Molecular Microbiology (2006) **59**(1), 212–230

doi:10.1111/j.1365-2958.2005.04922.x
First published online 18 October 2005

Fibronectin-binding proteins of *Staphylococcus aureus* mediate activation of human platelets via fibrinogen and fibronectin bridges to integrin GPIIb/IIIa and IgG binding to the FcγRIIa receptor

J. Ross Fitzgerald,[1†] Anthony Loughman,[1] Fiona Keane,[1] Marian Brennan,[2] Morris Knobel,[1] Judy Higgins,[1] Livia Visai,[3] Pietro Speziale,[3] Dermot Cox[2] and Timothy J. Foster[1*]

[1]*Department of Microbiology, Moyne Institute of Preventive Medicine, University of Dublin, Trinity College, Dublin, Ireland.*

[2]*Department of Clinical Pharmacology, School of Pharmacy, Royal College of Surgeons in Ireland, Dublin 2, Ireland.*

[3]*Department of Biochemistry, University of Pavia, Viale Taramelli 3/B, Pavia 27100, Italy.*

Summary

Staphylococcus aureus **is a leading cause of infective endocarditis (IE). Platelet activation promoted by *S. aureus* resulting in aggregation and thrombus formation is an important step in the pathogenesis of IE. Here, we report that the fibrinogen/fibronectin-binding proteins FnBPA and FnBPB are major platelet-activating factors on the surface of *S. aureus* from the exponential phase of growth. Truncated derivatives of FnBPA, presenting either the fibrinogen-binding A domain or the fibronectin-binding BCD region, each promoted platelet activation when expressed on the surface of *S. aureus* or *Lactococcus lactis*, indicating two distinct mechanisms of activation. FnBPA-promoted platelet activation is mediated by fibrinogen and fibronectin bridges between the A domain and the BCD domains, respectively, to the low affinity form of the integrin GPIIb/IIIa on resting platelets. Antibodies recognizing the FnBPA A domain or the complex between the FnBPA BCD domains and fibronectin were essential for activation promoted by bacteria expressing the A domain or the BCD domain respectively. Activation was inhibited by a monoclonal antibody (IV-3) specific for the FcγRIIa IgG receptor on platelets. We propose that the activation of quiescent platelets by bacteria expressing FnBPs involves the formation of a bridge between the bacterial cell and the platelet surface by (i) fibronectin and fibrinogen interacting with the low affinity form of GPIIb/IIIa and (ii) by antibodies specific to FnBPs that engage the platelet Fc receptor FcγRIIa. Platelet activation by *S. aureus* clinical IE isolates from both the exponential and stationary phases of growth was completely inhibited by monoclonal antibody IV-3 suggesting that the IgG–FcγRIIa interaction is of fundamental importance for platelet activation mediated by this organism. This suggests new avenues for development of therapeutics against vascular infections.**

Introduction

Staphylococcus aureus is a leading cause of infective endocarditis (IE) (Fowler *et al*., 2005). An important step in the development of IE is the interaction of bacteria with platelets (Sullam *et al*., 1996). This results in the formation of platelet-bacteria thrombi on the surface of heart valves, which develop into vegetative growths characteristic of IE. There is evidence that virulence in endovascular infections correlates with the ability to bind to and cause aggregation of platelets (Herzberg *et al*., 1990; 1992; Sullam *et al*., 1996).

Bacteria-mediated aggregation of platelets occurs in two stages. Firstly, bacterial cells interact with resting platelets causing activation and stimulation of intracellular signalling which leads to activation of the platelet integrin glycoprotein GPIIb/IIIa. This results in platelet aggregation mediated by GPIIb/IIIa binding to fibrinogen. Several distinct mechanisms of bacterial activation of platelets have been described. Some strains of *Streptococcus sanguis* can interact directly with the platelet von Willebrand factor receptor, GPIb (Kerrigan *et al*., 2002; Plummer *et al*., 2005), while others require IgG and complement to bind to the bacterial surface allowing a dual interaction with the platelet via a complement receptor and an IgG–FcγRIIa interaction (Sullam *et al*., 1988; Ford *et al*., 1996; 1997; Loughman *et al*., 2005). In contrast, *Helicobacter pylori* binds indirectly to GPIb via a von Willebrand factor bridge

Accepted 21 September, 2005. *For correspondence. E-mail tfoster@tcd.ie; Tel. (+353) 1 608 2014; Fax (+353) 1 679 9294. †Present address: Centre for Infectious Diseases, The Chancellor's Building, New Royal Infirmary, University of Edinburgh, Edinburgh EH16 4SB, Scotland, UK.

but activation also requires an IgG bridge to the platelet FcγRIIa receptor (Byrne *et al*., 2003).

