Iron regulated surface determinant B (IsdB) promotes Staphylococcus aureus adherence to and internalization by non-phagocytic human cells

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Iron regulated surface determinant B (IsdB) promotes *Staphylococcus aureus* adherence to and internalization by non-phagocytic human cells.

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Summary

Staphylococcus aureus is a human pathogen that causes invasive and recurring infections. The ability to internalize into and persist within host cells is thought to contribute to infection. Here we report a novel role for the well characterized iron regulated surface determinant B (IsdB) which we have shown can promote bacterial adherence to 293T, HeLa cells and platelets independently of its ability to bind haemoglobin. IsdB bound to the active form of the platelet integrin $\alpha_{IIb}\beta_3$, both on platelets and when the integrin was expressed ectopically in CHO cells. IsdB also promoted bacterial invasion into human cells. This was clearly demonstrated with bacteria lacking fibronectin-binding proteins (FnBPs), which are known to promote invasion in the presence of fibronectin, However, IsdB also contributed significantly to invasion by cells expressing FnBPs in the presence of serum. Thus IsdB appears to be able to interact with the broader family of integrins that bind ligands with the RGD motif and to act as a back up mechanism to promote interactions with mammalian cells.

Introduction

Staphylococcus aureus is a human commensal that colonizes the moist squamous epithelium of the anterior nares. Approximately 20% of the population are persistently colonized while for the remainder the association is transient (van Belkum *et al.*, 2009). The organism is also an opportunistic pathogen that can cause many infections ranging from superficial skin lesions to invasive infections such as endocarditis, osteomyelitis and septic arthritis (Lowy, 1998).

S. aureus can express on its surface a range of proteins that promote binding to components of the extracellular matrix, notably fibrinogen (Fg), fibronectin (Fn),

elastin and collagen (Hauck *et al.*, 2006; Speziale *et al.*, 2009). Fibrinogen- and fibronectin - binding proteins can also promote interactions with host cells by using the plasma proteins to provide bridges to host cell integrin receptors ($\alpha_5\beta_1$ – Fn ; $\alpha_{IIb}\beta_3$, Fn, Fg). The integrins are heterodimers formed by non-covalent association of α and β subunits. They bind to components of the extracellular matrix and in some cases to counter-receptors on adjacent cells. They are essential for cell adhesion in tissue maintenance, embryonic development and haemostasis (Harburger *et al.*, 2009).

Until recently *S. aureus* was regarded as an extracellular pathogen. However it is now clear that the bacterium can adhere to and become internalized by a range of host cells that are not normally phagocytic. This has been demonstrated in cell culture with endothelial cells, epithelial cells, osteoblasts, fibroblasts and keratinocytes (Edwards *et al.*, 2010; Ellington *et al.*, 1999; Massey *et al.*, 2001; Sinha *et al.*, 1999; Sinha *et al.*, 2005; von Eiff *et al.*, 2001). The fibronectin-binding proteins (FnBPs) were the first bacterial proteins to be shown to promote internalization. The FnBPs bind to the N-terminal domain of Fn. The host proteins act as a bridge between the bacterial cell and the $\alpha_5\beta_1$ integrin which occurs on a wide range of host cell types. The integrin binds to the RGD-containing domain of Fn that is located towards the C-terminus. A single FnBP protein has tandemly-arrayed ligand binding domains which attract several molecules of Fn. Binding of FnBP to $\alpha_5\beta_1$ results in clustering of the integrin which triggers intracellular signalling events leading to local actin rearrangements and bacterial invasion into a phagocytic vesicle (Edwards *et al.*, 2010; Schroder *et al.*, 2006; Sinha *et al.*, 1999; Sinha *et al.*, 2005).

Other mechanisms of internalization are available to *S. aureus* since mutants that lack FnBPs are often not completely deficient in invasion. One such “back up” mechanism is provided by the autolysins Atl which can bind directly to the heat shock

cognate protein Hsc70 and indirectly to host integrins to promote uptake (Hirschhausen *et al.*, 2010).

Studies on *S. aureus* adhesion and internalization have been performed with bacterial cells grown in rich laboratory media containing iron. This contrasts with *in vivo* conditions where all iron is sequestered, approximately 95% of it in haem and haemoglobin (Otto *et al.*, 1992a; Otto *et al.*, 1992b). The lack of available iron *in vivo* inactivates the Fur repressor leading to up-regulation of a number of genes (Allard *et al.*, 2006) among which are those that encode the iron regulated surface determinant (Isd) proteins, the primary role of which is to acquire the essential nutrient iron from host haemoglobin (Mazmanian *et al.*, 2003). These include IsdA, IsdB and IsdH that are anchored to cell wall peptidoglycan by sortase A and are exposed on the cell surface. Each contains structurally conserved near iron transporter (NEAT) motif(s) that bind haemoglobin and haem (Grigg *et al.*, 2011; Grigg *et al.*, 2010; Krishna Kumar *et al.*, 2011). Extracted haem is then transported into the cytoplasm. The surface-exposed Isd proteins are multifunctional. IsdA binds to several different host proteins including cytokeratin 10, and contributes to bacterial adhesion to desquamated nasal epithelial cells and to nasal colonization (Clarke *et al.*, 2009; Clarke *et al.*, 2004; Corrigan *et al.*, 2009b). It also binds lactoferrin which helps to protect bacteria from the toxic effects of the host protein (Clarke *et al.*, 2008; Clarke *et al.*, 2007). IsdH enhances the inactivation of the complement opsonin C3b and contributes to evasion of the host's innate immune defences (Smith *et al.*, 2011; Visai *et al.*, 2009). Our earlier work showed that IsdB can promote *S. aureus* adhesion to platelets (Miajlovic *et al.*, 2010b). The interaction was blocked by specific inhibitors of the platelet integrin which implied that a direct interaction between the bacterial surface protein and the integrin was involved. The objective of the current study was

to study in more depth the interaction between IsdB and $\alpha_{IIb}\beta_3$ and to investigate if IsdB could bind to other integrins and perhaps promote bacterial internalization.

Results

IsdB promotes adhesion to human cells as well as platelets

Our previous study which showed that IsdB expressed on the surface of *S. aureus* could promote platelet adhesion and aggregation was conducted with strain Newman mutants lacking the Fg-binding proteins ClfA and ClfB, both of which can interact indirectly with the platelet integrin $\alpha_{IIb}\beta_3$ by forming Fg bridges. Newman does not express FnBP adhesins, which can also interact with platelets *via* plasma molecules bridges, because of stop codons in the 3' ends of the *fnbA* and *fnbB* genes (Grundmeier *et al.*, 2004). Here we extended the study of IsdB to strain SH1000 which expresses each of the aforementioned surface proteins. A deletion mutation in *isdB* was isolated in SH1000 and SH1000 *clfAclfBfnbAfnbB* by allele exchange using pKOR1. All strains of SH1000 and Newman used in this study were validated by Western immunoblotting and bacterial adhesion assays to immobilized ligands following growth in the iron-limited medium RPMI (Fig. 1). Cell wall-anchored proteins were solubilized with lysostaphin and analyzed by Western immunoblotting probing with anti-IsdB serum. This showed that Newman and SH1000 expressed IsdB at similar levels and that the protein was absent in the *isdB* mutants. IsdA was expressed at the same level in all strains (Fig. 1A). SH1000 strains were probed for expression of FnBPs by Fn-affinity blotting with biotinylated Fn which showed they were expressed at similar levels by SH1000 and SH1000 *isdB* (Fig. 1A). Both Newman and SH1000 adhered dose-dependently and saturably with similar profiles to immobilized Fg while mutants lacking the Fg - binding adhesins (Newman *clfAclfB*;

SH1000 *clfAclfBfnbAfnbB*) did not (Fig. 1B). SH1000 adhered to immobilized Fn but Newman (like SH1000 *clfAclfBfnbAfnbB*) did not adhere because of the defects in its *fnb* genes (Fig. 1C). RPMI-grown Newman *isdB* and SH1000 *isdB* bound to Fn and Fg at levels similar to those of the wild-type strains indicating that IsdB expression does not contribute detectably binding to these ligands (Fig. 1BC).

We hypothesized that IsdB might promote interactions with other human cells as well as platelets. Bacterial strains were grown in RPMI and immobilized in microtitre dishes. Washed human platelets, 293T cells and HeLa cells were added to the wells and adherent cells were estimated by measuring intracellular acid phosphatase. BSA and the extracellular matrix proteins Fn and Fg served as controls (Fig. 2ABC). Platelets bound to Fg- but not to Fn-coated wells (Fig. 2A). In contrast HeLa and 293T cells bound to Fn-coated wells, but not to those with Fg (Fig. 2BC), most likely employing Fn-binding integrins. We confirmed our previous finding that platelets adhered to *S. aureus* Newman cells in an IsdB-dependent manner (Fig. 2A). This involves the platelet integrin $\alpha_{IIb}\beta_3$. Newman and SH1000 bound to 293T and HeLa cells unlike the *isdB* mutant, which implies that IsdB can interact with these cells in similar manner, perhaps also using integrins (Fig. 2BC). *S. aureus* SH1000 and SH1000 *clfAclfBfnbAfnbB* bound to the 293T and HeLa cells at similar levels. Binding of mammalian cells to the *isdB* mutants was significantly reduced. It should be pointed out that these experiments were performed in the absence of serum and hence Fn which would otherwise promote binding *via* FnBPs. Addition of the RGD peptide reduced the level of 293T and HeLa cell binding to *S. aureus* Newman to the same level as the *isdB* mutant (Fig. 3AB) which suggests that IsdB can interact with "RGD" integrins such as $\alpha_5\beta_1$. Addition of RGD did not inhibit the binding to *isdB*

mutant of strain Newman suggesting that the residual adherence does not involve the integrins.

