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Direct interaction of iron-regulated surface determinant IsdB of *Staphylococcus aureus* with the GPIIb/IIIa receptor on platelets

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The interaction of bacteria with platelets is implicated in the pathogenesis of endovascular infections, including infective endocarditis, of which *Staphylococcus aureus* is the leading cause. Several *S. aureus* surface proteins mediate aggregation of platelets by fibrinogen- or fibronectin-dependent processes, which also requires specific antibodies. In this study *S. aureus* was grown in iron-limited medium to mimic *in vivo* conditions in which iron is unavailable to pathogens. Under such conditions, a *S. aureus* mutant lacking the known platelet-activating surface proteins adhered directly to platelets in the absence of plasma proteins and triggered aggregation. Platelet adhesion and aggregation was prevented by inhibiting expression of iron-regulated surface determinant (Isd) proteins. Mutants defective in IsdB, but not IsdA or IsdH, were unable to adhere to or aggregate platelets. Antibodies to the platelet integrin GPIIb/IIIa inhibited platelet adhesion by IsdB-expressing strains, as did antagonists of GPIIb/IIIa. Surface plasmon resonance demonstrated that recombinant IsdB interacts directly with GPIIb/IIIa.

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen that colonizes the desquamated epithelium of the anterior nares. At sites of infection such as wounds, ulcers or abscesses, *S. aureus* can gain access to the bloodstream, resulting in bacteraemia. Increased use of intravascular devices and invasive procedures in hospitals has led to an increased incidence of bacteraemic infections (Moreillon & Que, 2004). Several endovascular infections, including infective endocarditis, result from the interaction of bacteria with platelets. *S. aureus* is the leading cause of infective endocarditis, which is characterized by the build-up, on heart-valve surfaces, of vegetative bodies consisting of bacteria, fibrin and aggregated platelets (Moreillon & Que, 2004; Mylonakis & Calderwood, 2001).

The ability of *S. aureus* and other bacteria to adhere to and aggregate platelets is thought to contribute to the development of endovascular infections. Aggregation of platelets by bacteria is the result of a multi-step process. Bacteria interact with receptors on the platelet surface either directly or indirectly through bridging molecules such as fibrinogen. Initial adhesion of bacteria to platelets can result in subsequent platelet activation, which is characterized by signalling events and calcium oscillations within the platelet. Upon activation, the major platelet integrin GPIIb/IIIa undergoes conformational changes that allow it to bind avidly to fibrinogen and fibronectin in solution (Calvete, 1999). Aggregation of platelets occurs when adjacent platelets interact with the γ-chain of the bivalent fibrinogen molecule, cross-linking platelets into aggregates.

The interaction between *S. aureus* and platelets is complex and involves multiple factors (O'Brien *et al.*, 2002). Several surface-expressed proteins can stimulate platelet aggregation. These include the fibrinogen-binding proteins clumping factors A and B (ClfA and ClfB), and the bifunctional fibronectin-fibrinogen binding proteins FnBPA and FnBPB (Fitzgerald *et al.*, 2006b; Loughman *et al.*, 2005; Miajlovic *et al.*, 2007). Recent studies have shown that, under high shear rates such as those seen in small arteries and arterioles, protein A and ClfA are crucial for platelet aggregation (Kerrigan *et al.*, 2008; Pawar *et al.*, 2004).

Binding of fibrinogen or fibronectin by surface proteins effectively coats *S. aureus* with these two ligands, allowing it to engage the low-affinity form of the GPIIb/IIIa on resting platelets. Activation of platelets requires specific antibodies

Abbreviations: GFP, gel-filtered platelets; PRP, platelet-rich plasma; WP, washed platelets.

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to the bacterial surface proteins to engage platelet receptor FcγRIIa and trigger intracellular signalling events (Loughman *et al.*, 2005; Miajlovic *et al.*, 2007). A slower complement-dependent mechanism of activation was detected under circumstances where the major proaggregatory surface proteins were missing or defective (Ford *et al.*, 1996; Loughman *et al.*, 2005).

All studies on the interaction of *S. aureus* with platelets have been performed with bacterial cells grown in rich laboratory media replete with iron. *In vivo, S. aureus* has restricted access to iron and expresses iron-regulated surface determinant (Isd) proteins to capture haem from haemoglobin and transport it into the cell (Skaar *et al.*, 2004; Skaar & Schneewind, 2004). Two of the Isd proteins, IsdA and IsdH, are known to have other biological functions. IsdA interacts with an array of host proteins, and expression of IsdA by *S. aureus* also confers resistance to the innate defences of the human skin (Clarke *et al.*, 2004, 2007, 2009). IsdH has been shown to play a role in evasion of phagocytosis as a result of accelerated degradation of the serum opsonin C3b (Visai *et al.*, 2009). It is reasonable to predict that these major surface components of bacteria grown *in vivo* might also interact with platelets. This study investigated platelet adhesion and aggregation mediated by *S. aureus* strain Newman grown in an iron-deficient medium. Using *S. aureus* mutants defective in various surface proteins, it was shown that IsdB binds to platelets by a direct interaction with the platelet integrin GPIIb/IIIa.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were grown in at 37 °C with shaking (200 r.p.m.) in RPMI 1640 (Sigma) to create iron-poor conditions. *Lactococcus lactis* was grown in GM17 medium with 3.2 ng nisin ml^{-1}. The following antibiotics (Sigma) were added to the media as required: chloramphenicol (Cm) at 10 μg ml^{-1}, erythromycin (Em) at 10 μg ml^{-1} and tetracycline (Tc) at 2 μg ml^{-1}.