Platelet aggregation stimulated by bacteria is preceded by a lag phase, which is the time taken for activation to occur after bacterial cells are mixed with platelets. This is called the lag time. For *S. aureus* Newman cells in the stationary phase of growth, the lag time is between 1 and 1.5 min whereas for a mutant lacking clumping factor A it is 8–10 min (O'Brien *et al*., 2002). The lag time reflects the time required for bacteria to bind to platelets and trigger the activation signal. This is longer than that seen with soluble agonists. Differences in lag times may reflect differences in the affinity of bacterial surface components for platelet receptors and the density of adhesins on the bacterial cell surface. Thus, the lag time is commonly used to measure the relative contribution of specific bacterial factors to platelet activation (Kerrigan *et al*., 2002; O'Brien *et al*., 2002; Byrne *et al*., 2003; Loughman *et al*., 2005). The aggregation response is all or nothing with maximum aggregation achieved each time. Agents that interfere with the platelet aggregation process rather than the bacterial–platelet interaction (e.g. GPIIb/IIIa antagonists) reduce the aggregation response rather than the lag time. Thus, when studying the response to inhibitors of platelet function the lag time is typically constant and the percentage platelet aggregation is measured (Kerrigan *et al*., 2002; Byrne *et al*., 2003; Loughman *et al*., 2005).

Despite the potential importance of *S. aureus*-induced platelet activation in the pathogenesis of invasive endocarditis, it was only recently that the first steps were taken in elucidating the mechanisms involved at the molecular level. We showed that surface proteins clumping factors ClfA and ClfB, and to a lesser extent the serine-aspartate repeat protein E (SdrE) could mediate activation of platelets (O'Brien *et al*., 2002). However, this study was conducted exclusively with the laboratory strain Newman grown to stationary phase and the molecular mechanisms were not elucidated. In the current study, we investigated a strain that is more representative of IE isolates than strain Newman and analysed cells from the exponential phase of growth, which are likely to better represent bacteria from *in vivo* blood stream infection.

Our group recently investigated the molecular basis of platelet activation promoted by ClfA, a fibrinogen binding protein that is expressed predominantly in the stationary phase of growth (Loughman *et al*., 2005). We showed that ClfA promoted rapid activation of platelets that required both a fibrinogen bridge to the platelet integrin GPIIb/IIIa, as well as ClfA-specific antibodies that linked the surface protein to the IgG Fc receptor FcγRIIa. Pawar *et al*. (2004) reported that protein A may also be an important mediator of platelet activation by *S. aureus* under conditions of high shear, probably through a vWF protein cross-bridge with the platelet receptor GP1b.

The fibronectin-binding proteins FnBPA and FnBPB are expressed predominantly during the exponential phase of growth (Saravia-Otten *et al*., 1997). They are multifunctional, mediating bacterial binding to fibrinogen, fibronectin and elastin (Wann *et al*., 2000; Roche *et al*., 2004). FnBPA-mediated binding to fibronectin occurs through 11 fibronectin-binding segments contained mostly within the B, C and D domains by a β-zipper mechanism (Schwarz-Linek *et al*., 2003) (Fig. 1). The BCD region of the protein is unfolded and has no discernible secondary structure. Binding of FnBPA to the N-terminal type I domains of fibronectin results in the formation of neo-epitopes for antibodies that only recognize FnBPA when it is bound to fibronectin (Casolini *et al*., 1998). Furthermore, *in vitro* studies showed that a single FnBPA molecule bound two molecules of fibronectin (Matsuka *et al*., 2003).

Binding to fibrinogen is mediated through the N-terminal A domain by a mechanism analogous to the interaction of ClfA with the C-terminal residues of the γ-chain (Wann *et al*., 2000). Importantly, FnBPA was previously shown to be a virulence factor in experimental endocarditis (Kuypers and Proctor, 1989; Que *et al*., 2001; 2005). A recent study (Heilmann *et al*., 2004) reported that FnBPA, but not FnBPB, could cause platelet aggregation when expressed in *Staphylococcus carnosus*, but the mechanism of activation was not elucidated.

The work described here was undertaken to identify the factors expressed by *S. aureus* during exponential growth that mediate platelet activation and to characterize the molecular mechanisms involved. Using bacterial cells that were grown to exponential phase, experiments with mutants of *S. aureus* strains Newman and P1 that were defective in several surface proteins including FnBPA and FnBPB, as well as *Lactococcus lactis* expressing FnBPA or FnBPB, showed that both FnBPs could stimulate activation of platelets. By analysing bacteria expressing either the fibronectin-binding domain or the fibrinogen-binding domain of FnBPA, two distinct mechanisms of platelet activation were discovered.

Results

Platelet activation by S. aureus *mutants deficient in expression of surface proteins*

In order to identify the surface protein(s) responsible for stimulating platelet activation by *S. aureus* cells in the exponential phase of growth, mutants deficient in the expression of various surface proteins were constructed and tested for their ability to cause platelet aggregation (Fig. 2). The laboratory strain Newman used in our previous study (O'Brien *et al*., 2002) was compared with strain P1 which adheres strongly to extracellular matrix proteins and may better represent clinical isolates. The lag time to aggregation was measured for surface protein-deficient