IsdB binds to the high affinity form of integrin $\alpha_{IIb}\beta_3$

Our previous studies indicated that *S. aureus* cells can bind to platelets by a direct interaction between IsdB and the platelet integrin (Miajlovic *et al.*, 2010b). The integrin undergoes complex conformational changes from the bent (low affinity) conformation on resting platelets to the extended (high affinity) state on activated platelets (Ma *et al.*, 2007). To identify the conformation required for binding to IsdB we expressed and purified a recombinant form of IsdB with a 6 x His tag. The purity and integrity of the protein was indicated by the single band in SDS-PAGE and by the ability of the protein to bind to immobilized haemoglobin dose-dependently and saturably (Fig. S1). We then studied the protein's ability to bind to platelets prior to and after activation with Thrombin Receptor Activating Peptide (TRAP) which converts the $\alpha_{IIb}\beta_3$ integrin to the high affinity conformation (Coutinho *et al.*, 2007). Fluorescently labelled recombinant IsdB bound at a two-fold higher level to the activated platelets than the untreated washed platelets, indicating its preference for the high affinity conformation of the integrin (Fig. 4AB). There was no binding of platelets to fluorescently labelled recombinant SasG, a surface protein of *S. aureus* which was used as a control (data not shown).

To confirm that IsdB interacts with the high affinity conformation of the platelet integrin $\alpha_{IIb}\beta_3$ we used a CHO-K1 cell line stably expressing human wild-type $\alpha_{IIb}\beta_3$ and the N305T mutant locked in the high affinity conformation by an N-glycosylation site introduced into the interface between the β3 I-like and hybrid domains (Luo *et al.*, 2003; Luo *et al.*, 2004). Fibrinogen, IsdB and BSA were

immobilized in microtiter dishes and cell attachment to and spreading on immobilized ligands was analysed using the xCELLigence system. This instrument performs quantitative real-time measurements of impedance in microtiter wells containing electrodes. The baseline impedance is measured in the absence of the cells. When cells stick to the electrode surface they act as isolators inducing an increase in the electrical impedance. If more cells attach or if cells spread there will be an increase in the impedance which corresponds to higher Cell Index (CI) values. Cells expressing the wild-type $\alpha_{IIb}\beta_3$ integrin in the presence of 2 mM Ca^{2+}, which stabilizes the low affinity conformation of the integrin, did not attach to or spread on surfaces coated with IsdB, fibrinogen or BSA (Fig. 5A). Cells expressing the high affinity form of $\alpha_{IIb}\beta_3$ (N305T) attached to fibrinogen and IsdB but not BSA (Fig. 5B). Consistent with previous observations, this result indicates that IsdB interacts with the high affinity form of $\alpha_{IIb}\beta_3$. The attachment to and spreading of the high affinity mutant was stronger on surfaces coated with fibrinogen than with IsdB suggesting a higher affinity of the integrin for the plasma protein than IsdB (Fig. 5B).

We also compared the attachment of the CHO-K1 $\alpha_{IIb}\beta_3$ (N305T) mutant to recombinant IsdH and IsdA proteins. The cells attached strongly to IsdB, weakly to IsdA and there was no binding to IsdH (Fig. 5C). The attachment to and spreading on IsdB was more than 2.5 fold greater than on IsdA (Fig. 5C). This is consistent with IsdB located on the bacterial cell surface providing more specific attachment for the high affinity $\alpha_{IIb}\beta_3$ than other Isd proteins. The possible contribution of IsdA to the adhesion of *S. aureus* to human cells was investigated. Iron-starved *S. aureus* Newman *isdA* was bound by platelets, 293T and HeLa cells at levels similar to the wild-type Newman (data not shown) indicating that the contribution of IsdA to adhesion is not significant.

IsdB expression contributes to S. aureus invasion

Adherence of *S. aureus* to host cells is a prerequisite for invasion. *S. aureus* uses fibronectin-binding proteins A (FnBPA) and B (FnBPB) to bind to fibronectin which attaches to epithelial and endothelial cells by the β1 integrins, such as $\alpha_5\beta_1$. This bridging of bacteria and host cell integrins triggers bacterial uptake by host cell driven processes involving actin remodelling, focal adhesion kinase and Src family kinases (Agerer *et al.*, 2005; Sinha *et al.*, 2005).

As shown above, iron-starved *S. aureus* Newman can adhere to human cells in an IsdB-dependent fashion (Fig. 2ABC). To evaluate internalization of *S. aureus* Newman into human host cells we performed gentamicin protection assays. Initially, strains Newman wild-type and Newman *isdB* grown to stationary phase in RPMI were added to 293T or HeLa cell monolayers. The invasion was performed in an iron-restricted medium lacking serum. Approximately 15% of the *S. aureus* Newman inoculum was able to invade the cells (Fig. 6AB) whereas only 7% of Newman *isdB* was recovered (Fig. 6A). This indicates that IsdB promotes invasion of *S. aureus* into cells in the absence of Fn. Electron microscopy confirmed that S. *aureus* Newman and SH1000 *clfAclfBfnbAfnbB* had successfully invaded the 293T cells in the absence of serum (Fig. 6B and 7B). No disruption of the kidney embryo cells was seen upon the bacterial uptake. Internalized bacteria resided in the cytoplasm, sometimes surrounded by lysosomal membranes. Many dividing cells were seen suggesting active intracellular growth (Fig. 6B and 7B).

Most if not all *S. aureus* strains express FnBPs which provide a potent mechanism for promoting invasion when serum (and hence Fn) is present (Edwards *et al.*, 2010). Thus we sought to determine if IsdB contributes to invasion of wild-type FnBP-expressing bacteria in the presence of Fn. Firstly, in the absence of serum both

SH1000 and SH1000 *clfAclfBfnbAfnbB* expressing IsdB, were found to invade at similar levels (43% and 40% of input inocula, respectively) while SH1000 *isdB* and SH1000 *clfAclfBfnbAfnbBisdB* were significantly less efficient at approximately 24% of the input inocula (Fig. 7A). In the presence of serum 93% of the SH1000 wild-type inoculum was taken up whereas the SH1000 *clfAclfBfnbAfnbB* mutant was much less effective at ca. 46% which was similar to SH1000 wild-type in the absence of Fn. This demonstrates that FnBPs and Fn are required for the highest levels of invasion. Comparing SH1000 to SH1000 *isdB* indicated a small but statistically significant reduction in invasion when IsdB was missing (80% uptake compared to 93%). SH1000 *clfAclfBfnbAfnbBisdB* was reduced to 27% compared to SH1000 *clfAclfBfnbAfnbB* (46%) (Fig. 7A). These results show that IsdB promotes invasion by SH1000 both in the absence of and the presence of functional FnBPs and serum fibronectin.

Invasion was also performed with staphylococcal strains that had been grown in iron replete TSB, conditions that repress expression of Isd proteins. Invasion of human cells by *S. aureus* Cowan and *S. carnosus* has been studied previously (Hirschhausen *et al.*, 2010) and these strains were used as controls. Bacteria were incubated with monolayers of 293T cells that had been grown in DMEM supplemented with serum. One hundred % of the *S. aureus* Cowan input inoculum became internalized whereas *S. carnosus* did not invade detectably (Fig. 8). *S. aureus* SH1000 wild type and SH1000 *isdB* invaded at very similar levels (ca. 80% of input inocula) (Fig. 8) indicating that invasion attributed to IsdB expression reported above is indeed dependent on iron starvation and hence induction of IsdB.

Complementation of SH1000 isdB

In order to complement the *ΔisdB* mutant in SH1000, the wild-type *isdB* gene was amplified and cloned into the anydrotetracycline (aTc)-inducible expression vector pRMC2 (Corrigan *et al.*, 2009a). Both the pRMC2*isdB* and the empty vector were transformed into SH1000 *isdB*. Expression of IsdB was verified by Western immunoblotting of proteins solubilised from the bacterial cell wall of cells that had been grown in aTc (Fig. 9A).

To determine if IsdB - mediated invasion could be restored in the *isdB* mutant expression of IsdB was induced in *S. aureus* SH1000 *isdB* (pRMC2*isdB*) and the ability of bacteria to become internalized by 293T cells was measured and compared to SH1000 wild-type, SH1000 *isdB* and SH1000 *isdB* (pRMC2). *S. aureus* wild-type was internalized at 9% of the inoculum, in contrast to SH1000 *isdB* where ca. 3.5 % was taken up. The presence of pRMC2*isdB* restored the level of invasion of *S. aureus isdB* to that of the wild-type while in *S. aureus* SH1000 *isdB* (pRMC2) invasion occurred at approximately 3% of the input inoculum, a similar level as the *isdB* mutant (Fig. 9B). It should be noted that the lower levels of invasion compared to those in Figure 7 are the result of anhydrotetracycline present in the infection medium. The results demonstrated that IsdB - mediated invasion can be restored in *S. aureus ΔisdB* by expression of IsdB from the inducible plasmid.

We then investigated if expression of IsdB in a heterologous host is sufficient to enable internalization. IsdB was expressed from the nisin-inducible plasmid pNZ8048 in *L. lactis* NZ9000 (Fig. S2). *L. lactis* NZ9000 carrying the empty vector pNZ8048 and the plasmid free *L. lactis* NZ9000 host strain were used as controls. No invasion was observed by any of the strains (data not shown) indicating that expression of IsdB alone is not sufficient to promote bacterial internalization.