Construction of *S. aureus* strains. A frameshift mutation was constructed in the *isdB* gene by replacing bases 10–14 with a *Bam*HI restriction site. An upstream *isdB* sequence of 745 bp was PCR amplified with primers 5′-GTCCTGCAGATTCTTACATTAGCT-GACGCA-3′ and 5′-GGCGGATCCTTTGTTCATGTTGTAGAAA-CA-3′ and a downstream sequence of 904 bp was amplified with primers 5′-GAAGGATCCAAAAGAATTTAAATCATTTTA-3′ and 5′-CGCTCTAGAATCTTGAATTTTATTTAATTC-3′. When the two fragments were ligated at the *Bam*HI site, a +1 frameshift was created. The ligated fragments were cloned between the *Pst*I and *Xba*I sites of plasmid pBluescript II-SK. This construct was then ligated with pTSermC using the *Xba*I sites in both plasmids to form pHM1. The temperature-sensitive shuttle plasmid bearing the *isdB* mutation was transduced with phage 85 into Newman *clfA* and Newman *clfA isdA*. Growth of these strains at a restrictive temperature of 44 °C resulted in integration of the pHM1 plasmid into the chromosomal *isdB* gene. Plasmid excision was encouraged by growth of single integrants without selection at 28 °C followed by three cycles of growth in drug-free broth alternately at 44 °C and at 28 °C to enrich for plasmid-free excisants. Single colonies were isolated on drug-free agar and screened for sensitivity to erythromycin. Mutants were validated by PCR amplifying 1.6 kb *isdB* genomic DNA, which was subsequently digested with *Bam*HI. Strains containing the desired mutation had a novel *Bam*HI restriction site present in *isdB*. Western

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
S. aureus		
DU5999	Newman *clfA5*, frameshift mutation in *clfA*	Fitzgerald *et al.* (2006b)
DU6000	Newman *clfA5*, *clfB*::*lacZ*[Em^r]	Fitzgerald *et al.* (2006b)
Newman *isdA*	Newman deletion of *isdA*	Clarke *et al.* (2007)
Newman *clfA isdA*	Newman *clfA5*, deletion of *isdA*	This study
Newman *clfA isdB*	Newman *clfA5*, frameshift mutation in *isdB*	This study
Newman *clfA isdA isdB*	Newman *clfA5*, frameshift mutation in *isdA* and *isdB*	This study
Newman *clfA clfB isdA*	Newman *clfA5*, frameshift mutation in isdA, *clfB*::Tc^R	This study
Newman *clfA clfB isdB*	Newman *clfA5*, *clfB*::*lacZ* Em^R, frameshift mutation in *isdB*	This study
Newman *clfA clfB isdA isdB*	Newman *clfA5*, *clfB*::*lacZ* Em^R, frameshift mutation in *isdA* and *isdB*	This study
Newman *clfA clfB isdH*	Newman *clfA5*, *clfB*::Tc^R, *isdH*::Em^R	This study
L. lactis		
NZ9800	Derivative of MG1363, Tn*5276*, Δ*nisA*	Kuipers *et al.* (1993)
Plasmids		
pQE30	*E. coli* cloning vector, Amp^R	Stratagene
pQE30 *isdB*	pQE30 encoding residues 48–480 of IsdB	This study
pBluescript II SK	*E. coli* cloning vector, Amp^R	Stratagene
pBlueΔ*isdB*	pBluescript II SK containing *isdB* DNA with frameshift mutation, Amp^R	This study
pTS*ermC*	*S. aureus* plasmid containing temperature-sensitive replicon, Em^R	Fitzgerald *et al.* (2006b)
pJH1	Temperature-sensitive plasmid containing frameshift *clfA*5, Em^R	Fitzgerald *et al.* (2006b)
pHM1	Shuttle plasmid containing pBlueΔ*isdB* and pTS*ermC*, Amp^R/Em^R	This study
pNZ8037*clfB* Q235A	pNZ8037*clfB* with point mutation at Q235A, Cm^R	Miajlovic *et al.* (2007)

immunoblotting was also carried out to validate strains. Similarly, a frameshift mutation was introduced into the *clfA* gene in strain Newman *isdA* using pJH1, resulting in strain Newman *clfA isdA*.

To construct Newman *clfA clfB isdA* a *clfA*::Em^R mutation and a *clfB*::Tc^R mutation were transduced into strain Newman *isdA*. Strain Newman *clfA clfB isdB* was constructed by transducing *clfB*::Em^R into strain Newman *clfA isdB*. The *clfB*::Em^R mutation was transduced into Newman *clfA isdA isdB* to create Newman *clfA clfB isdA isdB*. Finally, strain Newman *clfA clfB isdH* was constructed by transducing *isdH*::Em^R and *clfB*::Tc^R into strain Newman *clfA*.

Western immunoblotting. Cultures of *S. aureus* grown in RPMI for 18 h at 37 °C were washed twice in PBS and adjusted to an OD_{600} of 10 in 250 µl of 20 mM Tris (pH 8), 10 mM $MgCl_2$ containing 30 % (w/v) raffinose. Complete EDTA-free protease inhibitor cocktail (Roche) and lysostaphin (200 µg ml^{-1}; AMBI) were added to the cells and incubated at 37 °C for 10 min. Protoplasts were sedimented by centrifugation at 5000 r.p.m. for 10 min (Hartford *et al.*, 2001). The cell-wall fraction was separated on 7.5 % (w/v) polyacrylamide gels, transferred onto PVDF membranes (Roche) and blocked in 10 % (w/v) skimmed milk (Marvel). Membranes were probed with polyclonal anti-IsdB and anti-IsdA at a dilution of 1 : 5000 and with polyclonal anti-IsdH antibodies at a dilution of 1 : 10 000.