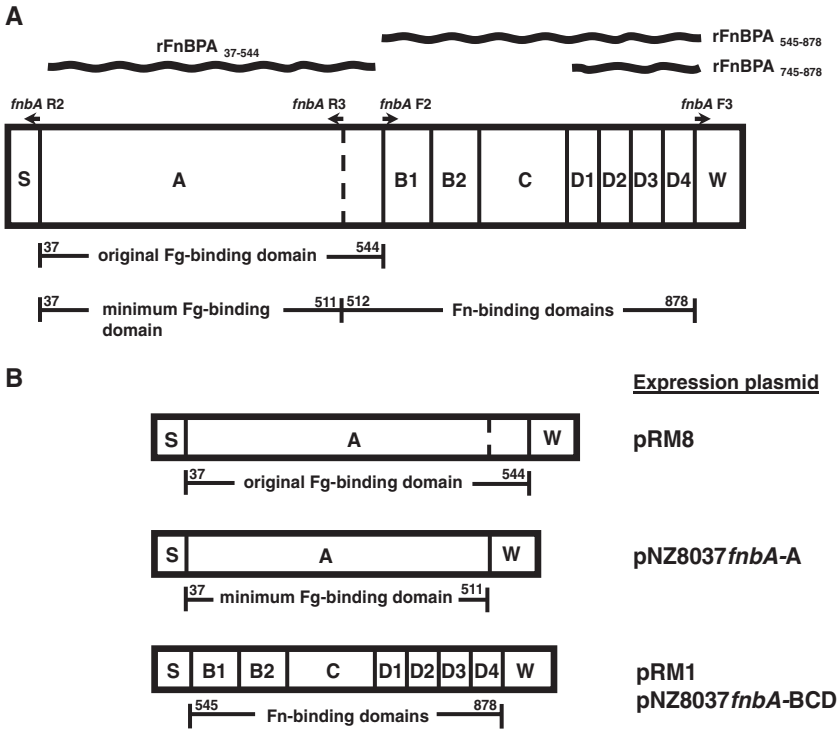


Fig. 1. Schematic diagram showing the domain structure of FnBPA and FnBPA truncates.
A. S, signal sequence; W, wall-spanning and peptidoglycan-anchoring domain. The original and redefined A domain and fibronectin-binding B, C and D domains are indicated. The entire *fnbA* gene is encoded by the *S. aureus* expression vector pFNBA4 and in the *L. lactis* vector pNZ8037*fnbA*. Positions of primers used in constructing *L. lactis* (pNZ8037*fnbA*) deletions are indicated. The extent of recombinant proteins used in this study are shown by the upper wavy lines.
B. Schematic representations of the truncated FnBPA proteins encompassing either the Fg-binding A domain (pRM8 or pNZ8037*fnbA*-A) or the Fn-binding BCD domain (pRM1or pNZ8037*fnbA*-BCD).

mutants and compared with the wild-type strain in platelet-rich plasma (PRP).

Wild-type *S. aureus* Newman cells from the exponential phase of growth had a lag time to aggregation of 3.7 ± 0.3 min compared with stationary phase cells which have a lag time of 1.5 ± 0.25 min (O'Brien *et al.*, 2002). The *clfA* mutant grown to exponential phase had a longer lag time of 6.3 ± 0.2 min ($P < 0.01$; Fig. 2). Although ClfA is expressed maximally during stationary phase (O'Brien *et al.*, 2002; Bischoff *et al.*, 2004; J. Higgins and T.J.F., unpubl. data), where it is the dominant pro-aggregatory surface component, these data also suggest that ClfA contributes to activation by exponential phase Newman cells. The introduction of mutations in the *clfB*, *spa* and *sdrE* genes into the *clfA* mutant strain marginally increased the lag time to aggregation (Fig. 2) indicating a minor role for these proteins.

However, introduction of mutations causing defects in ClfA, ClfB, SdrE and Spa into strain P1 had no effect on the short lag time to activation (1.5–2 min) mediated by the wild-type strain (Fig. 2), indicating that a different protein(s) is responsible for the rapid platelet aggregation phenotype during exponential growth. Possible candidates are the fibronectin-binding proteins FnBPA and FnBPB because they are predominantly expressed during exponential growth (Saravia-Otten *et al.*, 1997) and have been implicated in the pathogenesis of IE (Kuypers and Proctor, 1989; Que *et al.*, 2001; 2005). Accordingly, we transduced the linked *fnbA* and *fnbB* mutations into strain P1, which expresses high levels of FnBPs (Roche *et al.*, 2004), resulting in an FnBP-deficient mutant (P1 *fnbAfnbB*) and platelet aggregation assays were performed with exponentially growing cells. Because strain Newman does not express FnBPs on its cell surface

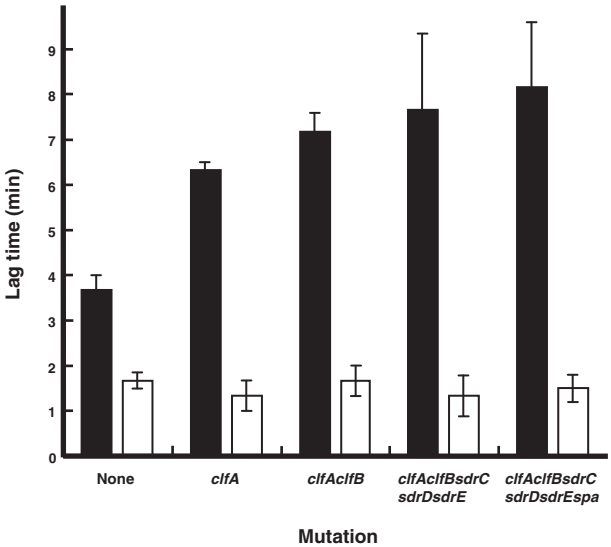


Fig. 2. Platelet aggregation by *S. aureus* strains and their surface protein-deficient mutants. Lag time to platelet aggregation by *S. aureus* strain Newman derivatives (black columns) and *S. aureus* strain P1 derivatives (white columns) grown to exponential phase. Washed bacterial cells were added to PRP samples and the lag times to aggregation were determined. Derivatives of strain Newman and P1 lacking the surface proteins ClfA, ClfB, SdrC, SdrD, SdrE and SpA as indicated were constructed as described in the *Experimental procedures* section.

