are indicated (Student's *t* test; *, $p < 0.05$).

S. aureus IsdB captures Vn from plasma

To investigate if *S. aureus* cells expressing IsdB can recruit Vn from plasma, bacteria were incubated with human plasma and the amount of Vn captured was quantified by Western blotting and densitometry. *S. aureus* expressing IsdB captured approximately 4-fold more Vn than the *isdB* mutant. Thus, it can be concluded that IsdB expression is important for *S. aureus* to capture Vn from human plasma. However, it must be pointed out that a substantial amount of Vn bound to the *isdB* mutant, indicating that other Vn receptors are operational on the surface of *S. aureus* cells (Fig. 6).

Vn mediates adherence to and invasion of HeLa and HUVEC monolayers

To investigate the role of the Vn-binding activity of IsdB in promoting adherence of *S. aureus* SH1000 to host cells, bacteria were grown to the stationary phase in BHI or RPMI medium and then tested for attachment to HeLa and human umbilical vein endothelial cell (HUVEC) monolayers (Fig. 7, A and C) in the absence of Vn. No adherence of the strain grown under either condition was observed. Conversely, the addition of exogenous human Vn to the cell monolayers promoted a high level of adherence of the RPMI-grown but not the BHI-grown bacteria. Almost complete inhibition of adherence was observed when the assays were performed in the presence of an integrin $\alpha_v\beta_3$ -binding mAb or the specific $\alpha_v\beta_3$ inhibitor cilengitide, suggesting the involvement of integrin $\alpha_v\beta_3$. To examine whether IsdB/Vn is also involved in the staphylococcal invasion of HeLa cells or HUVECs (Fig. 7, B and D), BHI- and RPMI-grown bacteria were incubated with cell monolayers in the presence or

absence of Vn and then tested for internalization. We found a moderate level of invasion only by RPMI-grown bacteria in the presence of Vn, while no internalization was observed with BHI-grown bacteria even in the presence of Vn. In confirmation of this, neither adherence nor invasion was observed with the *isdB* mutant (Fig. 7). Together, these data suggest that for promoting efficient adherence and gaining entry of bacteria into HeLa cells or HUVECs, *S. aureus* can use Vn as a molecular bridge between surface-expressed IsdB and the $\alpha_v\beta_3$ integrin.

Staphylococcal adherence to HeLa and HUVEC cell lines was similar, while bacterial invasion of HUVECs was 50-fold more effective than that of the HeLa cell line. Variation in expression levels of the $\alpha_v\beta_3$ integrin on the two cell types may contribute to the difference in pathogen invasiveness.

Notably, a persistent, low level of bacterial invasion was observed when bacteria grown in RPMI were incubated with HeLa and HUVECs in the absence of Vn, suggesting that other internalization mechanisms are operational.

Discussion

In our search for *S. aureus* MSCRAMMs with Vn-binding activity, here we show that *S. aureus* IsdB interacts with Vn. As previously demonstrated, IsdB is strongly upregulated under conditions of iron restriction and, in accordance with this property, Vn binding was strictly related to the expression of IsdB by bacterial strains. Bacteria bind Vn when grown under iron starvation conditions and not in an iron-rich medium. Moreover, binding of Vn by bacteria was mostly expressed by cells in the stationary phase of growth.

To characterize this binding to IsdB, we purified and used multimeric Vn, the conformational form of the protein found in the ECM. Notably, we found that bacteria expressing IsdB also captured Vn from plasma, where the monomeric form of the protein prevails. Thus, it seems that IsdB binds to both conformational states of Vn. This is reminiscent of the situation observed with *Moraxella catarrhalis* and *Haemophilus influenzae*, which interact with both isoforms of Vn (23).

The interaction of IsdB with Vn was confirmed using several approaches, including far Western blotting, ELISA, and SPR. Binding of IsdB to Vn was specific and saturable and exhibited a K_D in the nanomolar range, which is comparable to that recorded for high-affinity CWA protein–host protein interactions (49–52).

To narrow down the Vn binding site(s) of IsdB, the NEAT₁ and NEAT₂ domains were recombinantly produced and tested for their ability to bind the ligand. The individual modules interacted with an affinity for Vn comparable to that of the full-length IsdB, suggesting that each module represents the minimal motif required for efficient interaction of IsdB with Vn and that IsdB potentially can bind two Vn molecules. The finding that both domains interact with Vn is particularly intriguing, in view of the fact that the two NEAT domains of IsdB have very low sequence identity (about 12%) and that they are functionally different. In fact, the NEAT₁ domain is involved in Hb binding while NEAT₂ plays a role in heme extraction from α and β chains of Hb (53). In view of the discovery that even NEAT motifs with low sequence identity show Vn-binding