Preparation of platelet-rich plasma. Platelet-rich plasma (PRP) was prepared as described previously (Loughman *et al.*, 2005). Briefly, human blood was drawn into a syringe containing 3.2 % (w/v) sodium citrate. Whole blood was centrifuged at 150 ***g*** for 10 min. The top layer, consisting of PRP, was removed. Platelet aggregation was measured using light transmission aggregometry. Aggregation of platelets occurred after a variable period of time referred to as the lag time. This time reflects the time taken for aggregation to occur after bacteria and platelets come into contact. Overall percentage aggregation was also measured.

Preparation of washed platelets. Blood was drawn into a syringe containing acid-citrate-glucose (ACD, 25 mM citric acid, 75 mM sodium citrate, 135 mM D-glucose). Following preparation of PRP the pH of the platelets was adjusted to 6.5 using ACD. Prostaglandin E1 (1 µM; Sigma) was added to prevent activation of platelets during centrifugation. PRP was centrifuged at 720 ***g*** for 10 min. The supernatant was carefully removed and discarded. The platelet pellet was resuspended in 1 ml fresh JNL buffer (6 mM D-glucose, 130 mM NaCl, 9 mM $NaHCO_3$, 10 mM sodium citrate, 10 mM Tris, 3 mM KCl, 0.8 mM KH_2PO_4 and 0.9 mM $MgCl_2$; pH 7.4) and diluted to obtain a platelet count of 3×10^8 platelets ml^{-1}.

Preparation of gel-filtered platelets. Washed platelets (WP) were passed through a 10 ml Sepharose 2B column (Sigma). Gel-filtered platelets (GFP) were collected and diluted to obtain a platelet count of 3×10^8 platelets ml^{-1}. WP and GFP were supplemented with 2 mM $CaCl_2$.

Removal of IgG from commercial supplies of fibrinogen and from human serum. IgG present in commercial fibrinogen and in serum was removed by passage through a column of protein A coupled to Sepharose (Amersham Biosciences). Depletion of IgG was confirmed by ELISA using protein A-peroxidase (Sigma). Human serum was diluted 1 : 20 and analysed by SDS-PAGE to confirm loss of IgG.

Inactivation of complement proteins in human serum. Complement components were inactivated in human serum by heating to 56 °C for 30 min.

Platelet adhesion assay. Cultures of bacterial strains were pelleted by centrifugation, washed in PBS and resuspended to an OD_{600} of 1. Wells of microtitre plates were coated with 100 µl bacteria and incubated for 16 h at 4 °C, washed with PBS and blocked with 1 % BSA (w/v; 100 µl) for 90 min at 37 °C. Wells were washed with JNL buffer and 50 µl WP was added. Plates were incubated at 37 °C for 40 min and washed subsequently with JNL buffer. Adherent platelets were measured with a lysis buffer containing a substrate for acid phosphatase [100 mM sodium acetate, 0.1 % (v/v) Triton X-100, 10 mM *p*-nitrophenol phosphate (Sigma)]. Absorbance at 405 nm was read in an ELISA plate reader. Equal coating of plates with *S. aureus* strains was confirmed by fixing adherent cells with 25 % (v/v) formaldehyde and staining with of 0.5 % (w/v) crystal violet.

Platelet aggregation. Bacterial cells were washed with PBS, resuspended to an OD_{600} of 1.6 and 25 µl washed cells was added to 225 µl PRP in siliconized flat-bottom glass cuvettes (BioData). PRP and bacteria were incubated with stirring (900 r.p.m.) in an aggregometer (Bio-Data) at 37 °C. Light transmission was monitored for 25 min. Inhibitors tested were anti-GPIIb/IIIa (Abciximab, Eli Lily) antibodies at a 1/100 dilution, anti-FcγRIIa (Medarex) antibodies at a 1/50 dilution and anti-GPIb monoclonal antibody (AN51, Dako) at a 1/50 dilution. RGD peptide and tirofiban (Merck) were used at 10 mM and 2 µM respectively. GFP were supplemented with purified fibrinogen at a final concentration of 0.5 mg ml^{-1}. Pooled human IgG (Baxter) was added to GFP to a final concentration of 2 µg ml^{-1}. Twenty-five microlitres of human serum was added to a final volume of 225 µl GFP.

GPIIb/IIIa-binding assay. Microtitre plates were coated for 16 h with 100 µg ml^{-1} of GPIIb/IIIa (Calbiochem). Plates were washed with PBS and blocked with 1 % (w/v) BSA for 2 h at 37 °C. Bacteria were stained with a fluorescent stain (Cyber), adjusted to an OD_{600} of 0.5 and added to GPIIb/IIIa-coated plates. Following a 40 min incubation at 37 °C, plates were washed with PBS and fluorescence units read at 485–535 nm. Values were adjusted by subtracting binding to BSA.

Purification of recombinant IsdB. DNA encoding residues 48–480 of IsdB was amplified from the genomic DNA of strain Newman using primers F*isdB* 5′-GGCCATGGATCCACAAATACAGAAG-CACAACCAAA-3′ and R*isdB* 5′-GGCCATCCTGCAGAGTAGC-TTCCTTCTTAGCTGA-3′. The *isdB* coding sequence was cloned between the *Bam*HI and *Pst*I sites in pQE30. pQE30 *isdB* was transformed into *Escherichia coli* strain TOPP3 and IsdB expression was induced with 1 mM IPTG. Recombinant rIsdB 48–480 was purified by Ni^{2+} affinity chromatography as described previously (O'Connell *et al.*, 1998).