(Grundmeier *et al*., 2004) it was not included in the *fnb* mutagenesis experiments.

Staphylococcus aureus P1 wild type and DU5998 (P1 *fnbAfnbB*) expressing either FnBPA or FnBPB from multicopy plasmids adhered strongly to immobilized fibronectin in a dose-dependent and saturable manner, whereas DU5998 (P1 *fnbAfnbB*) was defective in binding (Fig. 3A). Ligand affinity blots of cell wall extracts confirmed that *S. aureus* P1 expressed a fibronectin-binding protein with an apparent molecular weight of >175 kDa (Fig. 3B) which was absent in the *fnbAfnbB* mutant. P1 strains expressing FnBPs from multicopy plasmids expressed approximately fourfold more protein than the wild-type strain, as judged by densitometry analysis. Breakdown of native FnBPs in strain P1, as well as plasmid expressed FnBPs, was observed, likely due to bacterial proteases (Signäs *et al*., 1989; Jönsson *et al*., 1991).

Strain DU5998 (P1 *fnbAfnbB*) exhibited a much longer lag time to platelet aggregation (9.25 ± 0.6 min; $n = 3$) than P1 wild type (1.2 ± 0.2 min; $n = 3$; $P < 0.001$; Fig. 3C). This strongly suggests that FnBPA and/or FnBPB are important determinants of platelet aggregation by P1 cells during exponential growth. To confirm this hypothesis, multicopy plasmids expressing either FnBPA or FnBPB were introduced into strain Newman and into the *fnbAfnbB* mutant of strain P1 (DU5998). For DU5998 the lag time was shortened from 9.25 ± 0.7 min to 1.3 ± 0.1 and 1.8 ± 0.2 min by complementing expression of FnBPA or FnBPB respectively ($n = 3$; $P < 0.001$). In strain Newman, expression of FnBPA and FnBPB reduced the lag time to aggregation from 3.7 ± 0.3 min to 1.2 ± 0.1 and 1.3 ± 0.1 min respectively ($n = 3$; $P < 0.001$). The longer lag time of strain Newman compared with P1 in exponential growth is likely to be due to the absence of FnBPs. These data clearly demonstrate that both fibronectin-binding proteins A and B are potent pro-aggregatory surface proteins.

To demonstrate that the aggregation response was the result of platelet activation, *S. aureus* P1 and its derivatives were tested for the ability to stimulate cytosolic calcium flux in platelets, a marker of platelet activation. The P1 wild-type strain and the complemented DU5998 strains expressing either FnBPA or FnBPB stimulated rapid activation, whereas the FnBP-defective DU5998 did not (Fig. 3D). This suggests that the rapid aggregation associated with FnBP-expression is the result of genuine platelet activation, and not agglutination.

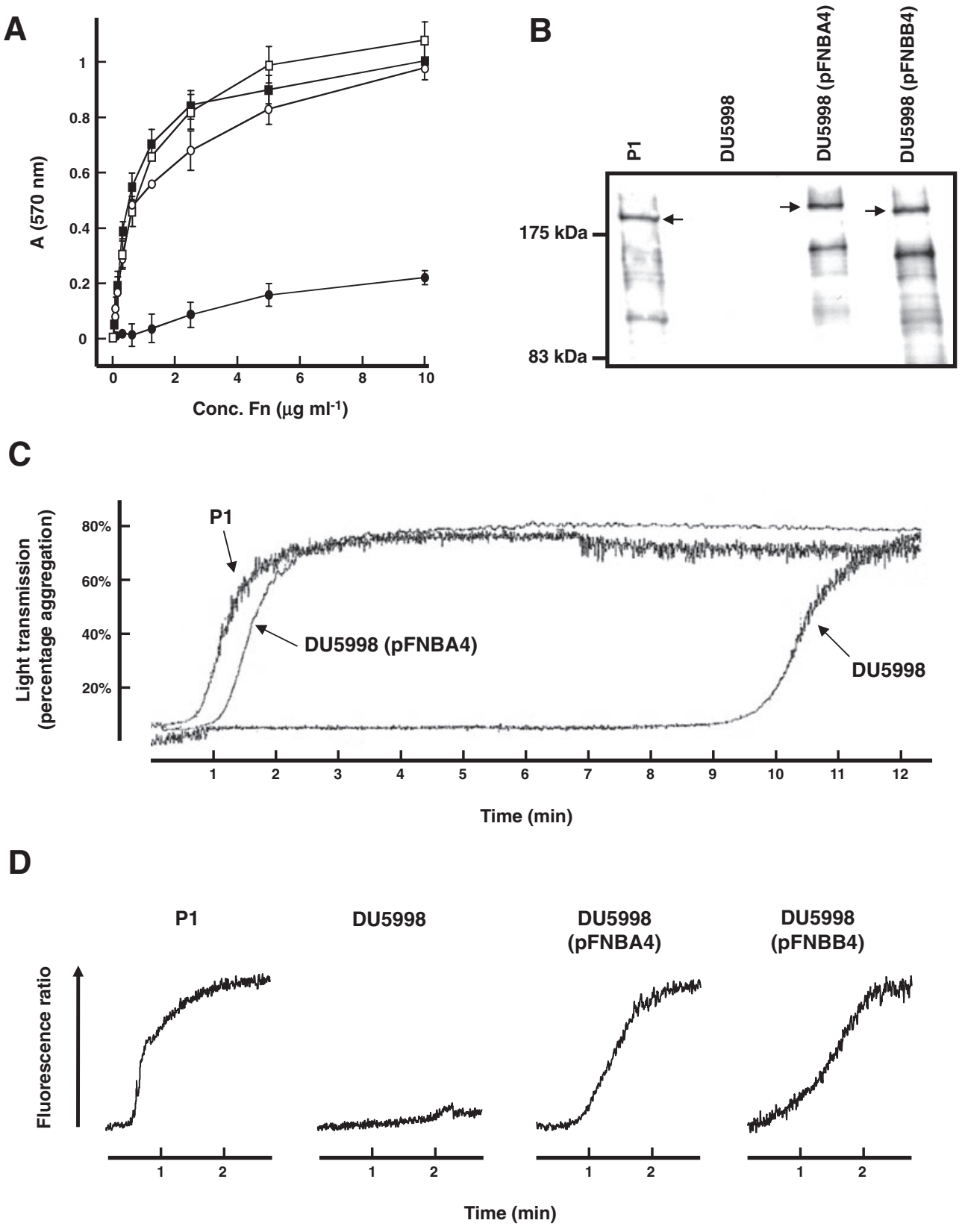
Expression of fibronectin-binding proteins in L. lactis