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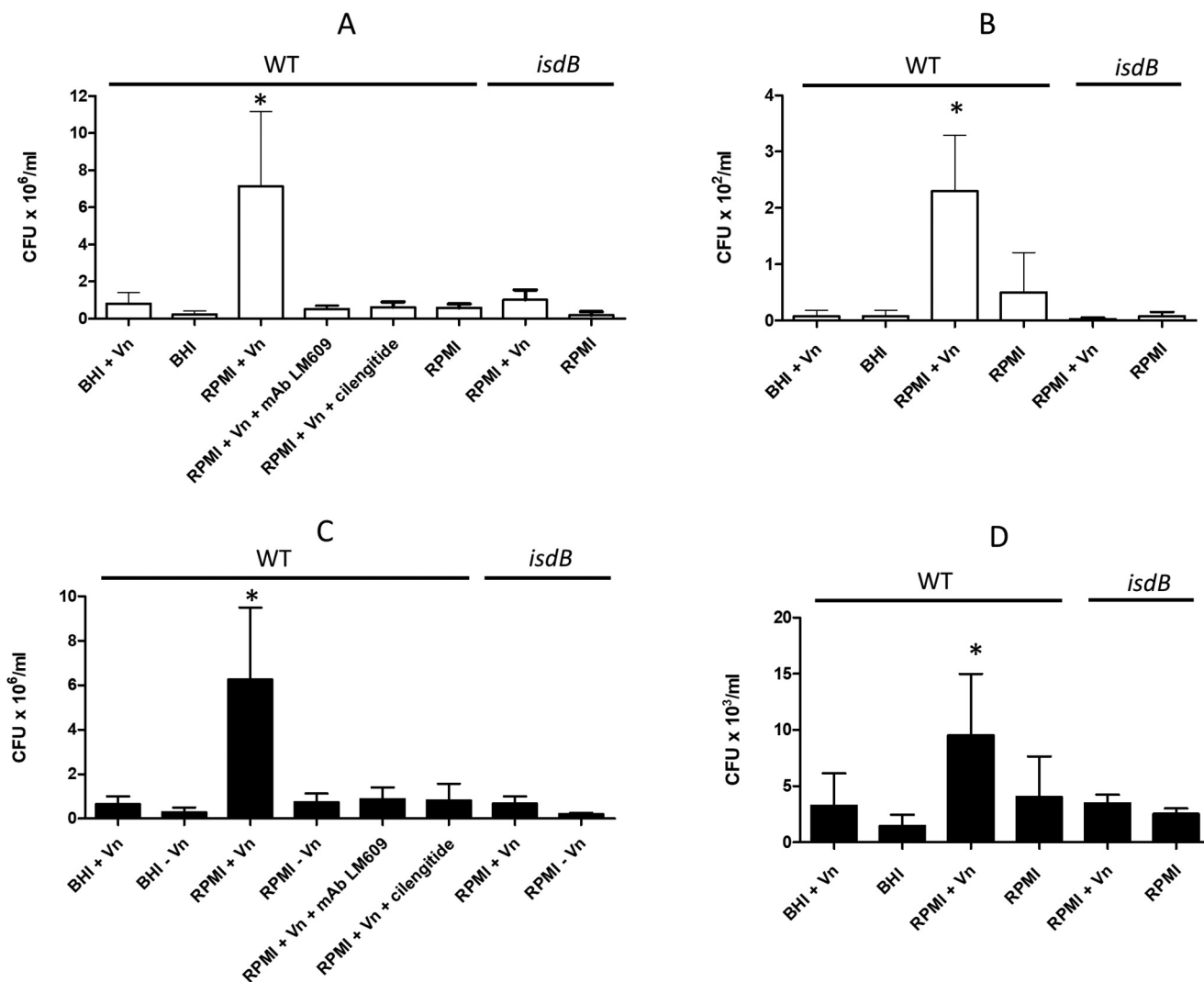


Figure 7. Adhesion and internalization of *S. aureus* cells. Bacterial strains were grown overnight in BHI or RPMI medium, added to HeLa (A and B) or HUVEC (C and D) cell monolayers, and tested for adhesion (A and C) or internalization (B and D). The experiments were performed in DMEM for 2 h at 37 °C. The effects of $\alpha_v\beta_3$ mAb LM609 and cilengitide on bacterial adherence are also reported. The inocula and adherent and internalized bacteria were quantified by viable counting. Error bars show S.D. of the means from three independent determinations performed in triplicate with similar results (Student's *t* test; *, $p < 0.05$).

activity, other Isd proteins could exhibit Vn-binding activity. The presence of additional Vn receptors on the staphylococcal surface is consistent with the finding that the *isdB* mutant is capable of capturing Vn from human plasma and by the observation that other non-Isd surface proteins of *S. aureus*, such as Atl, have Vn-binding activity (26, 27). In apparent contradiction with these observations, IsdB seems to be the major Vn receptor when material released from bacterial cells was tested by far Western blotting (Fig. 2). This could be due to a higher level of expression of IsdB and/or to a higher affinity of IsdB for Vn compared with that of other potential Vn binders.