IsdB residues involved in binding to haemoglobin

IsdB is the major haemoglobin (Hb) receptor of *S. aureus*. It uses a conserved mechanism to capture Hb that involves the aromatic loop (β1-β2) on the N-terminal NEAT domain (Krishna Kumar *et al.*, 2011). Residues critical for coordinating capture of the αHb subunit are located in that loop. Three of these residues were substituted (Y165A H166E Y167A) both in the recombinant IsdB protein expressed from pQE30 and also in the chromosomally-expressed Isd protein (after mutations were introduced into the *isdB* gene of Newman *clfAclfB* by allelic exchange). Recombinant IsdB (Y165A H166E Y167A) did not bind to human Hb compared to the wild-type protein which bound dose-dependently and saturably with a half maximum binding concentration of 30 nM (Fig. S1). The Newman *clfAclfBisdB* (Y165A H166E Y167A) mutant both adhered to and invaded 293T cells at the same levels as the parental (IsdB wild-type) strain (Fig. 10A and 10B) indicating that residues involved in Hb binding are not involved in the interaction with human cells.

IsdB promotes the adhesion of MCF-7 cells to Fn and Vn

We monitored the attachment of breast cancer MCF-7 cells to immobilized integrin ligands (fibronectin or vitronectin) or BSA after addition of recombinant IsdB, RGD peptide or BSA. The real-time measurement was performed using the xCELLligence as described above. Microtiter wells were coated with ligands, blocked with BSA and equal numbers of MCF-7 cells (2×10^4) were added to each well. The MCF-7 cells bound strongly and spread on fibronectin and vitronectin as indicated by the increasing cell index (CI) values. In contrast only small CI increase was seen in wells coated with BSA (Fig. 11, data not shown). Addition of soluble BSA did not influence the binding of MCF-7 cell to immobilized ligands (Fig. 11ABC). Upon

addition of the RGD peptide inhibition of the attachment to vitronectin and fibronectin was observed (Fig. 11ABD) indicating that the peptide bound to integrins and obstructed the binding of the cells onto the immobilized integrin ligands. In contrast when recombinant IsdB (at 5 μM) was added an increase in adhesion and spreading of the cells on the immobilized fibronectin and vitronectin was observed. This effect was not seen in wells coated with BSA, suggesting that IsdB specifically modulated the activity of integrins that bind to fibronectin and vitronectin (Fig. 11). Neither IsdA nor IsdH had a similar effect (Fig. S3). These results indicate that IsdB promotes the binding of cells to ligands by potentiating the activity of integrins.

Discussion

Studies using various cell culture models demonstrated that *Staphylococcus aureus* can successfully invade cells that are not normally phagocytic and can persist within the cells without causing damage (Garzoni *et al.*, 2009). The first mechanism of *S. aureus* invasion to be described involves the use of fibronectin-binding proteins and plasma Fn to provide a bridge to host cell integrins. This mechanism is sufficient to trigger bacterial uptake as demonstrated *in vitro* and *in vivo* by *L. lactis* expressing FnBPA which invades endothelial cells both in culture and in the endothelium lining heart valves during endocarditis infection (Que *et al.*, 2005). However this mechanism is clearly not the sole means by which S. *aureus* invades host cells. For instance in the mouse mastitis model an *S. aureus fnbAfnbB* mutant was still able to invade mammary gland epithelial cells, indicating that uptake could be mediated by other factors (Brouillette *et al.*, 2003) possibly Eap or Atl (Haggar *et al.*, 2003; Hirschhausen *et al.*, 2010).

Here we have identified a role for the *S. aureus* IsdB protein in promoting adhesion to and internalization by non-professional phagocytes. IsdB is a member of a group of *S. aureus* proteins that are up-regulated under iron-restricted conditions that pertain during growth *in vivo* and which may contribute to the ability of this pathogen to infect mammalian hosts (Allard *et al.*, 2006).

The IsdB protein has been previously shown to mediate *S. aureus* adhesion to platelets by interacting with the platelet integrin $\alpha_{IIb}\beta_3$ (Miajlovic *et al.*, 2010b). Here we have demonstrated that the interaction requires the high affinity conformation of the integrin. Recombinant IsdB bound to TRAP-activated platelets where the surface-located integrin has been converted to the high affinity state. Furthermore, CHO cells expressing a mutant of $\alpha_{IIb}\beta_3$ (N305T) which is locked in the high affinity conformation (Luo *et al.*, 2003) bound to immobilized rIsdB and to fibrinogen, whereas cells expressing wild-type $\alpha_{IIb}\beta_3$ did not adhere. Adhesion of washed platelets to immobilized *S. aureus* expressing IsdB (Miajlovic *et al.*, 2010b) seemed to occur at a higher level than to immobilized fibrinogen. This method does not differentiate between the affinity of interacting components and their avidity and therefore may misrepresent the 'strength' of the interaction. To analyze binding in more detail we performed quantitative real-time measurements of the binding of CHO cells expressing $\alpha_{IIb}\beta_3$ N305T to rIsdB and fibrinogen. The xCELLigence method allowed us to compare the strength of the binding of cells to the ligands which indicated a higher affinity for the fibrinogen than IsdB.

Expression of IsdB promoted adhesion of *S. aureus* to the human kidney embryo 293T cells and breast cancer epithelial HeLa cells. These cells adhered to iron-starved *S. aureus* Newman and SH1000 better than to the *isdB* deficient mutants. We initially studied adhesion in the absence of fibrinogen and fibronectin which

eliminated any contribution from FnBPs or Clf proteins, both of which require host proteins to act as bridging ligands. Adhesion could be inhibited by the RGD peptide which binds at the interface between the α and β subunits of 'RGD integrins' suggesting that IsdB could interact with integrins other than $\alpha_{IIb}\beta_3$. This group of integrins are promiscuous receptors, with the β3 subunit involved in binding to a large number of extracellular matrix and soluble ligands in plasma. The family comprises five α_V integrins, two β1 integrins (α_5, α_8) and the platelet integrin $\alpha_{IIb}\beta_3$. They have some ligands in common but differ in affinity due to differences in the ability of the RGD domain to bind to the different α-β pockets (Harburger *et al.*, 2009).

The expression of IsdB also promotes invasion of *S. aureus* into 293T and HeLa cells. Enhanced levels of invasion by *S. aureus* Newman and SH1000 grown under iron limited conditions occurred both in the presence of and absence of FnBPs or plasma fibronectin, suggesting that IsdB may provide a back-up mechanism of uptake. It is noteworthy that IsdB contributed significantly to *S. aureus* internalization even when fibronectin and FnBPs were present. The contribution of IsdB to invasion is dependent on the conditions of bacterial growth and infection, because when cultured in iron-containing media *S. aureus* SH1000 and SH1000 *isdB* were internalized at equal levels.

IsdB and IsdH are both receptors for haemoglobin. Purified recombinant IsdB and IsdH proteins bind to human haemoglobin with similar affinities in the low nanomolar range (Dryla *et al.*, 2007; Pishchany *et al.*, 2010). However, IsdB was shown to be more important in binding to human haemoglobin by *S. aureus* cells and is therefore considered to be the major haemoglobin receptor (Torres *et al.*, 2006). IsdB was also found to bind preferentially to human haemoglobin rather than mouse haemoglobin (Pishchany *et al.*, 2010). Despite this specificity towards human

haemoglobin, several studies have demonstrated that IsdB is an important virulence factor that promotes sepsis and abscess formation in murine models of infection (Cheng *et al.*, 2009; Kim *et al.*, 2010; Torres *et al.*, 2006). In contrast, IsdH did not contribute to virulence in the same models (Kim *et al.*, 2010). Perhaps the role of IsdB in virulence could be indicative of another role for the protein. The ability to bind to haemoglobin and to mediate bacterial adherence to and invasion of cells do not seem related. *S. aureus* Newman *clfAclfB* expressing a mutant form of IsdB (Y165A H166E Y167A) that is unable to bind to haemoglobin could still adhere to and be internalized by the 293T cells to the same extent as the isogenic strain expressing wild-type IsdB. This alteration to IsdB might be used to differentiate between these two functions of the protein during *in vivo* studies to analyze its contribution to *S. aureus* virulence.

Interestingly, when added to a suspension of human epithelial cells, recombinant IsdB promoted binding to the integrin ligands fibronectin and vitronectin. This suggests that soluble IsdB may interact with the integrins to enhance or stabilize their binding with RGD-containing ligands. We speculate that there may be a connection between this phenomenon and the ability of surface-bound IsdB to promote fibronectin-dependent invasion by *S. aureus,* particularly because expression of IsdB was shown to increase *S. aureus* invasion in the presence of FnBPs and fibronectin. The conformation of the receptors changes to the active form during seeding and upon their contact of the cells with their RGD ligands. IsdB has a lower affinity for integrins than their natural ligands and only binds to the active form. We propose that soluble rIsdB binds to and stabilizes the active conformation of integrins. The interaction is weak and the integrins remain in the active conformation transiently allowing them to adhere more efficiently to the immobilized ligands. However,

multiple copies of the low affinity IsdB protein attached to the bacterial cell surface cooperate to promote adhesion to and invasion of bacteria into host cells.

IsdB-mediated invasion was restored in the SH1000 *isdB* mutant when IsdB was expressed from an inducible plasmid which confirms that the phenotype is IsdB-dependent. In contrast, expression of IsdB in the surrogate host *L. lactis* did not promote internalization by 293T cells suggesting that the IsdB is not sufficient and requires other factors. It is possible that other factors expressed by *S. aureus* could trigger a conformational change in the integrin or IsdB may be part of a protein complex on the cell surface that enhances integrin binding.

In summary this study has confirmed our previous report that IsdB binds to platelets by engaging the integrin $\alpha_{IIb}\beta_3$ and has shown that that active conformation of the integrin is required. Furthermore IsdB binds to human cells expressing other RGD integrins and can promote bacterial attachment and invasion. IsdB-mediated adherence and invasion occurs independently of haemoglobin binding. These studies help explain the important contribution of IsdB to virulence of *S. aureus* and underpin its use as a vaccine candidate antigen.