In order to investigate the role of FnBPs in platelet activation, the *fnbA* and *fnbB* genes were cloned into the nisin-inducible expression vector plasmid pNZ8037. *L. lactis* NZ9800 expressing FnBPA (pNZ8037*fnbA*) adhered strongly to immobilized fibronectin (Fig. 4A) and fibrinogen (Fig. 4B) in a dose-dependent and saturable manner, suggesting that FnBPA was expressed in a fully functional form. Similar results were obtained for *L. lactis* expressing FnBPB (data not shown). *L. lactis* (pNZ8037*fnbA*) stimulated platelet aggregation with a similar lag time to *S. aureus* strains expressing FnBPA (Fig. 4D). In contrast, *L. lactis* (pNZ8037*fnbB*) caused platelet aggregation after a longer lag time (3.8 ± 2.5 min, $n = 3$) suggesting that FnBPB may not be as potent a pro-aggregatory factor as FnBPA or that expression of the protein by *L. lactis* is lower than FnBPA. The *L. lactis* host strain carrying just the expression plasmid pNZ8037 did not stimulate aggregation in any experiments performed (Fig. 4D).

Fibronectin-binding protein A mediates platelet aggregation by two different pathways

The definition of the functional domains of FnBPA has recently been revised (Schwarz-Linek *et al*., 2003). Originally, the protein was divided into the A domain which binds fibrinogen, and domains B, C and D (Fig. 1). Fibronectin-binding activity was previously assigned only to the D repeats (Signäs *et al*., 1989) but recently, multiple repeated fibronectin-binding domains were identified between regions A and D (Massey *et al*., 2001; Schwarz-Linek *et al*., 2003). Indeed, we have observed that a recombinant protein comprising the originally defined region A (residues 37–544) carries a single fibronectin-binding domain which is functional (unpubl. data). Deletion mutants expressing truncated derivatives of FnBPA on the surface of *S. aureus* have been constructed (Massey *et al*., 2001). Plasmid pRM8 expresses on the *S. aureus* cell surface a truncated derivative of FnBPA comprising only the fibrinogen-binding A domain (FnBPA-A) and a single fibronectin-binding domain but lacking region BCD, whereas pRM1 lacks the A domain and expresses only domains BCD (FnBPA-BCD). They were introduced into a mutant of *S. aureus* strain 8325-4 defective in *fnbA* and *fnbB* (Massey *et al*., 2001). FnBPA-A supported adherence to fibrinogen but not to fibronectin (despite the presence of a single fibronectin-binding domain, which must be cryptic when the truncated protein is expressed on the cell surface), while FnBPA-BCD supported adherence to fibronectin but not to fibrinogen. In both cases adhesion occurred with the same affinity as that seen with bacteria containing the parental plasmid pFNBA4 (Massey *et al*., 2001).

Strains were grown to exponential phase and tested for their ability to stimulate platelet aggregation. Strain DU5883 (*S. aureus* 8325-4 defective in *fnbA* and *fnbB*) promoted activation with a lag time of 8.3 ± 0.3 min



whereas DU5883 carrying pFNBA4, pRM1 or pRM8 exhibited rapid lag times of 1–1.3 min; $P < 0.0001$ (Fig. 4C). This demonstrates that both the fibrinogen-binding A domain and the fibronectin-binding BCD domains of FnBPA can independently promote platelet aggregation when expressed in *S. aureus*.