Surface plasmon resonance. Surface plasmon resonance (SPR) was performed using the BIAcore X100 system (GE Healthcare). GPIIb/IIIa (Enzyme Research Laboratories) was covalently immobilized on CM5 sensor chips using amine coupling. This was performed using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), followed by *N*-hydroxysuccinimide (NHS) and ethanolamine hydrochloride, as described by the manufacturer. GPIIb/IIIa (100 µg ml^{-1}) was dissolved in 10 mM sodium acetate (with 1 mM $MgCl_2$ and 1 mM $CaCl_2$) at pH 4.5 and then immobilized on the flow cell at a flow rate of 30 µl min^{-1} in HEPES-buffered saline. On another flow cell, the dextran matrix was treated as described above but without GPIIb/IIIa present to provide an uncoated reference flow cell. Increasing concentrations of rIsdB were flowed over immobilized GPIIb/IIIa and the reference flow cell at a rate of 5 µl min^{-1}.

The sensorgram data presented were subtracted from the corresponding data from the reference flow cell. The response generated from injection of buffer over the chip was also subtracted from all sensorgrams. Data were analysed using the BIAevaluation software

version 3.0. The equilibrium dissociation constant (K_D) was obtained by globally fitting the data using the Langmuir 1 : 1 binding model.

Statistical analysis. The data presented by this study represent the means ± SD of three experiments unless otherwise stated. The unpaired *t*-test was used to determine the significance of differences in aggregation between strains, with significance defined as $P<0.05$.

RESULTS

Platelet adhesion and aggregation mediated by iron-starved *S. aureus*

S. aureus Newman mediates adhesion to and aggregation of platelets using surface proteins ClfA and ClfB. In strain Newman the FnBPA and FnBPB surface proteins are not expressed on the cell surface due to nonsense mutations at the 3′ ends of the genes and therefore do not contribute to platelet aggregation (Grundmeier *et al.*, 2004). In order to investigate the possibility that Isd proteins adhere to and aggregate platelets in the absence of known proaggregatory surface proteins, strain Newman with mutations in *clfA* and *clfB* was studied.

Microtitre dishes were coated with Newman *clfA clfB* that had been grown in the iron-restricted medium RPMI. Washed platelets (WP) adhered strongly to the bacteria at a level twofold higher than to control wells coated with immobilized fibrinogen ($P<0.0001$, Fig. 1a). Adherence of platelets to bacteria occurred without the addition of exogenous fibrinogen. Growth of bacteria in RPMI with 50 μM $FeCl_3$ eliminated adhesion of WP to immobilized bacteria ($P<0.0001$). These results suggest that direct adherence of strain Newman to platelets is dependent on proteins induced by iron starvation.

Platelet aggregation by RPMI-grown Newman *clfA clfB* was measured by light transmission with an aggregometer. Aggregation occurred with a mean lag time of 5.6 ± 0.67 min at an overall percentage aggregation of 60 ± 4 %. Growth of Newman *clfA clfB* in RPMI supplemented with 50 μM $FeCl_3$ eliminated platelet aggregation (Fig. 1b). These results indicate that proteins induced by iron starvation can also trigger platelet aggregation possibly by the direct interaction shown in Fig. 1(a).

Newman *isd* mutants

In order to determine whether the surface-exposed Isd proteins IsdA, IsdB or IsdH interact with platelets, mutants of Newman *clfA clfB* that lacked each of these proteins were constructed. The mutants were studied by Western immunoblotting to ensure that each lacked the relevant Isd protein and still produced the other Isd proteins.

The 38 kDa IsdA protein could not be detected in solubilized cell-wall extracts of Newman *clfA clfB isdA* whereas IsdB and IsdH proteins could be detected (Fig. 2). Strain Newman *clfA clfB isdB* expressed 38 kDa IsdA and 150 kDa IsdH proteins, whereas IsdB was absent. Newman *clfA clfB isdA isdB* lacked IsdA and IsdB, whereas IsdH was detected. IsdH was not expressed by Newman *clfA clfB isdH* whereas IsdA and IsdB were present (Fig. 2).

Platelet adhesion and aggregation mediated by Newman *isd* mutants

Bacterial strains were grown in RPMI for 16 h at 37 °C. Cells were washed and added to microtitre plates and tested for their ability to support binding of WP. Platelets adhered avidly to strain Newman *clfA clfB*. Mutations in

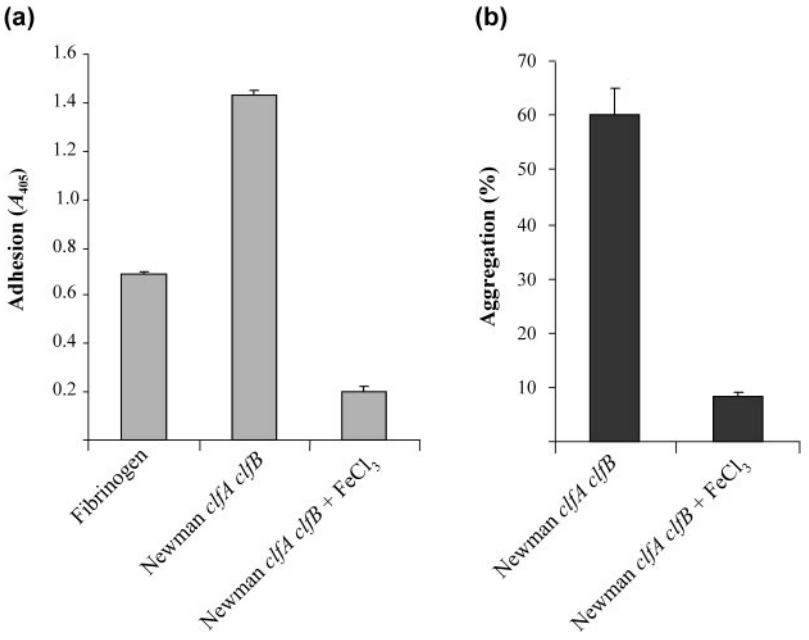

Fig. 1. Platelet adhesion and aggregation mediated by RPMI-grown *S. aureus*. Newman *clfA clfB* was grown in RPMI with or without the addition of 50 μM $FeCl_3$. (a) Washed platelets were added to microtitre wells coated with bacteria or 50 μg fibrinogen ml^{-1}. Adherent platelets were lysed and detected with a substrate for intracellular acid phosphatase. Results are means ± SD. (b) Washed bacterial cells were added to PRP in an aggregometer. Results are means ± SD of light transmission (percentage aggregation) observed after 25 min.