Experimental procedures

Growth conditions, chemicals, bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were grown in RPMI-1640 medium (Sigma) (Moore *et al.*, 1967) to create iron-restricted conditions (Yuan *et al.*, 1995). Bacteria were pre-cultured in 10 ml of RPMI medium overnight and subsequently diluted 1 : 50 into 20 ml of RPMI and grown to stationary phase (for 16 h) achieving an OD_{600} ~1. Stationary phase *S. aureus* grown in RPMI expresses FnBPs and clumping factors unlike the bacterium

grown in rich laboratory media where FnBPs and ClfB are missing (Miajlovic *et al.*, 2010a). To create iron-rich conditions bacteria were grown in tryptic soya broth (TSB) overnight. *E. coli* strains XL - 1 Blue and DC10B used for cloning plasmids were grown in Luria - Bertani (LB) broth. *E. coli* TOPP10 used for protein expression was grown in LB broth. *L. lactis* was grown in M17 medium supplemented with 0.5% glucose (GM17). All reagents were from sigma unless stated otherwise. The following antibiotics were added to the media as required: ampicillin (Amp) at 100 μg ml $^{-1}$, chloramphenicol (Chm) at 10 μg ml $^{-1}$, tetracycline (Tet) at 2 μg ml $^{-1}$, erythromycin (Erm) at 20 μg ml $^{-1}$.

Cell culture

The human epithelial cervical cancer (HeLa), the human embryo kidney 293T and the Chinese hamster ovary (CHO-K1) cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS; Lonza). Media for cells stably expressing the $\alpha_{IIb}\beta_3$ integrin variants were additionally supplemented with 1 mg ml $^{-1}$ of geniticin sulphate (G418). The human breast adenocarcinoma (MCF-7) cell line was cultured in MEM/F12 (1:1) medium supplemented with 12.5 mM HEPES, 2 mM glutamine (Lonza), 5% FBS, 1 μg ml $^{-1}$ insulin, 0.5 μg ml $^{-1}$ hydrocortisone and 50 ng ml $^{-1}$ epidermal growth factor. Cells were split once every 2-3 days using Trypsin-EDTA.

Preparation of washed platelets

Platelets were isolated from human blood as described previously (Miajlovic *et al.*, 2010b).

Construction of ΔisdB

DNA fragments comprising sequences located 500bp upstream from the start codon of *isdB* gene and 500bp downstream from the stop codon were amplified from genomic DNA of *S. aureus* SH1000 and cloned into pKOR1 (Bae *et al.*, 2006; Monk *et al.*, 2012). Plasmids were electroporated into *S. aureus* SH1000 *and S. aureus* SH1000 *clfAclfBfnbAfnbB* (Lofblom *et al.*, 2007). Allelic exchange was performed by homologous recombination initiated by switching the bacterial growth temperature from that optimal for plasmid replication (28^{o}C) to the non-permissive temperature of 43^{o}C. The detailed protocol was described previously (Bae *et al.*, 2006; Monk *et al.*, 2012).

IsdB (Y165A/H166E/Y167A)

Mutations creating the amino acid substitutions were introduced in pQE30*isdB* and pKOR1*isdB* by sequential primer overlap mutagenesis with Phusion High-Fidelity Polymerase. Primers used for each step are listed as 1-6 in Supplementary Table 1. Complementary forward and reverse primers incorporating a mutation were extended by PCR to produce mutated plasmids. PCR products were digested with *Dpn*I for 1.5 h at 37°C to eliminate methylated parental DNA and then transformed into *E. coli* strain XL-1 Blue. Chimaeric plasmids were validated by PCR and sequencing.

pKOR1*isdB* (Y165A H166E Y167A) was employed to introduce the amino acid substitutions into the chromosomally expressed IsdB by alleleic exchange (Bae *et al.*, 2006).

Inducible expression of IsdB

The *isdB* gene was amplified and cloned into pRMC2 using primers 13-14 in Supplementary Table 1. Plasmids were cloned in *E. coli* DC10B and subsequently electroporated into *S. aureus* SH1000 *isdB*. pRMC2*isdB* allows anhydrotetracycline (aTc) inducible expression of the full length IsdB protein in *S. aureus*. To induce expression aTc (1 μg ml^{-1}) was added to exponentially growing cultures.

Expression of IsdB by *Lactococcus lactis* was induced by adding nisin (300 μg ml^{-1}) to exponentially growing cultures of *L. lactis* NZ9000 (pNZ8048 *isdB*) and incubating for 16 h.

Western immunoblotting

Expression of cell wall-associated proteins was measured in *S. aureus* strains growing in RPMI for 18 h at 37°C. The cultures were harvested by centrifugation (4,000 × g, 10 min), washed in phosphate buffer saline (PBS) and adjusted to an OD_{600} of 10. Cell wall peptidoglycan was solubilised by lysostaphin digestion (Miajlovic *et al.*, 2010b). Cell wall fractions were separated by SDS-PAGE and electroblotted onto PVDF membranes (Roche) for 1 h at 100 V using a wet transfer cell (Bio-Rad). Membranes were incubated for 1 h at 37°C, or overnight at 4°C in TS buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 10 % skimmed milk (Marvel). Rabbit anti-IsdB serum (Miajlovic *et al.*, 2010b), rabbit anti-IsdA (Miajlovic *et al.*, 2010b) serum or biotinylated fibronectin were diluted in 10% Marvel TS and incubated with the membranes for 1 h or 2 h, at room temperature with shaking. Unbound antibodies or fibronectin were removed by washing three times for 15 min with TS buffer. The HRP - conjugated secondary reagents (goat anti-rabbit IgG or streptavidin) were then incubated with the membranes for 1 h at room

temperature with shaking. Unbound secondary reagent was removed by washing three times with TS buffer and developed with chemiluminescent substrate (LumiGlo; New England Biolabs) and visualised using an ImageQuant LAS imager (GE Healthcare).

Purification of recombinant proteins IsdB, IsdH and IsdA proteins

His-tagged IsdB, IsdB (Y165A/H166E/Y167A), IsdA and GST-tagged IsdH proteins were purified by nickel - affinity chromatography using HisTrap HP (GE Healthcare) columns or glutathione - affinity chromatography using GSTrap FF columns (GE Healthcare) as described previously (O'Connell *et al.*, 1998). Proteins were dialysed against PBS, concentrated by ultrafiltration (Amicon Ultra, EMD Millipore) and stored at -70^{o}C. Protein concentrations were estimated by BCA Protein Assay Reagent (Thermo Scientific Pierce).

Binding of fluorescently labelled recombinant proteins to platelets

Fluorescein isothiocyanate (FITC) labelling of the recombinant proteins was performed using FluoReporter FITC Protein Labeling Kit (Invitrogen) according the manufacturer's instructions. To activate platelets Thrombin Receptor Activating Peptide (TRAP) at 10 μM was added to a platelet suspension immediately before the experiment. 2 x 10^6 of resting or TRAP - activated platelets were incubated with equimolar amounts of proteins (200 nM) for 30 minutes at 37^{o}C. Bound IsdB was detected by flow cytometry.

Bacterial adherence to immobilized ligands

Microtiter plates were coated with doubling dilutions of fibrinogen or fibronectin in PBS overnight at 4 ^{o}C. Control wells contained 5 % BSA in PBS. After

washing with PBS the plates were blocked for 2 h at 37°C with 5 % (w/v) BSA in PBS. After washing three times, 100 µl of a bacterial suspension (OD_{600} of 1 in PBS) was added to each well, and the plates were incubated for 2 h at 37 °C. After washing three times, adherent cells were fixed by adding 100 µl of 25 % formaldehyde and incubating at room temperature for at least 20 min. The plates were then washed and stained with crystal violet for 2 min. After three subsequent washes with PBS 100 µl of 5 % (v/v) acetic acid was added to the wells to solubilise the crystal violet. Absorbances were read in Multiscan ELISA plate reader at 570 nm.

Cells adhesion assays

Adherence of human cells to immobilized bacteria was performed as described previously (Claro *et al.*, 2011; Miajlovic *et al.*, 2010b). Control experiments were performed to confirm that all bacterial used could be immobilized in the microtiter wells at the same level (data not shown).

Bacterial cells were grown in RPMI, pelleted by centrifugation, washed in PBS and resuspended to an OD_{600} of 1. Wells of NUNC microtitre plates were coated with 100 µl of bacteria in triplicate and plates were incubated for 2 h at 37°C. Controls included 1% BSA, fibrinogen and fibronectin at 50 µg ml $^{-1}$ in PBS. Following incubation microtitre wells were washed 3 times with PBS. 1% BSA (100 µl) was added to wells and plates were incubated for 2 h at 37°C. Wells were washed three times with 100 µl of JNL (platelets) or PBS (293T and HeLa cells) buffer. Washed platelets (50 µl), 293T cells or HeLa cells (100 µl) at 2 x 10^6 per ml in JNL (platelets) or PBS (293T or HeLa cells) supplemented with 1.8 mM $CaCl_2$ were added to wells. Plates were incubated at 37°C for 40 min and washed three times with JNL (platelets) or PBS (293T or HeLa cells) buffer containing $CaCl_2$. Adherent platelets

were detected by using a lysis buffer containing a substrate for acid phospatase (100 mM Na acetate, 0.1 % (v/v) Triton-X-100, 10 mM *p*-nitrophenol phosphate. Plates were incubated in the dark for 2 h at 37°C. Sodium hydroxide (20 µl of 1 M) was added to each well to stop the reaction and the absorbance at 405 nm was read in an ELISA plate reader (Labsystems).