In order to test this further we expressed the equivalent FnBPA truncates in *L. lactis* using the nisin-inducible vector plasmid pNZ8037. The ability of *L. lactis* NZ9800 carrying plasmid pNZ8037*fnbA* expressing the full-length FnBPA protein, pNZ8037*fnbA*-A (expressing the shorter revised domain A variant) and pNZ8037*fnbA*-BCD (expressing a truncate lacking domain A) to adhere to immobilized fibrinogen and fibronectin was measured. *L. lactis* (pNZ8037*fnbA*) and *L. lactis* (pNZ8037*fnbA*-BCD) adhered to immobilized fibronectin with similar affinities, while *L. lactis* (pNZ8037*fnbA*-A) did not (Fig. 4A). *L. lactis* (pNZ8037*fnbA*-BCD) did not bind immobilized fibrinogen, whereas both *L. lactis* (pNZ8037*fnbA*) and *L. lactis* (pNZ3087*fnbA*-A) bound strongly (Fig. 4B). *L. lactis* (pNZ3087*fnbA*) adhered slightly more strongly to fibrinogen than *L. lactis* (pNZ3087*fnbA*-A).

Lactococcus lactis expressing wild-type FnBPA or its truncated derivatives mediated platelet aggregation with short lag times (1–1.2 min) similar to the *S. aureus* variants described above (Fig. 4D). These data indicate that FnBPA does not require any additional *S. aureus*-specific surface components to stimulate platelet aggregation.

To confirm that *L. lactis* expressing the FnBPA variants stimulated platelet activation, calcium flux experiments were performed. Representative calcium flux traces are shown in Fig. 4E, demonstrating that *L. lactis* strains expressing the FnBPA truncates, as well as the full length protein, caused a calcium flux in FURA-2-AM labelled platelets. *L. lactis* carrying the pNZ8037 expression vector did not stimulate activation (Fig. 4E). This confirms the suggestion that platelet activation mediated by FnBPA-expressing bacteria occurs through either the fibrinogen-binding A domain or the fibronectin-binding BCD domains.

Inhibition of FnBPA-mediated platelet activation

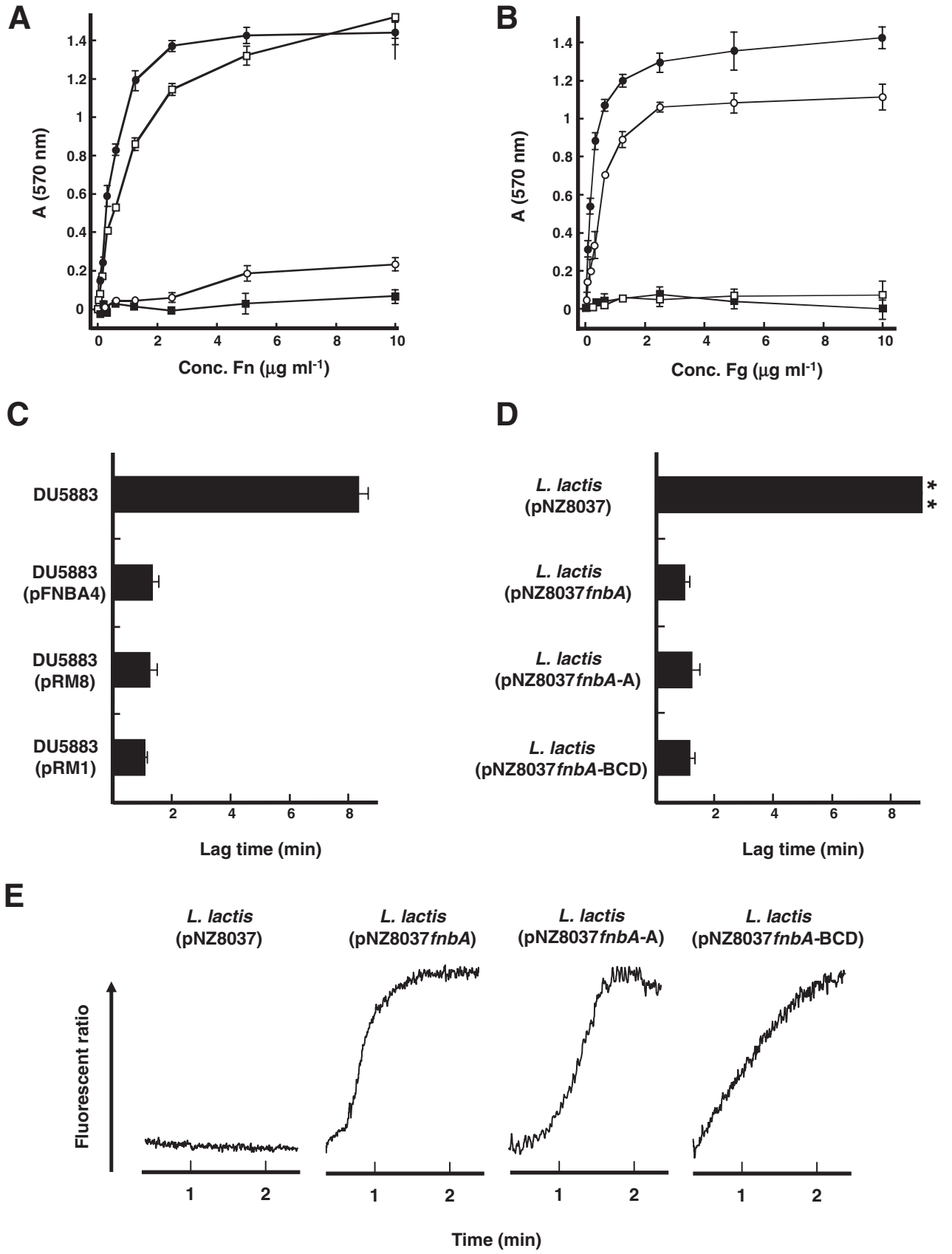
In order to investigate the possible role of fibronectin in promoting activation of platelets by bacteria expressing FnBPs, a recombinant protein corresponding to the fibronectin-binding D1-D4 repeat region of FnBPA (rD1-D4) was purified and incubated with PRP before platelet activation with *S. aureus* DU5883 expressing full length FnBPA or the truncates. rD1-D4 completely inhibited platelet aggregation by *S. aureus* expressing FnBPA-BCD, presumably by binding and sequestering fibronectin in plasma, but did not inhibit aggregation by strains expressing either full length FnBPA or the A domain alone (Fig. 5).