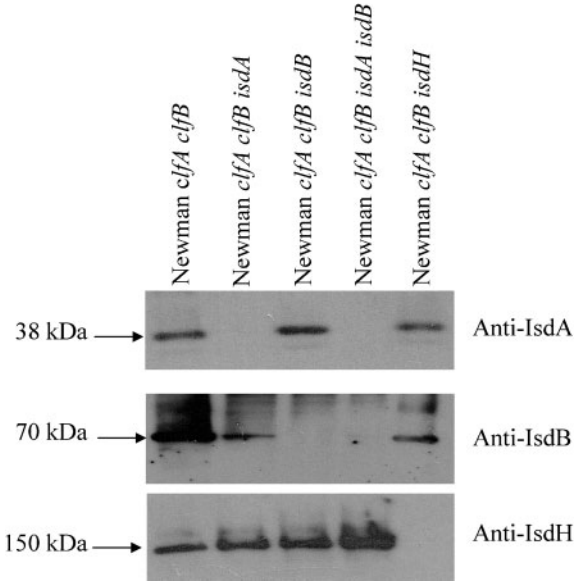

Fig. 2. Characterization of Newman *isd* mutants. The strains indicated were grown to stationary phase in RPMI. Cell-wall proteins were solubilized by lysostaphin digestion, separated on 7.5 % SDS-PAGE gels and electroblotted onto PVDF membranes. Membranes were probed with polyclonal anti-IsdA, anti-IsdB and anti-IsdH and bound antibodies were detected with HRP-conjugated protein A-peroxidase.

either *isdA* or *isdH* had little or no effect on adhesion of platelets (Fig. 3a). In contrast, strain Newman *clfA clfB isdB* was unable to support adherence of WP ($P<0.0001$). Eliminating IsdA did not significantly lower adhesion ($P=0.3609$; Fig. 3a). These results suggest that IsdB is the protein responsible for adhesion to platelets under iron-limiting conditions.

Newman *clfA clfB* cells that had been grown under iron-restricted conditions caused platelet aggregation in PRP with a lag time of 5.6 ± 0.67 min. Mutants lacking either IsdA or IsdH were able to promote aggregation of platelets, with no significant differences in the percentage aggregation or lag time to aggregation ($P=0.46$ and $P=0.68$, respectively). In contrast, the *isdB* mutant failed to cause platelet aggregation ($P=0.0002$; Fig. 3b). The mutant lacking both IsdB and IsdA was also unable to support platelet aggregation. IsdB appears to be crucial for both adhesion to and aggregation of platelets by iron-starved *S. aureus* cells.

Plasma proteins required for platelet aggregation mediated by IsdB

Lag times to aggregation for Newman *clfA clfB* grown in RPMI varied with plasma donor, with a mean lag time of 5.6 ± 0.67 min. Longer lag times are often associated with complement-mediated platelet activation (Ford *et al.*, 1996; Loughman *et al.*, 2005). To further investigate the

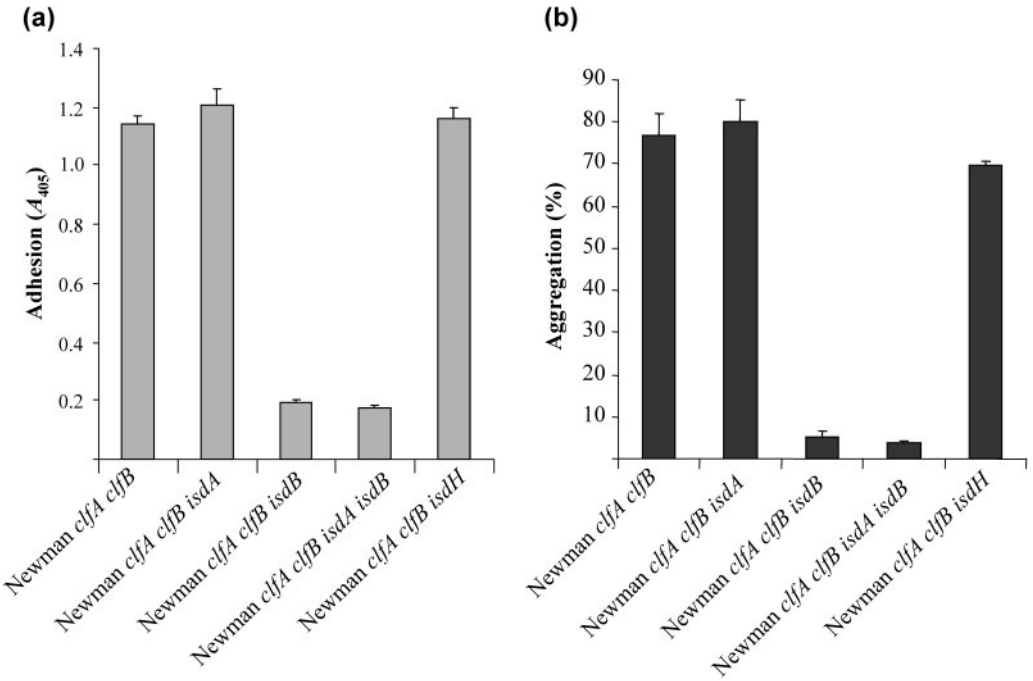

Fig. 3. Platelet adhesion and aggregation mediated by Newman *isd* mutants. (a) WP were added to microtitre wells coated with bacterial cells. Adherent platelets were lysed and detected with a substrate for intracellular acid phosphatase. Results are means ± SD. (b) Washed bacterial cells were added to PRP in an aggregometer. Results are means ± SD of light transmission (percentage aggregation) observed after 25 min.