Cell invasion assays

HeLa or 293T cells were seeded into 6-well invasion plates at 1.5 x 10^6 cells per well in DMEM with 10% FBS 24 h prior to the experiment and incubated at 37^{o}C in 5 % CO_2. Confluent monolayers that formed overnight were washed twice before the experiment with 2 ml of RPMI with or without 10 % FBS serum (as stated). Bacteria were grown for 18 h in RPMI or differently if stated, harvested by centrifugation (4,000 × g, 10 min), washed with RPMI and diluted in RPMI to the concentration of 6 x 10^7 cfu per ml. The infection step was performed by adding 1 ml of the bacterial suspension to each well and incubating at 37^{o}C in 5 % CO_2 for 2 h. After 2 h infection bacterial suspensions were removed from each well of the invasion plate and 2 ml of killing medium (DMEM 10 % FBS supplemented with 100 µg ml^{-1} gentamicin and 20 µg ml^{-1} of lysostaphin) was added to each well and incubated for 1 h at 37 ^{o}C in 5 % CO_2. After 1 h the killing medium was removed and each well was washed with 2 ml of PBS. 1 ml of lysing solution (1 % TritonX-100, 0.1 % SDS in PBS) was added and pipetted up and down for 10 min before plating on agar to estimate the viable count of intracellular bacteria. The percentage internalization represents the ratio of intracellular bacteria compared to the inoculum. The inoculum was also incubated at 37 ^{o}C in 5 % CO_2 for 2 h before being plated on agar.

Real-time cell adhesion and spreading analysis

The xCELLigence system was used according to manufacturer's instructions. The following system components were used: a Real-Time Cell Analyzer (RTCA) computer with integrated software, a station for the RTCA plates and disposable E-plates. E-plates were inserted into RTCA stations and remained in a standard tissue culture incubator. The E-plates are microtiter plates with glass bottoms containing capillary gold electrodes covering each well. They allow sensitive detection of the attachment of cells with relatively uniform distribution of the electric field. The voltage applied on electrodes during RTCA measurements was approximately 20 mV. The impedance was measured at three different frequencies (10, 25 or 50 kHz). The baseline was measured after buffer was added to wells in the absence of cells. Subsequently cells were added to the wells and may become attached causing an increase in the electrical impedance. The proliferation or spreading of the cells results in further increases of the impedance. The xCELLigence calculates these changes as dimensionless values called Cell Index (CI).

Cell attachment and spreading on immobilized ligands was performed as follows. 100 µl of soluble ligands in PBS were immobilized in E-plate wells at 37^{o}C for 2 h. The wells were washed three times with PBS and blocked with 100 µl of 3 % BSA in PBS solution for 2 h at 37^{o}C. Wells were washed three times with PBS and 100 µl of PBS (CHO-K1 cells) or medium (MCF-7 cells) was added to each well and the baseline impedance was measured. Various cell lines were being prepared in the meantime. Detached cells were washed in PBS and adjusted to 2 x 10^5 cells per ml in PBS with 2 mM $CaCl_2$ (CHO-K1 cells) or medium supplemented with recombinant proteins, IsdB at 5 µM, RGD 20 µM or BSA at 3 %. After the baseline was measured, 100 µl of cell suspensions were added to wells and measurement continued over time.

The xCELLigence software was used to obtain the average of measurements performed in triplicates, the traces of impedance versus time and the slope changes (slope/h).

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Figure legends

Fig. 1. Characterization of *S. aureus* Newman and SH1000 mutants. The strains indicated were grown to stationary phase in RPMI. **(A)** Cell wall proteins were solubilised by lysostaphin digestion, separated by SDS-PAGE and analyzed by Western immunoblotting using rabbit anti-IsdB, rabbit anti-IsdA or biotinylated fibronectin as primary detectors and HRP - protein A or HRP – streptavidin respectively, as secondary detectors. **(BC)** Bacterial adherence was performed in microtiter plates coated with various concentrations of either **(B)** fibrinogen or **(C)** fibronectin and incubated with RPMI-grown bacterial strains (as indicated). After binding and fixing the cells were stained with crystal violet and the optical density measured at 570nm. Values represent the means of triplicate wells. Binding assays were preformed three times with similar results.

Fig. 2. Adhesion of human platelets, 293T and HeLa cells to *S. aureus.* Wild-type Newman and SH1000 along with mutants were grown in RPMI and immobilized in microtiter plates. **(ABC)** BSA (at 3 % w/v), fibronectin (Fn) and fibrinogen (Fg) at 50 μg ml^{-1} were added to control wells. Platelets (white columns), 293T (vertical lines) and HeLa (grey columns) cells were washed and added to microtitre wells and incubated for 1 h at 37 ^{o}C. Adherent cells were lysed and intracellular acid phosphatase measured. Experiments were performed three times. Statistically significant differences are indicated (Student's two tailed t test, *P<0.05, **P<0.03).

Fig. 3. Inhibition of 293T and HeLa cells adhesion to *S. aureus*. Newman strain was grown in RPMI to stationary phase and immobilized in microtiter wells. 293T and

HeLa cells were washed and incubated with RGD peptide at 20 μM, BSA or PBS for 10 min at 37 °C before being added to the wells. Adherent cells were lysed and intracellular acid phosphatase measured. Experiments were performed three times. Statistically significant differences are indicated (Student's two tailed t test, *P<0.05).

Fig. 4. Flow cytometric analysis of IsdB protein binding to platelets. **(A)** Representative flow cytometry traces of recombinant fluorescein isothiocyanate (FITC) labelled IsdB protein binding to isolated and washed platelets (WP) or WP activated by Thrombin Receptor Activating Peptide (WP TRAP). Traces: WP and WP (dark grey), FITC-IsdB (light grey, broken line) and FITC-IsdB bound WP or FITC-IsdB bound to WP TRAP (black). **(B)** Percentage of IsdB bound to WP or WP TRAP. Binding assays was preformed three times with similar results.

Fig. 5. Real-time measurement of attachment and spreading of CHO-K1 cells expressing human $\alpha_{IIb}\beta_3$ variants on immobilized ligands. **(A** and **B)** Bovine serum albumin (BSA), recombinant IsdB and fibrinogen (Fg) or **(C)** glutathione S-transferase (GST), IsdH-GST, IsdA and IsdB. Proteins were immobilized in microtiter wells, blocked with BSA and incubated with equal numbers of CHO-K1 cells expressing $\alpha_{IIb}\beta_3$ **(A)** wild-type or **(B** and **C)** (N305T) high affinity mutant. XCELLigence was used to measure impedance in wells. Attachment and spreading of cells led to increased impedance in the wells which corresponded to higher cell index (CI) values. Slope changes (slope/h) were calculated for CI traces within time points indicated by arrows. Experiments were performed three times with similar results. **(D)** Micrographs representing the levels of spreading of CHO $\alpha_{IIb}\beta_3$ (N305T) on

immobilized ligands: BSA, rIsdB and Fg. Rounded cells are regarded as non-spreading cells.

Fig. 6. *S. aureus* invasion into 293T and HeLa cells. **(A)** Gentamicin protection assay to quantify *S. aureus* Newman wild-type and Newman *isdB* mutant internalization by human cells. Infection was performed in RPMI for 2 h at 37 ^{o}C. Inocula and internalized bacteria were quantified by viable counting. The experiment was performed three times. Differences that are statistically significant are indicated (Student's two tailed t test, *P<0.03). **(B)** Transmission electron micrographs (TEM) of *S. aureus* Newman internalized by 293T cells. Cells were fixed with gluteraldehyde and processed for electron microscopy. Scale bar: 500 nm.

Fig. 7. Internalization of *S. aureus* SH1000 and mutants by 293T cells. **(A)** Gentamicin protection assay of RPMI-grown strains by 293T cells. Infection was performed in RPMI with or without added 10 % fetal bovine serum. The inocula and internalized bacteria were quantified by viable counting. The experiment was performed three times with similar results. Statistically significant differences are indicated (Student's two tailed t test, *P<0.05). **(B)** Transmission electron micrographs of RPMI-grown *S. aureus* SH1000 *clfAclfB* internalized into 293T cells in the absence of serum proteins. Scale bar: 2 μm.

Fig. 8. Internalization of *S. aureus* and *S. carnosus* by 293T cells. Bacterial strains were grown in TSB for 16 h and added to 293T cells in monolayers. The experiment was performed in DMEM supplemented with 10% serum for 2 h at 37 ^{o}C. The inocula and internalized bacteria were quantified by viable counting. (*) *S. carnosus* % of

internalization was < 1%. The experiment was repeated three times with similar results.

Fig. 9. Complementation of *S. aureus ΔisdB*. **(A)** Western immunoblotting analysis of IsdB expression by *S. aureus* SH1000, SH1000 *ΔisdB* and SH1000 *ΔisdB* carrying either pRMC2 or pRMC2*isdB*. Bacteria were grown in RPMI to exponential phase and supplemented with anhydrotetrycycline (aTc). Cultures were harvested and cell walls were solubilised by lysostaphin digestion and proteins separated by SDS-PAGE. Western immunoblotting analysis was performed using rabbit anti-IsdB and HRP - protein A. **(B)** Gentamicin protection assay to quantify the levels of internalization. Strains were grown as described above, harvested and incubated with 293T cells. Infection was performed in RPMI supplemented with 300 ng ml^{-1} of aTc for 2 h at 37^{o}C. The inocula and internalized bacteria were quantified by viable counting. The experiment was repeated three times with similar results. The experiment was performed three times. Statistically significant differences are indicated (Student's two tailed t test, *P<0.05).

Fig. 10. Adhesion and internalization by 293T cells of *S. aureus* expressing IsdB (Y165A/H166E/Y167A). **(A)** Adhesion of cells to immobilized *S. aureus* was performed as described in Figure 1. The experiment was performed three times. Statistically significant differences are indicated (Student's two tailed t test, *P<0.05). **(B)** Bacteria were grown as described above and used to infect 293T cells. Infection was performed in RPMI for 2 h at 37^{o}C. Inocula and internalized bacteria were quantified by viable counting. The experiment was performed three times. Differences are statistically significant. (Student's two tailed t test, *P<0.05).