In this context, it will be worth investigating the activity of IsdB from *Staphylococcus lugdunensis*, which contains NEAT₁ and NEAT₂, 60 and 56% identical to *S. aureus* IsdB NEAT₁ and NEAT₂ domains, respectively (40), and the Vn-binding by other bacterial species expressing Isd proteins, such as *Listeria monocytogenes* and *Bacillus anthracis*.

Increasing the ionic strength had a dramatic effect on Vn binding to IsdB, indicating that Vn/IsdB complex formation is

mainly driven by electrostatic interactions. Furthermore, the negatively charged heparin efficiently blocked the IsdB–Vn interaction, suggesting that the IsdB-binding region is located in the heparin-binding domain(s) of Vn. The inhibitory effect of heparin but not of heparan sulfate or chondroitin sulfate is in line with heparin possessing the highest negative charge density of any known biological macromolecule. In fact, although heparin and heparan sulfate are built up of linear chains of repeating disaccharide units, the average heparin disaccharide contains approximately 2.7 sulfate groups, whereas heparan sulfate contains ≥ 1 sulfate group per disaccharide (54).

Related to the finding that electrostatic bonds could be involved in Vn–IsdB interaction is the question of whether Vn and heme compete for the same binding site or have a different location on IsdB. The indication that IsdB uses only a hydrophobic pocket in the NEAT₂ domain to bind and extract heme (38) and our observation that Vn binds to both NEAT₁ and NEAT₂ domains suggest that the binding sites in IsdB for heme and Vn are separate.

Binding of *S. aureus* to Vn contributes to bacterial adhesion to mammalian cells, as demonstrated by attachment of IsdB-expressing staphylococci to HeLa and HUVEC monolayers. The inhibition of the process by an $\alpha_v\beta_3$ mAb and cilengitide strongly suggests that adhesion involves the formation of a Vn bridged complex between IsdB and the integrin. Vn also mediated the internalization of staphylococci into epithelial and endothelial cells. However, due to the modest contribution of Vn to the process, it is possible that Vn-dependent invasion could act as a backup mechanism that becomes operational in the context of tissues where Fn is poorly expressed or under conditions (specific growth medium and/or phase of growth) where bacteria express reduced levels of Fn-binding proteins FnBPA and FnBPB (55, 56).

Binding to Fn by FnBPs allows the formation of a bridge between bacterial cells and the $\alpha_5\beta_1$ integrin on host cells, and this is sufficient to trigger the bacterial uptake. This mechanism is widely acknowledged to be the main internalization process (57). Additional secondary invasion mechanisms have been reported, including, among others, the internalization mediated by EAP (58), Atl (31), and Lpl (59) proteins. Of note, the invasion assays performed in these studies have been carried out with experimental approaches and conditions (different cell lines and strains, absence/presence of serum) that significantly differ from the invasion pathway described in the present investigation. Thus, for an accurate analysis of the contribution of each mechanism to this multifactorial event, individual internalization processes should be evaluated and compared under identical standardized conditions.

It is unclear why the *isdB* deletion mutant, although it can express several Vn receptors (Fig. 6), does not adhere to and invade host cells (Fig. 7). If we hypothesize that non-IsdB receptors bind to Vn in a way that differs from IsdB, we can speculate that these receptors play a role other than adhesin/invasin in bacterial pathogenesis. For example, capture of Vn mediated by receptors could inhibit both assembly and deposition of the terminal complement complex on the bacterial surface. Alternatively, due to the plasminogen (PLG) binding ability of Vn (60), receptor-bound Vn could enhance plasminogen activation to plasmin, thereby contributing to the degradation of fibrin clots and bacterial spreading into the tissues.

IsdB binds directly to β_3 -containing integrins and promotes platelet activation and invasion of mammalian cells (48, 61). Consistent with this, we found a low level of invasion of both cell lines by bacteria grown in RPMI in the absence of Vn, suggesting that under these conditions the process can be driven by IsdB binding directly to integrins (48). As with many other MSCRAMMs, IsdB is a multivalent virulence factor and contributes in various ways to the pathogenesis of *S. aureus*. Examples of MSCRAMMs that bind to two or more ligands are clumping factor A (ClfA) that, in addition to binding to fibrinogen (62, 63), binds to complement factor I (64, 65), and clumping factor B (ClfB), another CWA protein that interacts with fibrinogen (66, 67), keratin 10 (68, 69), and loricrin (70, 71). These CWA proteins share similar mechanisms of binding (5). In parallel with this, the structural basis of Hb binding to IsdB NEAT motifs has been elucidated (72, 73). From this perspective, X-ray crystal structure analysis of the recombinant IsdB-

binding domain of Vn in complex with NEAT motifs could provide clues about the mechanism and function of this important CWA protein in bacterial colonization of and survival within the host. Purified IsdB elicits antibodies that block heme iron scavenging, provide partial protection against *S. aureus* bacteremia in animal models (74–76), and promote opsonophagocytosis of *S. aureus* (75, 77). Although vaccination of humans with IsdB resulted in failure (78, 79), in light of these new findings, the use of IsdB as a vaccine component warrants further investigation.