mechanism of platelet aggregation, complement was inactivated in human serum by heat treatment. Serum depleted of IgG was also utilized to determine the necessity for antibodies. *L. lactis* NZ9800(pNZ8037 *clfB* Q235A), expressing ClfB Q235A, was used as negative control in this assay. ClfB Q235A lacks the ability to bind to fibrinogen and therefore cannot activate platelets in a fibrinogen-dependent manner. Activation of platelets by *L. lactis* ClfB Q235A is complement dependent and requires IgG. This strain does not cause aggregation of gel-filtered platelets (GFP) in the presence of fibrinogen and complement-inactivated serum or IgG-depleted serum (Miajlovic *et al.*, 2007).

Both Newman *clfA clfB* and *L. lactis* ClfB Q235A were able to stimulate aggregation of GFP supplemented with fibrinogen and 10 % human serum (Fig. 4a). Adding heated serum or IgG-depleted serum to GFP did not support platelet aggregation mediated by *L. lactis* ClfB Q235A. In contrast, Newman *clfA clfB* was able to cause aggregation in GFP supplemented with heated serum and IgG-depleted serum. No significant difference in the percentage aggregation was observed between GFP supplemented with serum, heated serum or IgG-depleted serum ($P=0.1155$ and $P=0.2758$, respectively). Likewise, no significant difference was observed in the lag time to aggregation. Thus platelet aggregation mediated by Newman *clfA clfB* grown in iron-limiting conditions is not complement dependent and IgG does not seem to be necessary.

Further experiments were carried out to identify the mechanism of platelet aggregation. Iron-starved bacterial cells were added to GFP supplemented with purified fibrinogen lacking contaminating IgG present in commercial supplies. Strain Newman expressing IsdB was able to stimulate aggregation of GFP supplemented with fibrinogen alone (Fig. 4b). The addition of exogenous IgG decreased the mean lag time to aggregation from 4.33 ± 1.86 min to 2.33 ± 0.88 min. However, this difference was not statistically significant ($P=0.121$) due to considerable variations in lag time between donors. Strain Newman *clfA clfB isdB* was unable to cause activation of GFP supplemented with fibrinogen alone or with fibrinogen and IgG together.

Inhibition of platelet adhesion

Washed platelets were incubated with antibodies that are known to block receptors GPIIb/IIIa, FcγRIIa and GP1b prior to their addition to microtitre plates coated with bacteria. The anti-GPIIb/IIIa monoclonal antibody abciximab inhibited adherence of WP to RPMI-grown Newman *clfA clfB* ($P<0.0001$, Fig. 5a). Monoclonal antibodies IV3 and AN51, which block the FcγRIIa and GP1b, respectively, did not significantly inhibit adhesion of WP to Newman *clfA clfB* (Fig. 5a). This indicates that adherence of platelets to iron-starved *S. aureus* occurs via GPIIb/IIIa and does not involve GP1b or FcγRIIa.

In order to confirm that IsdB interacts with GPIIb/IIIa, inhibition experiments were carried out with GPIIb/IIIa antagonists. GPIIb/IIIa recognizes an RGD motif present in fibrinogen and fibronectin. Synthetic RGD peptide and tirofiban, a specific inhibitor of GPIIb/IIIa, were tested for

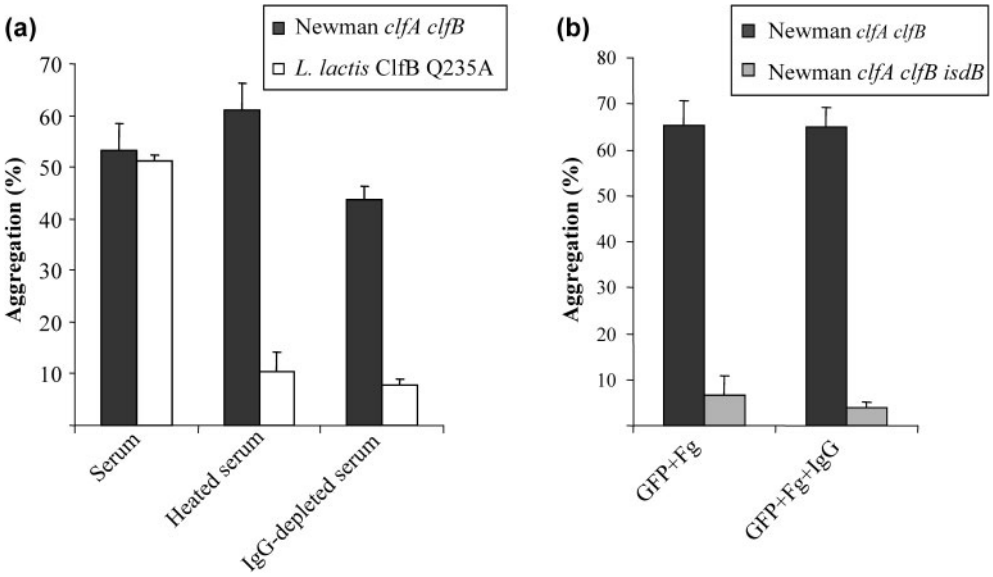

Fig. 4. Role of complement and IgG in platelet aggregation. (a) Strains *L. lactis* ClfB Q235A and Newman *clfA clfB* were washed and added to GFP supplemented with fibrinogen (Fg) and 10 % human serum (heated to 56 °C or IgG depleted). (b) Washed bacterial cells were added to GFP supplemented with 1 mg Fg ml^{-1}, with or without the addition of pooled human IgG. Results are means ± SD of light transmission (percentage aggregation) observed after 25 min.