Fig. 11. Real-time measurement of attachment and spreading of breast cancer epithelial MCF-7 cells. **(A)** Fibronectin (Fn), **(B)** vitronectin (Vn) or **(C)** bovine serum albumin (BSA) were immobilized in microtiter wells, blocked with BSA and incubated with equal numbers of MCF-7 cells supplemented with BSA (at 3%), IsdB (at 5 μM) or RGD peptide (at 20 μM). XCELLigence was used to measure impedance. Cell attachment and spreading led to increased impedance in which corresponded to higher cell index (CI) values. **(ABC)** Traces representing CI changes over-time. **(D)** Slope changes (slope/h) were calculated for CI traces within time points 30 min to 4 h.

Table 1. Strains and plasmids used in this study.

	Relevant characteristics	Reference or source
Strains		
S. aureus Newman	Widely used laboratory strain, defective in expression of FnBPA and FnBPB.	
S. aureus Newman *isdA*	*isdA*::Tn917 Erm^R.	(Clarke *et al.*, 2007)
S. aureus Newman *isdB*	Frameshift mutation in *isdB*.	(Miajlovic *et al.*, 2010b)
S. aureus Newman *clfAclfB*	Frameshift mutation in *clfA5*, frameshift mutation in *clfA*, *clfB::lacZ* Erm^R.	
S. aureus Newman *clfAclfBisdB*	Frameshift mutation in *clfA5 clfB::lacZ* Erm^R, Frameshift mutation in *isdB*.	
S. aureus Newman *clfAclfB isdB* (Y165A/H166E/Y167A)	Strain expressing IsdB Y165A/H166E/Y167A.	This study.
S. aureus SH1000	Laboratory strain. *rsbU* + derivative of *S. aureus* 8325-4.	(Horsburgh *et al.*, 2002)
S. aureus SH1000 *isdB*	*isdB* deletion.	This study.
S. aureus SH1000 *clfAclfBfnbAfnbB*	*clfA*::Tn917, *clfB*::Tet^R, *fnbA*::Tet^R, *fnbB*::Erm^R.	(O'Neill *et al.*, 2009)
S. aureus SH1000 *clfAclfBfnbAfnbBisdB*	*isdB* deletion in SH1000 *clfA clfB fnbA fnbB*.	This study.
S. aureus COWAN	Laboratory strain.	(Levinson *et al.*, 1983)
S. carnosus	Meat isolate.	(Schleifer *et al.*, 1982)
L. lactis NZ9000	*L. lactis* subsp. Cremoris MG1363 carrying nisRK two component system in the chromosome.	(Kuipers, 1998)
E. coli XL-1Blue	*E. coli* cloning host, Tet^R.	Stratagene
E. coli DC10B	*dcm* deficient *E. coli* DH10B, a *staphylococcal* cloning host allowing transformation of plasmids directly into *S.*	(Monk *et al.*, 2012)

	aureus.	
E. coli TOPP10	*E. coli* cloning and protein purification host.	Invitrogen
Plasmids		
pQE30	*E.coli* cloning and expression vector, Amp^R.	Stratagene
pQE30*isdB* (45-480)	pQE30 encoding residues 45 to 480 of IsdB.	(Miajlovic *et al.*, 2010b)
pQE30*isdA* (50-320)	pQE30 encoding residues 50 to 320 of IsdA.	
pQE30*isdB* (48-480) Y165A/H166E/Y167A	pQE30*isdB* (48-480) carrying point causing substitutions Y165A/H166E/Y167A.	This study.
pGEX-4t-2*isdH* (41-321)	pQE30 encoding residues 41 to 321 of IsdH.	(Visai *et al.*, 2009)
pRMC2	*E. coli* (Amp^R) - *S. aureus* (Chm^R) shuttle vector allowing aTc – inducible expression in *S. aureus.*	(Corrigan *et al.*, 2009a)
pRMC2*isdB*	Chm^R, plasmid for inducible expression of IsdA.	This study.
pNZ8037	*L. lactis* shuttle vector containing PnisA promoter, Chm^R for nisin-inducible expression of an insert.	(de Ruyter, 1996)
pNZ8037*isdB*	pNZ8048 encoding *isdB* insert, for controlled expression of IsdB in *L. lactis.*	This study.
pKOR1	*E. coli* (Amp^R) - *S. aureus* (Chm^R) shuttle vector for allelic replacement in *staphylococci.*	(Bae *et al.*, 2006)
pKOR1*ΔisdB*	For construction of deletion of *isdB* for *S. aureus* SH1000.	This study.
pKOR1*isdB* (Y165A/H166E/Y167A)	For construction of mutation causing substitutions of Y165A/H166E/Y167A into genomic *isdB*.	

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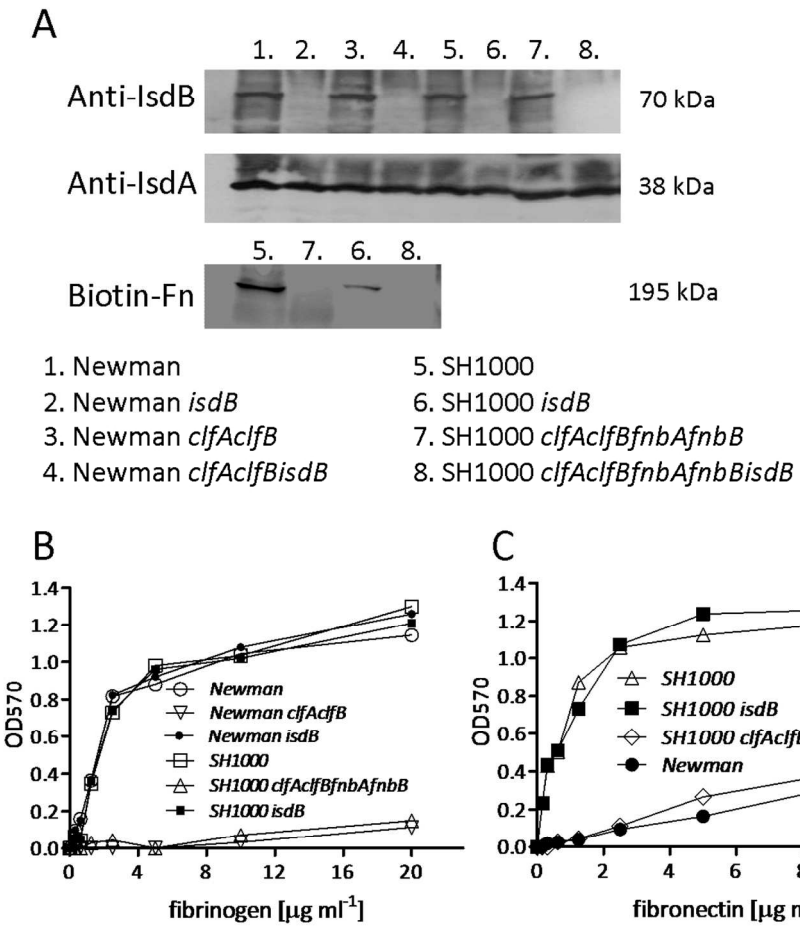

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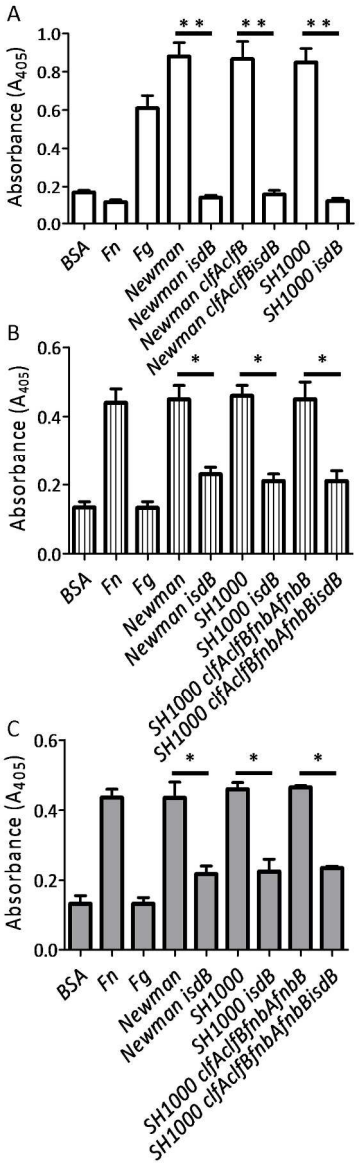

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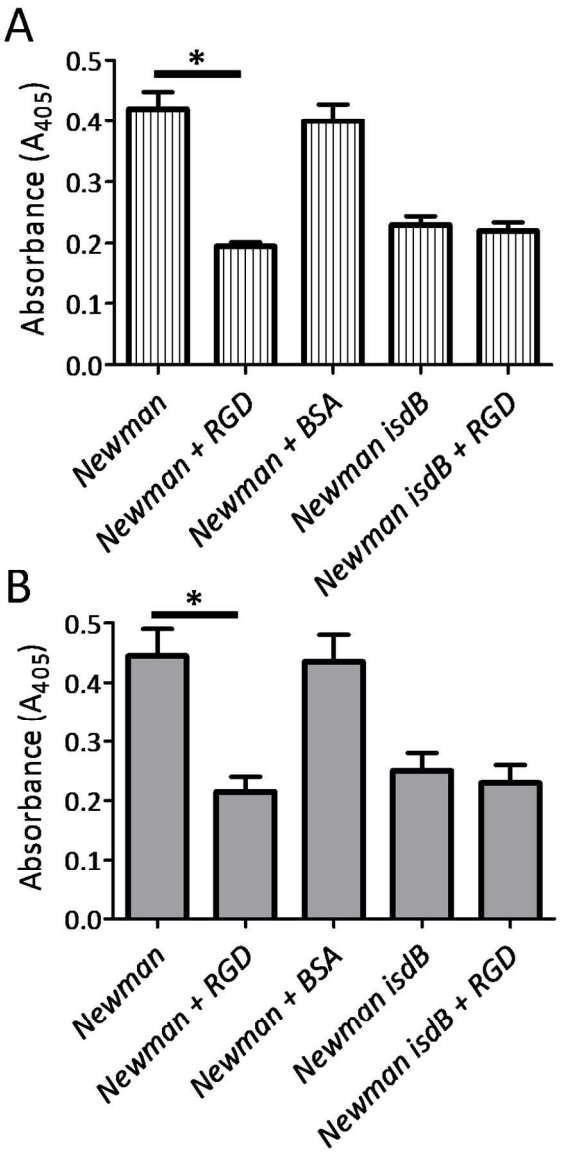