Experimental procedures

Bacterial strains and culture conditions

All strains used in this study are listed in Table 1. *S. aureus* cells (48, 80–84) were grown overnight in BHI (VWR International Srl, Milan Italy) or in RPMI 1640 (Sigma-Aldrich, MO, USA) medium at 37 °C with shaking. *L. lactis* cells carrying the expression vector alone (pNZ8037) (85) or harboring the *isdB* gene (pNZ8037::*isdB*) (48) were grown overnight in BHI medium supplemented with chloramphenicol (10 μ g/ml) at 30 °C without shaking. Cultures of *L. lactis* were diluted 1:100 in the same medium and allowed to reach exponential phase. Nisin (6.4 ng/ml) was added, and cultures were allowed to grow overnight as described above. In experiments where a defined number of cells were used, bacteria were harvested from the cultures by centrifugation, washed, and suspended in PBS at an optical density at 600 nm (OD₆₀₀) of 1.0. *Escherichia coli* strain XL1-Blue (Agilent Technologies, CA, USA) transformed with vector pQE30 (Stratagene, La Jolla, CA) or derivatives were grown in Luria agar and Luria broth (VWR) containing 100 μ g/ml ampicillin at 37 °C with shaking.

Construction of *S. aureus isdB* deletion mutant and *L. lactis* expressing *IsdB*

Construction of *Lactococcus lactis* expressing IsdB and construction of the *S. aureus isdB* deletion mutant were performed as reported in Table 1.

Plasmid and DNA manipulation

Plasmid DNA (Table 2) was isolated using the WizardPlus SV miniprep kit (Promega, WI, USA), according to the manufacturer's instructions, and transformed into *E. coli* XL1-Blue cells using standard procedures (86). Transformants were screened by restriction analysis and verified by DNA sequencing (Eurofins Genomics, Milan, Italy). Chromosomal DNA was extracted using the bacterial genomic DNA purification kit (Edge Biosystems, MD, USA). Cloning of IsdB NEAT₁-NEAT₂ (aa residues 48–480) was performed as reported by Miajlovic *et al.* (61). Cloning of IsdB NEAT₁ (aa residues 144–270) and IsdB NEAT₂ (aa residues 334–458) domains was performed following the NEBuilder[®] HiFi DNA assembly according to the manufacturer's instructions (New England Biolabs, MA, USA). The primers used to amplify IsdB NEAT₁ and IsdB NEAT₂ domains and the pQE30 vector (Table S1) were purchased from Integrated DNA Technologies (Leuven, Belgium). DNA

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Table 1
Bacterial strains

Bacterial strain	Relevant properties ^a	Reference or source
<i>S. aureus</i>		
LAC <i>spa</i>	Strain derivative of LAC* deficient in protein A; constructed by transduction of <i>spa</i> ::Kan ^r by phage 85 into strain LAC*	80
Newman <i>spa</i>	Strain containing a null mutation in protein A obtained through transduction from <i>S. aureus</i> 8325-4 <i>spa</i> ::Kan ^r (81) by generalized phage transduction using phage 85	82
8325-4 <i>spa</i>	<i>spa</i> gene inactivated by substituting part of the <i>spa</i> coding sequence for a DNA fragment specifying resistance to ethidium bromide. <i>In vitro</i> -constructed <i>spa</i> ::EtBrr substitution mutation was introduced into the <i>S. aureus</i> chromosome by recombinational allele replacement	81
SH1000	Laboratory strain. <i>rsbL</i> + derivative of <i>S. aureus</i> 8325-4 <i>rsbL</i> + derivative of <i>S. aureus</i> 8325-4	83
SH1000 <i>spa</i>	<i>spa</i> ::Tc ^r transduced from 8325-4 <i>spa</i> ::Tc ^r	84
SH1000 <i>isdB</i>	<i>isdB</i> gene deleted by allelic exchange	48
<i>L. lactis</i>		
NZ9800 (pNZ8037)	Expression vector with nisin-inducible promoter, Cm ^r	85
NZ9800 (pNZ8037:: <i>isdB</i>)	<i>isdB</i> gene cloned in pNZ8037, Cm ^r	48
<i>E. coli</i>		
XL1-Blue	<i>E. coli</i> cloning host	Stratagene

^a Cm^r, chloramphenicol resistance; Kan^r, kanamycin resistance; Tc^r, tetracycline resistance.

purification was carried out using the WizardSV gel and PCR clean-up system (Promega).