A monoclonal antibody (7C5) that recognizes the A domain of FnBPA and blocks adherence to immobilized fibrinogen (data not shown) completely inhibited platelet aggregation by bacteria expressing only FnBPA-A but did not affect platelet aggregation caused by cells expressing the full length FnBPA protein or the BCD region alone (Fig. 5). This strongly suggests FnBPA-A binds fibrinogen in order to stimulate activation.

Role of fibrin in platelet activation by FnBPA

A recent report found that soluble fibrin is the main mediator of *S. aureus* adhesion to activated platelets (Niemann *et al.*, 2004). To address the role of fibrin in platelet activation promoted by FnBPA, aggregation experiments were performed with PRP prepared from whole blood that had been treated with the thrombin inhibitor PPACK to prevent fibrin formation. Strain DU5998 (pFNBA4) stimulated aggregation of PRP prepared using PPACK with lag times of 3.2 ± 0.3 min; $72 \pm 6\%$ aggregation; $n = 3$. Similarly, *L. lactis* (pNZ8037*fnbA*) induced aggregation in PPACK PRP with lag times of 2.7 ± 0.3 min, $79 \pm 8\%$ aggregation, $n = 3$. Lag times to aggregation in PRP prepared from PPACK were slightly extended compared with PRP from the same donors that had been prepared using citrate (mean lag time of 1.8 ± 0.2 min; $75 \pm 10\%$ aggregation; $n = 5$). These data suggest that fibrin formation is not of major importance in platelet activation promoted by FnBPA.

Fig. 3. FnBPA and FnBPB mediate aggregation of human platelets in plasma.
A. Adherence of *S. aureus* P1 (closed squares), DU5998 (P1*fnbAfnbB*) (closed circles), DU5998 (pFNBA4) (open squares) and DU5998 (pFNBB4) (open circles) to immobilized fibronectin (0.04–10 μg ml^{-1}).
B. Cell wall-associated proteins of *S. aureus* P1, DU5998 (P1*fnbAfnbB*) and DU5998 expressing either FnBPA or FnBPB from multicopy plasmids pFNBA4 and pFNBB4 were released from stabilized protoplasts by lysostaphin digestion. Extracts were separated on 7.5% SDS-PAGE gels, electroblotted onto PVDF membranes, and probed with biotinylated human fibronectin (30 μg ml^{-1}). Membranes were washed and incubated with peroxidase-conjugated streptavidin and developed by chemiluminescence. The arrows indicate the position off the full length FnBP.
C. Representative aggregation trace obtained upon incubation of *S. aureus* P1 (lag time 1 min), DU5998 (lag time 10 min) and DU5998 (pFNBAA4) (lag time 1.2 min) in PRP. Cells of each strain were grown to exponential phase and washed in PBS prior to addition to PRP. Each strain stimulated comparable levels of aggregation (percentage aggregation), as measured by the degree of light transmission.
D. Stimulation of calcium flux by *S. aureus* P1 derivatives. Platelets were loaded with FURA-2-AM, and Ca^{2+}-mobilizing responses measured by fluorescence detection following excitation at wavelengths 340 and 380 nm, with emission at 510 nm. Increases in the fluorescent ratio indicate increases in intracellular Ca^{2+} characteristic of platelet activation.



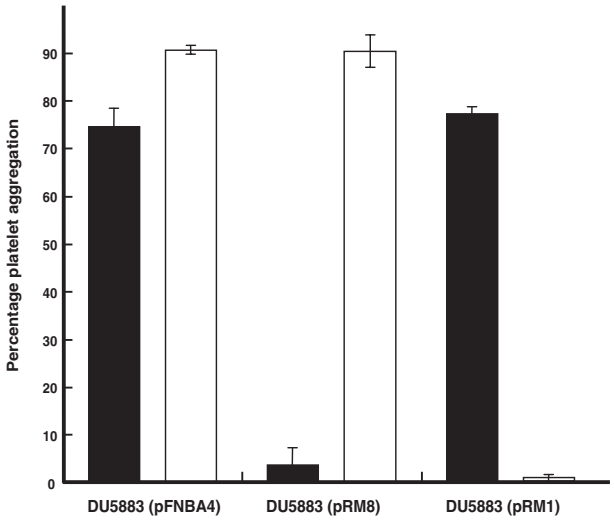


Fig. 5. Inhibition of FnBPA-mediated platelet activation. Percentage aggregation in PRP by *S. aureus* DU5883 complemented with plasmids expressing FnBPA or FnBPA truncates. Bacterial samples were incubated with the monoclonal antibody MAb 7C5 (20 μg ml^{-1}; closed columns) for 20 min before addition to PRP. PRP samples were incubated with rFnBPA 745–878 protein (Fig. 1A) (rD1-D4; 100 μg ml^{-1}, open columns) for 20 min before addition of bacterial cells.