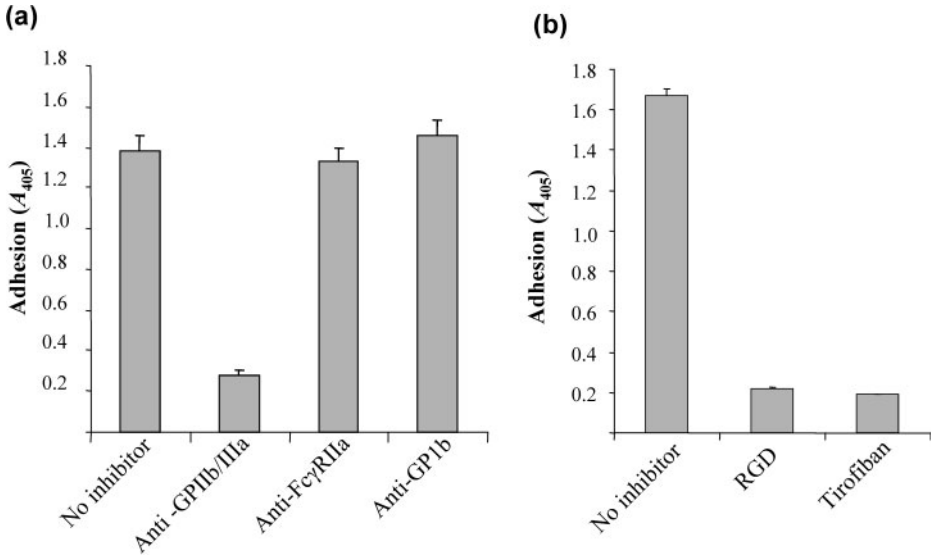

Fig. 5. Effect of platelet receptor inhibitors on adhesion. (a) WP were incubated for 15 min at 37 °C with inhibitory antibodies to platelet receptors and added to microtitre plates coated with RPMI-grown Newman *clfA clfB.* (b) WP were incubated for 15 min at 37 °C with RGD peptide or tirofiban and added to microtitre plates coated with RPMI-grown Newman *clfA clfB.* Adherent platelets were lysed and detected with a substrate for intracellular acid phosphatase. Results are means ± SD.

their ability to inhibit adhesion of platelets to iron-starved Newman *clfA clfB*. Both RGD peptide and tirofiban inhibited adhesion of platelets ($P<0.0001$ in both cases, Fig. 5b).

Direct interaction of IsdB and GPIIb/IIIa

Purified GPIIb/IIIa was coated onto microtitre plates. The ability of iron-starved Newman *clfA clfB* and Newman *clfA clfB isdB* to adhere to the immobilized integrin was measured. Newman *clfA clfB* adhered to immobilized GPIIb/IIIa but the *isdB* mutant failed to do so (Fig. 6a). Eliminating IsdB from Newman *clfA clfB* inhibited binding to GPIIb/IIIa ($P=0.0089$), confirming the direct interaction between these proteins.

Surface plasmon resonance was also used to demonstrate a direct interaction between IsdB and GPIIb/IIIa (Fig. 6b). Increasing concentrations of recombinant IsdB were flowed over a chip that had been coated with GPIIb/IIIa. The approximate dissociation constant (K_D) of the interaction was determined to be 405 ± 73.7 nM, indicating that IsdB directly interacts with GPIIb/IIIa with a high affinity.

DISCUSSION

The ability of *S. aureus* to adhere to and activate platelets is believed to be an important factor in the pathogenesis of cardiovascular infections, including infective endocarditis. Previous studies have established that *S. aureus* surface proteins ClfA, ClfB, FnBPA and FnBPB can promote activation of platelets (Fitzgerald *et al.*, 2006a). These studies were carried out with bacteria grown in complex iron-containing media. This study investigated platelet adhesion and aggregation mediated by bacteria that were grown in an iron-deficient medium to mimic more closely the *in vivo* environment.

Initial adhesion of bacteria to platelets is required to trigger subsequent platelet activation. A common mechanism for platelet activation has been identified for bacterial proteins capable of binding to the blood glycoproteins fibrinogen and fibronectin. ClfA and ClfB of *S. aureus* mediate adhesion to platelets via a fibrinogen bridge to platelet receptor GPIIb/IIIa (Loughman *et al.*, 2005; Miajlovic *et al.*, 2007). Under iron-restricted growth conditions the adhesion of *S. aureus* lacking these proteins to platelets was direct and did not require fibrinogen (Fig. 1a). Elimination of IsdB from the cell surface by growth in the presence of iron or by mutation of the *isdB* gene resulted in loss of platelet adhesion (Figs 1a and 3a). IsdH or IsdA were not required to promote platelet adhesion, as strains lacking these proteins were able to adhere at wild-type levels (Fig. 3a).

In order to identify the receptor for IsdB on the platelet surface, inhibitors of the major platelet receptors were utilized. Adhesion to platelets was only inhibited by function-blocking antibodies to receptor GPIIb/IIIa (Fig. 5a). GPIIb/IIIa is the most abundant receptor found on the platelet surface. It recognizes RGD motifs present in multiple ligands, including fibrinogen and fibronectin, to promote platelet adhesion at sites of thrombus formation. Tirofiban, a specific inhibitor of GPIIb/IIIa function, completely inhibited adhesion of platelets (Fig. 5b).