Expression and purification of recombinant proteins

Recombinant proteins IsdB NEAT₁-NEAT₂, IsdB-NEAT₁, and IsdB-NEAT₂ were expressed from pQE30 (Qiagen, Hilden, Germany) in *E. coli* XL1-Blue (Agilent Technologies, CA, USA). Overnight starter cultures were diluted 1:40 in Luria broth containing ampicillin (100 µg/ml) and incubated with shaking until the culture reached the exponential phase (optical density at 600 nm of 0.4–0.6). Recombinant protein expression was induced by the addition of 1 mM (final concentration) isopropyl 1-thio-β-D-galactopyranoside (IPTG) (Inalco, Milan, Italy) to the culture. After 4 h, bacterial cells were harvested by centrifugation and frozen at –80 °C. Cells were resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) containing 1 mM PMSF (Sigma-Aldrich) as the protease inhibitor and 20 µg/ml DNase (Sigma-Aldrich) and lysed by freezing with liquid nitrogen and subsequent defrosting with warm water. The lysis procedure was repeated twice. The cell debris was removed by centrifugation, and proteins were purified from the supernatants by Ni²⁺-affinity chromatography on a HiTrap chelating column (GE Healthcare, Buckinghamshire, UK).

Protein purity was assessed by 15% SDS-PAGE and Coomassie brilliant blue staining (Fig. S4A). A bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) was used to measure the concentration of purified proteins.

Reagents, proteins, and antibodies

BSA, hemoglobin, protease-free DNase I, skim milk, heparin, chondroitin sulfate, heparan sulfate, cilengitide, lysostaphin, trypsin, sheep anti-Vn polyclonal IgG, and HRP-conjugated rabbit anti-sheep IgG were purchased from Sigma-Aldrich. Human fibronectin (Fn) was purified from plasma by a combination of gelatin- and arginine-Sepharose affinity chromatography (87). The human Fn rabbit antibody was purchased from Pierce. Human fibrinogen (Fbg) was obtained from Calbiochem (Darmstadt, Germany). Collagen (Coll) type I was a generous gift of Professor R. Tenni (Department of Molecular Medicine, Pavia, Italy). Coll type I rabbit antibody was from Merck

(Rome, Italy). α_vβ₃ integrin mAb LM609 was from Abcam (Cambridge, UK). Vn mAb 8E6 was purchased from Merck. IsdB and Fbg antibodies were raised in mice by a routine immunization procedure using purified IsdB NEAT₁-NEAT₂ and Fbg as the antigen, respectively. Rabbit anti-mouse HRP-conjugated secondary antibody was purchased from Dako Cytomation (Glostrup, Denmark).

Human plasma

Normal human plasma was prepared from freshly drawn blood obtained from healthy volunteers with informed consent and permission of the ethical board of the University of Pavia (permit no. 19092013). The study also abides by the Declaration of Helsinki principles. After centrifugation, the plasma fraction was frozen in aliquots and stored at –20 °C.

Isolation of Vn from human plasma

Human Vn was purified from human plasma on a heparin-Sepharose Hi-TrapTM column (GE Healthcare) by following the protocol described by Akiyama (88). The method takes advantage of the observation that the monomeric plasma Vn must be activated or “opened” with 8 M urea before it can bind well to heparin. Thus, the plasma is first depleted of compounds such as fibronectin that bind specifically or nonspecifically to heparin-Sepharose and then treated with 8 M urea to activate the heparin-binding activity of Vn, which is subsequently bound to a heparin affinity column and eluted in a multimeric conformation.

The purity of the isolated Vn was checked with 12.5% (w/v) SDS-PAGE under reducing conditions and Coomassie brilliant blue staining (Fig. S4B, lane 1) and its concentration quantified with the BCA assay. The multimeric conformation of the isolated Vn was assessed by 12.5% PAGE performed in the absence of SDS (Fig. S4B, lane 2) and by determination of the reactivity with the mAb 8E6, which preferentially recognizes the multimeric, activated form of Vn (data not shown).

Vn binding to *S. aureus*

To test binding of Vn, microtiter wells were coated overnight at 37 °C with 100 µl/well of stationary-phase *S. aureus* cells

Table 2
Plasmids

Plasmid	Feature	Marker ^a	Source or reference
pQE30	<i>E. coli</i> vector for the expression of hexa-His-tagged recombinant proteins	Amp ^r	Qiagen
pQE30:: <i>isdB</i> NEAT ₁ -NEAT ₂	pQE30 encoding residues 48–480 of IsdB NEAT ₁ -NEAT ₂ protein	Amp ^r	61
pQE30::rIsdB-NEAT1 (144–270)	pQE30 derivative encoding the NEAT1 domain of IsdB NEAT ₁ -NEAT ₂ protein from <i>S. aureus</i> SH1000	Amp ^r	This study
pQE30::rIsdB-NEAT2 (334–458)	pQE30 derivative encoding the NEAT2 domain of IsdB protein from <i>S. aureus</i> SH1000	Amp ^r	This study

^a Amp^r, ampicillin resistance.

(OD₆₀₀ of 1) in PBS. The plates were washed with PBS. To block free protein binding sites, the wells were treated for 1 h at 22 °C with BSA (2%, v/v) in PBS. The plates were then incubated for 1 h with increasing concentrations of Vn (up to 250 ng/well). After several washings with PBS, 0.5 μg of anti-Vn sheep IgG in BSA (1%, v/v) was added to the wells and incubated for 90 min.