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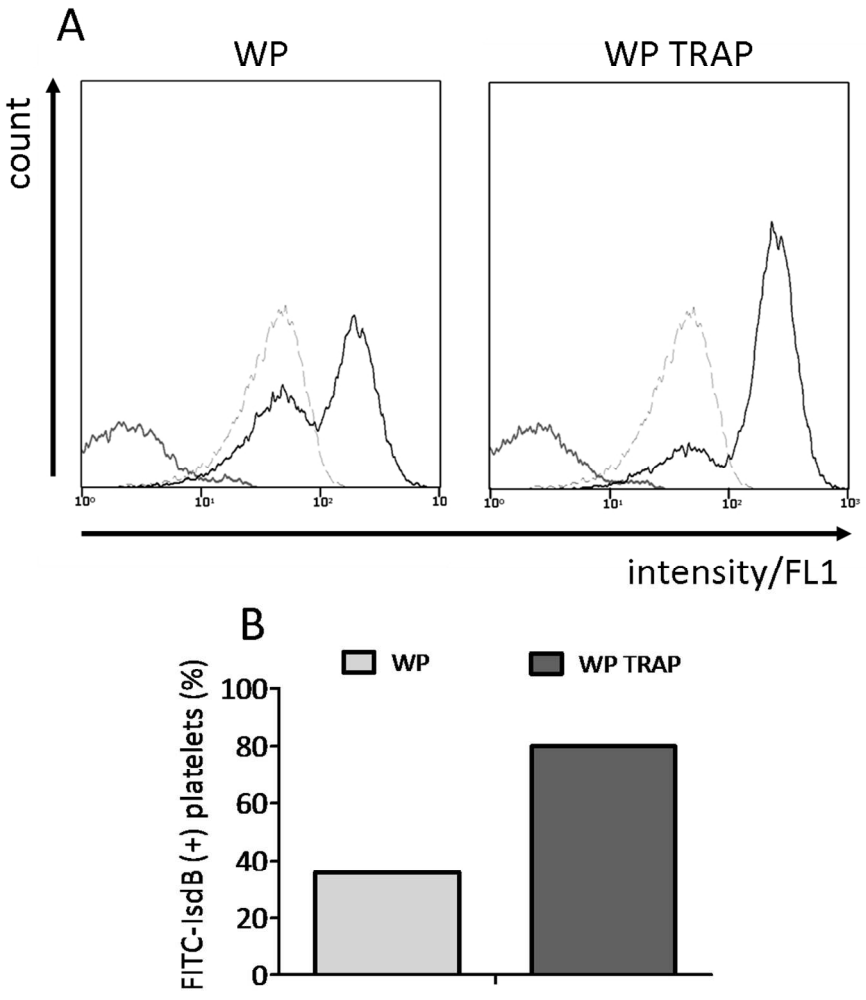

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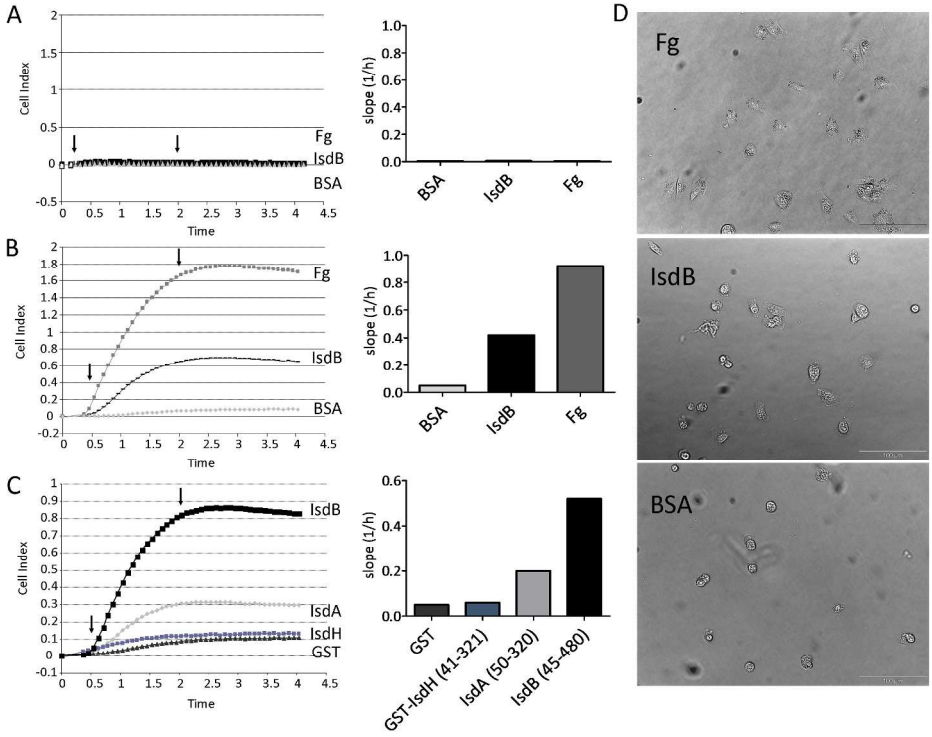

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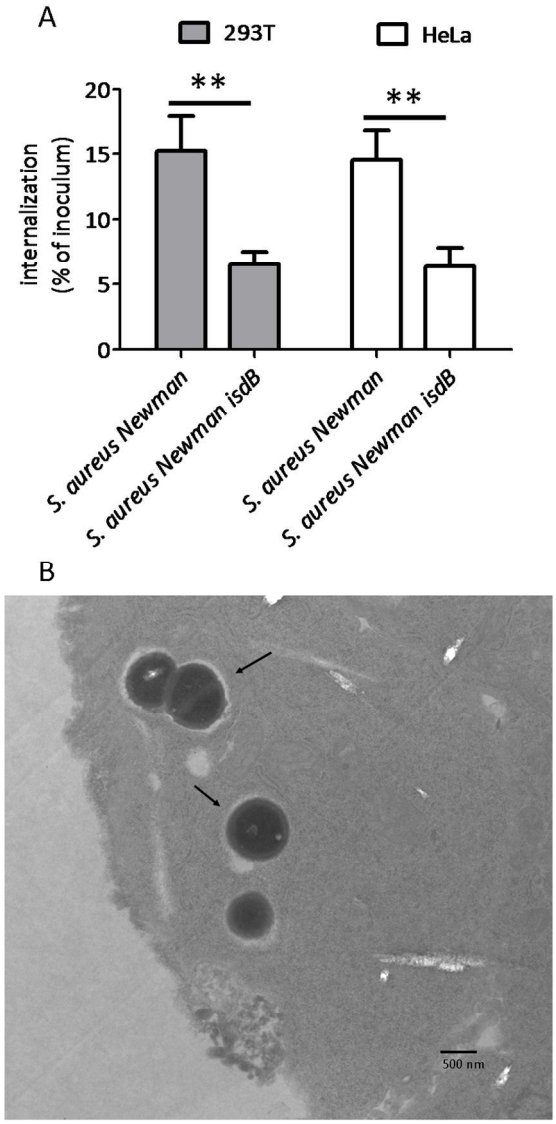

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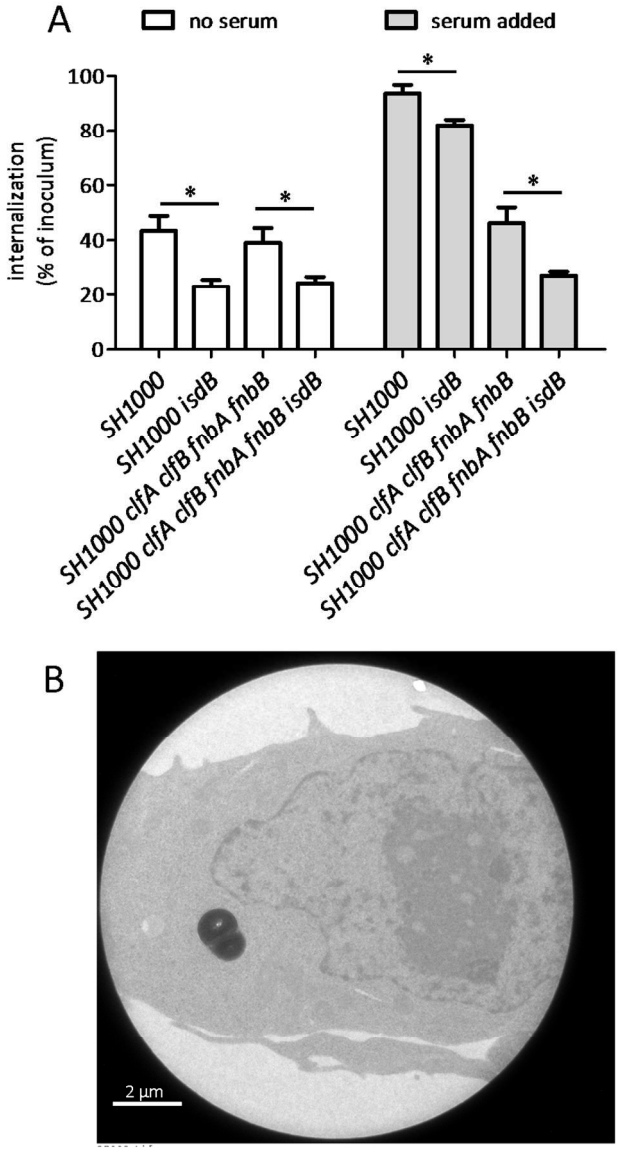