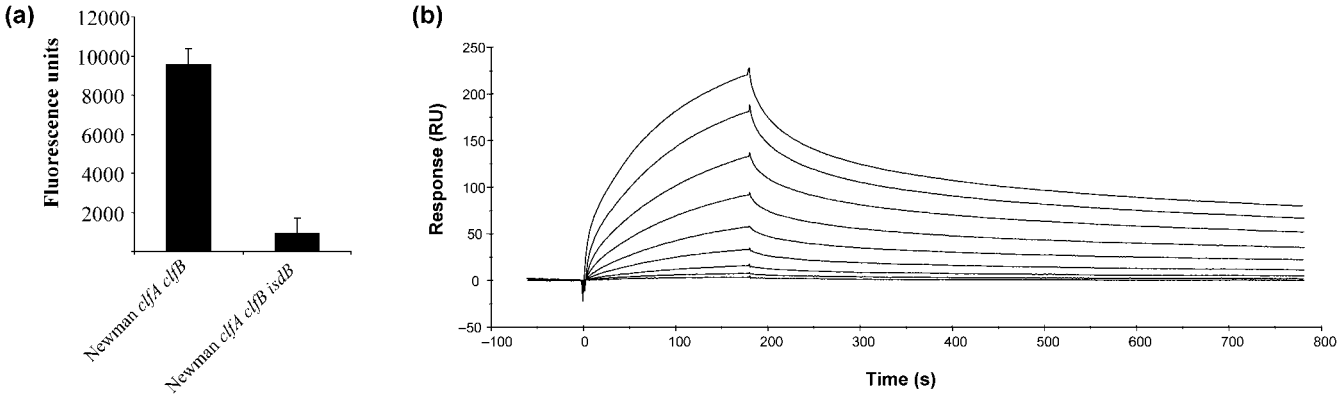

Fig. 6. Direct interaction of IsdB with GPIIb/IIIa. (a) Bacterial strains were fluorescently stained and incubated at 37 °C in microtitre plates coated with GPIIb/IIIa. Plates were washed and fluorescence units read at 485–535 nm (three readings in each of two experiments). Results are means ± SD. (b) GPIIb/IIIa was immobilized on the surface of a CM5 sensor chip. Sensorgrams of the binding to GPIIb/IIIa were obtained by passing increasing concentrations of rIsdB over the surface. Injections began at 0 s and ended at 180 s. Results shown are representative of two independent experiments.

Incubating platelets with soluble RGD peptide also inhibited adhesion of platelets to bacteria expressing IsdB, indicating that IsdB binds to the GPIIb/IIIa receptor on platelets.

To demonstrate that IsdB interacts directly with GPIIb/IIIa, it was shown that *S. aureus* strain Newman *clfA clfB* adhered to purified GPIIb/IIIa in an IsdB-dependent fashion (Fig. 6a). The direct nature of the interaction between IsdB and GPIIb/IIIa was confirmed by surface plasmon resonance (Fig. 6b). IsdB bound to GPIIb/IIIa with a high affinity (approximate K_D 405 ± 73.7 nM). Previous studies carried out with IsdH have shown that the residues in the fuctional NEAT I domain were crucial for both haemoglobin binding and evasion of phagocytosis (Visai *et al.*, 2009). The NEAT domain of IsdB does not have an integrin-binding RGD motif. Further studies will be carried out in this laboratory to characterize the GPIIb/IIIa-binding epitope in IsdB by substituting residues in the NEAT domains.

Aggregation of platelets following adhesion was dependent on expression of IsdB and occurred after a lag time of 5.6 ± 0.67 min. Longer lag times to activation are sometimes associated with complement-dependent platelet activation and reflect the time taken for complement fixation to occur on the bacterial cell surface. However, in this case aggregation was not complement dependent and occurred when complement proteins were lacking or inactivated (Fig. 4).

Bacteria expressing either clumping factors or fibronectin-binding proteins require specific IgG to trigger platelet aggregation. The only plasma factor necessary for platelet aggregation mediated by IsdB was fibrinogen, to allow cross-linking of platelets into aggregates (Fig. 4b). IgG did not seem to be directly required for aggregation since the addition of fibrinogen alone to GFP was sufficient to allow aggregation. However, there was a trend towards a faster lag time in the presence of IgG. This needs to be investigated further with more donors. It is possible that engagement of the Fc receptors on platelets by IgG will promote faster aggregation by triggering a second signalling event. The primary event is outside-in signalling mediated by IsdB binding to the low-affinity form of GPIIb/IIIa. To our knowledge, this is the first report of direct interaction of a *S. aureus* surface protein with GPIIb/IIIa. However, recent studies have shown that the platelet adhesion protein (PadA) of *Streptococcus gordonii* can directly bind to GPIIb/IIIa on platelets to promote adhesion but not aggregation (Petersen *et al.*, 2009). Other direct mechanisms of platelet aggregation have also been identified. *Streptococcus sanguis* expressing SrpA can mediate aggregation of platelets through direct binding to GP1b without the requirement for IgG (Kerrigan *et al.*, 2002).

IsdB-mediated platelet aggregation may be of relevance *in vivo*, particularly if other proaggregatory molecules are expressed at lower levels than when bacteria are grown in rich broth. Although ClfB was expressed at constant high levels from *S. aureus* grown in RPMI, expression of ClfA was dramatically reduced compared to that seen from cells grown in rich broth (data not shown). Induction of IsdB expression *in vivo* is likely to provide a mechanism of platelet aggregation that is independent of clumping factors and FnBPs. It will be interesting to determine if the Isd-promoted mechanism of platelet aggregation is of significance in bacteria that express clumping factors and FnBPs.

This study has identified a novel mechanism of platelet aggregation for *S. aureus* expressing Isd proteins, in which IsdB binds directly to the resting form of platelet integrin GPIIb/IIIa. Proteins such as IsdB whose expression is induced by the harsh growth environment *in vivo* may be

important in mediating platelet aggregation and thrombus formation in the human host.

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