The plates were washed and then incubated for 1 h with rabbit HRP-conjugated anti-sheep IgG diluted 1:1000. After washing, *o*-phenylenediamine dihydrochloride was added, and the absorbance at 490 nm was determined using an ELISA plate reader (Bio-Rad, CA USA).

Release of cell wall-anchored proteins from *S. aureus* and detection of Vn-binding activity

Lysostaphin digestion—To release cell wall-anchored proteins from *S. aureus*, bacteria were grown at 37 °C to exponential phase (OD₆₀₀ of 0.4–0.6) or stationary phase, either in RPMI or BHI medium. Cells were harvested by centrifugation at 7000 × *g* at 4 °C for 15 min, washed three times with PBS, and resuspended to an A₆₀₀ of 2.0 in lysis buffer (50 mM Tris-HCl, 20 mM MgCl₂, pH 7.5) supplemented with 30% raffinose. Cell wall proteins were solubilized from *S. aureus* by incubation with lysostaphin (200 μg/ml) at 37 °C for 20 min in the presence of protease inhibitors (Complete Mini; Sigma-Aldrich). Protoplasts were recovered by centrifugation at 6000 × *g* for 20 min, and the supernatants were taken as the wall fraction. The supernatant was concentrated by treatment with 20% (v/v) TCA at 4 °C for 30 min. The precipitated proteins were washed twice with ice-cold acetone and dried overnight.

SDS-PAGE and far Western blotting—Proteins released by lysostaphin digestion were boiled for 10 min in sample buffer (0.125 M Tris-HCl, 4% [w/v] SDS, 20% [v/v] glycerol, 10% [v/v] β-mercaptoethanol, 0.002% [w/v] bromophenol blue) and separated by 12.5% (w/v) SDS-PAGE. The gels were stained with Coomassie brilliant blue (Bio-Rad). For Western blotting, proteins were subjected to SDS-PAGE and electroblotted onto a nitrocellulose membrane (GE Healthcare). After blocking with 5% (w/v) skim milk (Sigma-Aldrich) in PBS overnight at 4 °C, the membrane was probed with 2 μg/ml of Vn in PBS for 1 h at 22 °C followed by anti-Vn sheep IgG (1:10,000) and with rabbit HRP-conjugated anti-sheep IgG (1:100). Finally, blots were developed using the ECL Advance Western blotting detection kit (GE Healthcare), and images of the bands were captured by an ImageQuant™ LAS 4000 mini-biomolecular imager (GE Healthcare).

MS analysis

Proteins released by lysostaphin digestion of bacterial cells were separated by 12.5% SDS-PAGE and stained with GelCode

blue stain reagent (Thermo Fisher Scientific, Waltham, MA, USA). The band marked by the *red arrow* (Fig. 2) was excised from the gel using a sterile scalpel and protein contained in the gel digested with sequencing grade trypsin (Sigma-Aldrich). Tryptic peptides were mixed with CHCA (alpha-cyano-4-hydroxycinnamic acid) and spotted onto the target plate. The spectra were acquired with a TOF/TOF 5800 system (AB SCIEX, Framingham, MT) using TOF/TOF Series Explorer acquisition software version 4.1.0. MS spectra were recorded in Reflecton positive mode (settings: fixed laser intensity, 3200 units; pulse rate, 400 Hz; total shots/spectrum, 1000; mass range (Da), 1000–4000 *m/z*). Calibration was performed using Peptide calibration Mix4 (LaserBio Laboratory, Sophia-Antipolis Cedex, France). MS-MS spectra of the most intensive MS signals were recorded in MS-MS positive mode (settings: fixed laser intensity, 3500 units; laser pulse rate, 1000 Hz; total shots/spectrum, 5000). MS-MS spectra were analyzed with ProteinPilot™ 5.0 software (AB SCIEX, Framingham, MT, USA) against the UniProtKB database updated 2 August 2016 using the following analysis parameters: (a) sample type, identification; (b) digestion, trypsin; (c) detected protein threshold [unused Protscore (Conf)], >0.05 (10%); (d) cysteine alkylation, iodoacetamide; (e) ID focus, biological modifications, amino acid substitutions; (f) FDR analysis, no; (g) taxonomy, no species.