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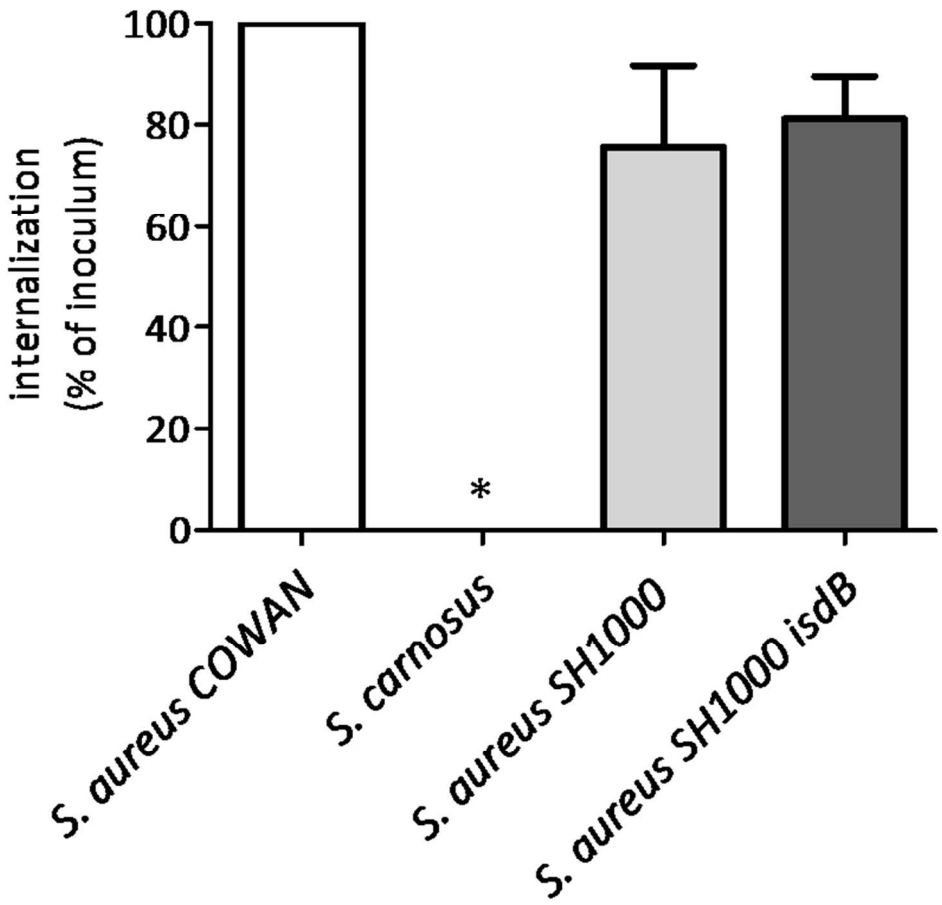

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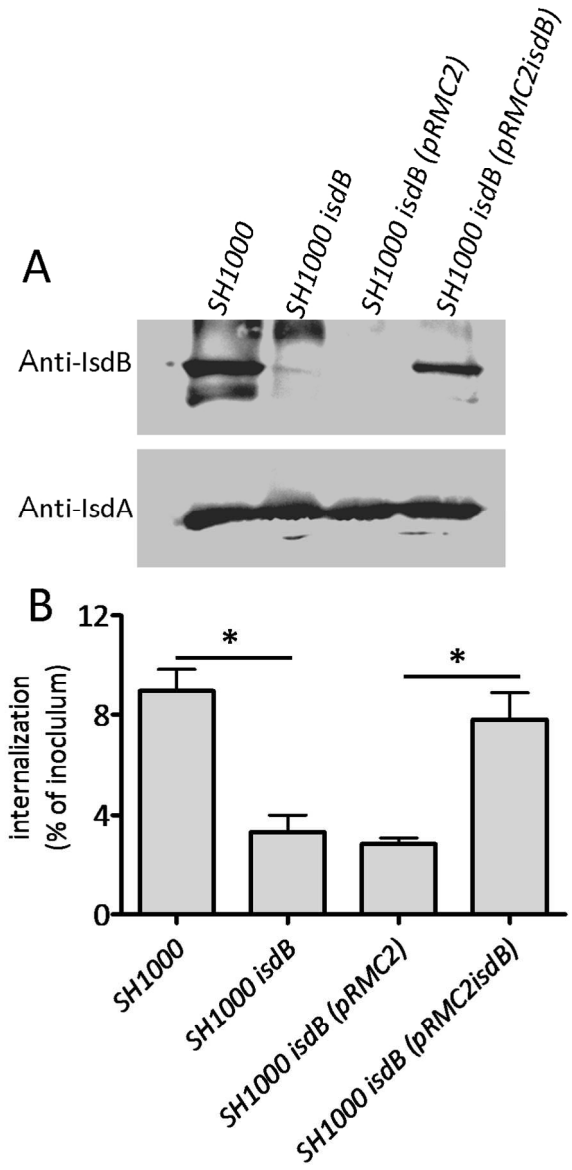

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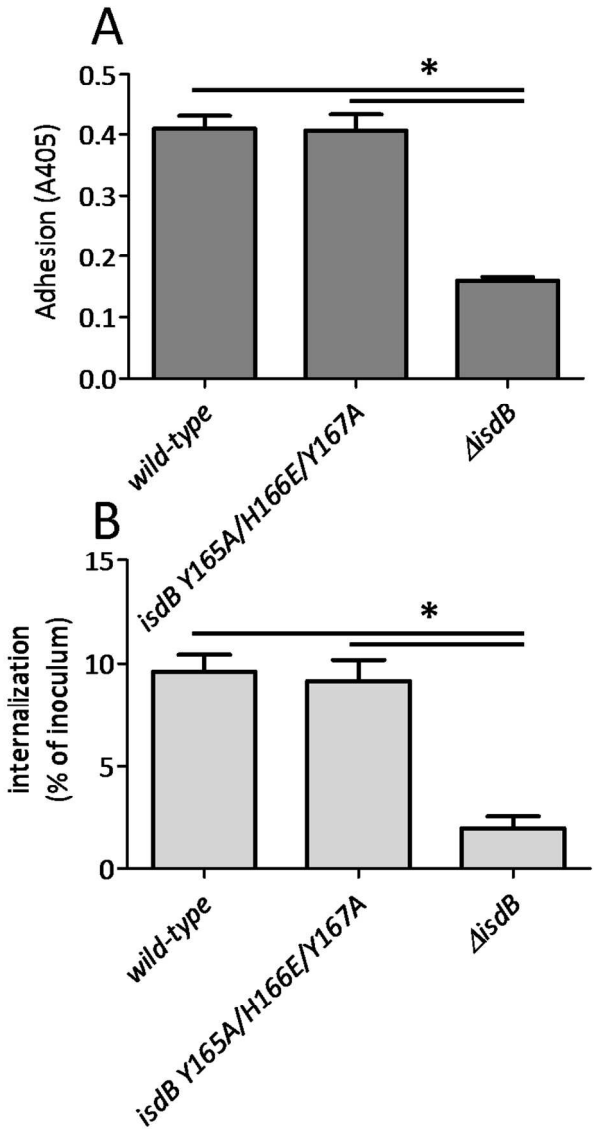

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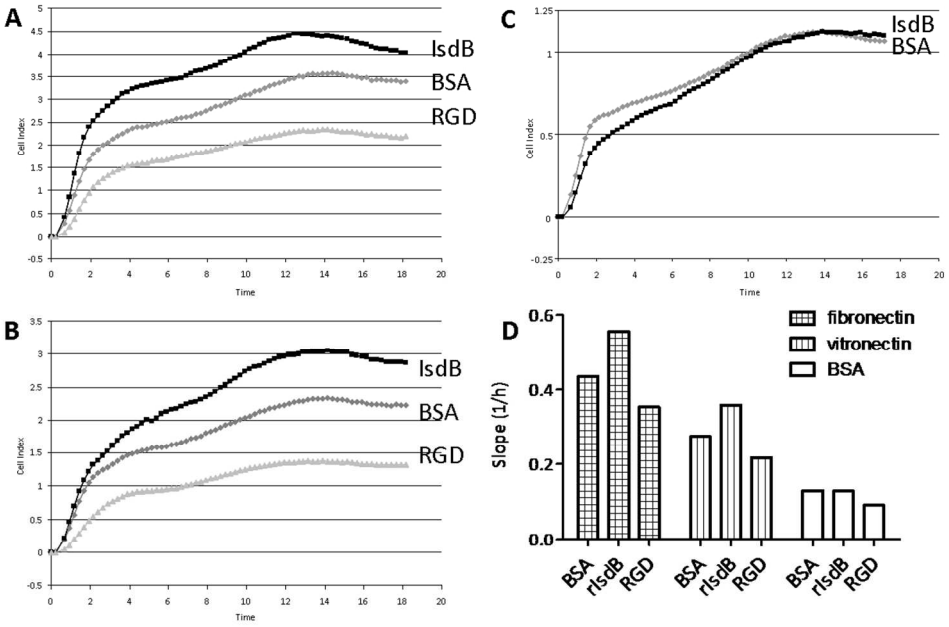

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