Determination of *isdB* gene expression by qRT-PCR

Total RNA was extracted from *S. aureus* SH1000 *spa* cells during the exponential (OD₆₀₀ of 0.4–0.6) and stationary phases of growth in BHI and RPMI media. Cells were harvested and total RNA was stabilized with RNeasy Protect bacterial reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was extracted using the Quick-RNA fungal/bacterial miniprep kit (Zymo Research, CA, USA) by following the manufacturer's recommendations. DNA was removed by DNase I treatment by using the TURBO DNA-free kit (Invitrogen, CA, USA). The RNA concentration was >100 ng/μl, and the A₂₆₀/A₂₈₀ ratio was >1.8. qRT-PCR was performed with an iTaq Universal SYBR Green one-step kit (Bio-Rad) using 4 ng of RNA in 20-μl volumes carried out on three replicates, and all reactions were performed in 20-μl volumes according to the manufacturer's protocol. The cDNA was analyzed using primers relative to the *isdB* coding sequence (Table S1). The conditions for thermal cycling were 50 °C for 10 min and 95 °C for 1 min, 35–40 cycles with 95 °C for 10 s, and then 60 °C for 10–30 s, followed by a slow increase of temperature by 0.5 °C per cycle to 95 °C, with a continuous measurement of the fluorescence. 4 ng of total RNA from each sample was used in a qRT-PCR experiment performed using a CFX Connect real-

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time PCR detection system (Bio-Rad) and an iTaq Universal SYBR Green one-step kit (Bio-Rad). Expression of *isdB* was analyzed using primers *isdBF* (GCAGGCGTTTTGTCTTTACC) and *isdBR* (GCCTAGCAAACCAACACCAT). Target gene expression was normalized using the reference gene *rpoC*, which was amplified using primers SAurpoCF (CCGCAC-CATCTGGTAAGATTAT) and SAurpoCR (GCTGTATCGG-CAAGACCTTTA). No-template and no-RT controls were run for each assay, and the specificity of each amplification product was verified using dissociation curve analysis. Standard curves were generated from serial dilutions of chromosomal DNA spanning at least six orders of magnitude. All reactions proceeded with 90% to 110% efficiency. Gene expression analysis was performed using the CFX Manager software (Bio-Rad). Three technical replicates were performed for each experiment.

Determination of expression level of *IsdB* by Western immunoblotting

Material released by lysostaphin digestion of SH1000 *spa* grown either in BHI and RPMI media to both mid-exponential and stationary phases was subjected to SDS-PAGE and electroblotted onto nitrocellulose membrane, as reported above. After blocking with skim milk, the membrane was sequentially incubated with mouse *IsdB* antibody (1:5000) and HRP-conjugated rabbit anti-mouse IgG (1:10,000) and developed as described above.

Detection of the Vn-binding activity of *IsdB*-expressing *L. lactis*

Release of cell wall-anchored proteins from L. lactis transformants by mutanolysin/lysozyme digestion and far Western blotting—*L. lactis* was grown to late-exponential phase in BHI broth, washed twice in PBS, and concentrated to an A_{600} of 40 in 1 ml 26% raffinose (Sigma-Aldrich) in 20 mM Tris-HCl, pH 8, 10 mM $MgCl_2$. Cell wall-associated proteins were released by incubation at 37 °C with occasional shaking with 500 U mutanolysin/ml and 200 μ g lysozyme/ml in the presence of protease inhibitors (Complete Mini; Sigma-Aldrich). Protoplasts were removed by centrifugation at $6000 \times g$ for 20 min, and the supernatant was concentrated as reported above. The interaction of Vn with *IsdB* was detected by SDS-PAGE followed by far Western blotting as reported above.

Vn binding to L. lactis by ELISA—To investigate the binding of Vn to *L. lactis*, microtiter wells were coated overnight at 37 °C with 100 μ l/well of bacterial cell suspensions (OD_{600} of 1) in PBS. After treating with BSA, the plates were incubated with 10 μ g/well Vn. Proteins bound to the bacterial cells were detected as described above.

Binding of Vn to *IsdB* by ELISA

The ability of immobilized recombinant *IsdB* proteins to bind to soluble Vn was determined using ELISA. Microtiter wells were coated overnight at 4 °C with 0.2 μ g/well of the appropriate *IsdB* protein in 0.1 M sodium carbonate, pH 9.5. The plates were washed with 0.5% (v/v) Tween 20 in PBS (PBST) and then treated for 1 h at 22 °C with BSA (2%, v/v) in

PBS. The plates were incubated for 1 h with increasing concentrations of soluble Vn in PBS. Bound Vn was detected by sheep anti-Vn IgG followed by rabbit HRP-conjugated anti-sheep IgG.

To determine the effect of ionic strength on the Vn-*IsdB* interaction, microtiter wells coated with 200 ng of *IsdB* NEAT₁-NEAT₂ were incubated with 500 ng/well of Vn in PBS containing increasing concentrations of NaCl. Complex formation was detected by incubation of the wells with antibodies as described above.

The effect of heparin, chondroitin sulfate, and heparan sulfate on the *IsdB* NEAT₁-NEAT₂-Vn interaction was examined by incubating *IsdB* NEAT₁-NEAT₂ immobilized onto microtiter plates (0.2 μ g/well) with 0.5 μ g/well of purified Vn in the presence of increasing concentrations of heparin, chondroitin sulfate, or heparan sulfate. Vn bound to *IsdB* NEAT₁-NEAT₂ was detected with antibodies as reported above.

SPR

To estimate the affinity of the interaction between Vn and *IsdB* NEAT₁-NEAT₂ or its domain NEAT₁ or NEAT₂, surface plasmon resonance (SPR) analyses were carried out using a multicycle injection strategy on a multiple flow cell Biacore X100 instrument (GE-Healthcare). *IsdB* NEAT₁-NEAT₂, NEAT₁, or NEAT₂ was covalently immobilized on a dextran matrix CM5 sensor chip surface in three different flow cells by using a protein solution (50 μ g/ml in 50 mM sodium acetate buffer, pH 4.5) in a 1:1 dilution with *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. The excess of the active groups on the dextran matrix was blocked using 1 M ethanolamine, pH 8.5. On the fourth flow cell, the dextran matrix was treated as described above but without any ligand to provide an uncoated reference flow cell. The running buffer used was PBS containing 0.005%, v/v, Tween 20. A 2-fold linear dilution series of Vn, in running buffer, was passed over the ligand at a flow rate of 30 μ l/min, and all the sensorgrams were recorded at 22 °C. Assay channel data were subtracted from reference flow cell data. The response units at the steady state were plotted as a function of Vn concentration and fitted to the Langmuir equation to yield the K_D values.

Capture of Vn by *S. aureus* cells

Stationary *S. aureus* strain SH1000 or the *isdB* mutant (10^8 cells/ml) were mixed with 1 ml of fresh human plasma for 30 min. Bacteria were then harvested by centrifugation, washed with PBS, and treated with the extraction buffer (125 mM Tris-HCl, pH 7.0, containing 2% SDS) for 3 min at 95 °C and then centrifuged at $10,000 \times g$ for 3 min. The supernatants were subjected to SDS-PAGE under reducing conditions, and the proteins were transferred to a nitrocellulose membrane. The membrane was sequentially probed for bound Vn with antibodies as described above. The band intensities were quantified with Quantity One software (Bio-Rad).

To analyze the capture of purified Vn by *S. aureus* SH1000 and its *spa* mutant, bacteria (1×10^8 cells/ml) were grown either in BHI or RPMI medium in the absence/presence of 1 mM

FeCl₃ and then incubated with purified Vn for 60 min. Bacterium-bound proteins were released from the bacterial surface by extraction buffer and subjected to SDS-PAGE and far Western blotting as reported above.

Cell culture infection with staphylococci and blocking experiments

HeLa cells were cultured in 24-well plates until reaching confluence in DMEM supplemented with 10% FBS. HUVECs were cultured as previously reported (89).

24 h before adherence or invasion assays, the medium was removed from each culture and replaced with serum-free medium. Overnight cultures of *S. aureus* SH1000 WT or the *isdB* mutant grown either in BHI or RPMI medium were suspended in DMEM without antibiotics and FBS to 1×10^7 cells/ml and added to the monolayers for 2 h at 37 °C in 5% CO₂. The effect of Vn on bacterial adhesion/invasion to monolayers was tested by pretreating the cells with 25 μg/ml of the protein 1 h prior to addition of the bacteria. To evaluate the role of α_vβ₃ integrin antibody and cilengitide on adhesion, monolayers were pretreated with Vn and then incubated with *S. aureus* SH1000 WT in the presence of 1 μg/ml of α_vβ₃ integrin LM609 mAb or 0.05 μM cilengitide. To determine bacterial adhesion, the infected cells were washed three times with Dulbecco's PBS, lysed, and plated on BHI agar for CFU counts. To enumerate internalized bacteria, the monolayers were further incubated for 2 h before cell lysis in medium supplemented with 100 μg/ml gentamicin to kill extracellular bacteria. Bacterial adherence and invasion are represented as recovered CFU/ml.

Statistical methods

Two group comparisons were performed by Student's *t* test. One-way analysis of variance, followed by Bonferroni's post hoc tests, was exploited for comparison of three or more groups. Analyses were performed using Prism 4.0 (GraphPad). Two-tailed *P* values of 0.05 were considered statistically significant.

Data availability

The MS proteomics data are available via ProteomeXchange with identifier PXD019371 (90). All other data supporting the findings of this study are available within the article and its supporting data.

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Abbreviations—The abbreviations used are: ECM, extracellular matrix; Aaa, autolysin/adhesin from *S. aureus*; Atl, autolysin; coll, collagen; EAP, extracellular adherence protein; Fn, fibronectin; Fbg, fibrinogen; Isd, iron-regulated surface determinant; Lpl, lipoprotein-like lipoprotein; NEAT, near iron transporter; MSCRAMM, microbial surface component recognizing adhesive matrix molecule; PLG, plasminogen; RU, response unit; SPR, surface plasmon resonance; Vn, vitronectin; OD, optical density; qRT-PCR, quantitative RT-PCR; CWA, cell wall-anchored; BHI, brain heart infusion; RPMI, Roswell Park Memorial Institute; HUVEC, human umbilical vein endothelial cell.

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