Interaction of L. lactis *expressing FnBPA with gel-filtered platelets (GFPs)*

In order to investigate if additional plasma protein(s) are required for activation of platelets by bacteria expressing FnBPA, platelets were purified from plasma by gel filtration (GFPs). Fibrinogen was added to GFP at physiological concentrations (1 mg ml^{-1}) because it is essential for platelet-platelet cross-linking during aggregation after platelet activation has been stimulated. Fibrinogen was purified by anion exchange chromatography to remove contaminating plasma proteins. *L. lactis* expressing full length FnBPA, the A domain or the BCD region were unable to stimulate activation of GFP in the presence of purified fibrinogen (Fig. 6). These data indicate that additional plasma factors are required.

When purified pooled human IgG was added to GFP and purified fibrinogen, FnBPA-expressing *L. lactis* cells caused aggregation to the same level as in PRP (> 70% aggregation; Fig. 6) and with the same rapid lag time of 1–2 min. Similar results occurred with *L. lactis* expressing the FnBPA A domain alone (Fig. 6). This suggests that antibodies are required along with fibrinogen for activation mediated by the A domain. Direct measurement of activation by *L. lactis* expressing only the A domain confirmed that both fibrinogen and IgG were required for activation (Fig. 7).

In contrast, a low level of aggregation of GFP in the presence of pure fibrinogen and IgG was stimulated by *L. lactis* (pNZ8037*fnbA*-BCD). However, rapid high-level aggregation occurred when purified human fibronectin was added (Fig. 6). Fibronectin did not stimulate activation in the absence of IgG. This suggests that bacteria expressing the BCD region of FnBPA are linked to a receptor on the platelet surface by a fibronectin bridge and that, in addition, IgG is required to stimulate activation.

FnBPA-specific antibodies are required for activation

In order to investigate if antibodies that are specific for the surface protein engaged in interaction with platelets are

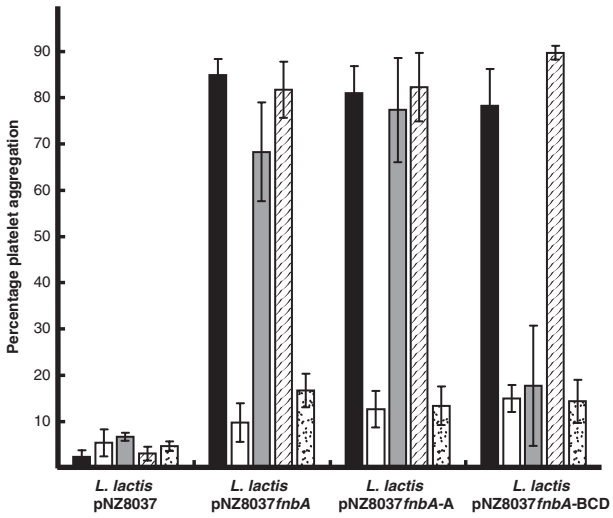


Fig. 6. Contribution of plasma proteins to FnBPA-mediated platelet activation. Percentage aggregation of gel-filtered platelets (GFP) by *L. lactis* expressing full length FnBPA or FnBPA truncates. Bacteria were incubated in GFP plus fibrinogen (blank columns); GFP plus fibrinogen and human IgG (grey columns); GFP plus fibrinogen, IgG and fibronectin (hatched columns) and GFP plus fibrinogen and fibronectin (stippled columns). Final concentrations of plasma proteins added to GFP samples were 1 mg ml^{-1} fibrinogen, 4 mg ml^{-1} pooled human IgG and 300 μg ml^{-1} fibronectin. Percentage aggregation for each strain in PRP (black columns) is also shown.

Fig. 4. The Fg-binding A domain and the Fn-binding BCD domains of FnBPA can independently activate platelets leading to platelet aggregation. A and B. Adherence to immobilized fibronectin (A) and fibrinogen (B) of *L. lactis* (pNZ8037) (closed squares), *L. lactis* (pNZ8037*fnbA*) (closed circles), *L. lactis* (pNZ8037*fnbA*-A) (open circles) and *L. lactis* (pNZ8037*fnbA*-BCD) (open squares).
C. Lag time to aggregation in PRP of *S. aureus* DU5883 (*fnbAfnbB*) and derivatives containing the multicopy plasmids pFNBA4 (full-length FnBPA), pRM8 (FnBPA A domain) and pRM1 (FnBPA BCD domain).
D. Lag time to aggregation in PRP of *L. lactis* expressing full-length FnBPA, FnBPA A domain or FnBPA BCD domain. Double asterisks indicate no aggregation occurred after 25 min incubation for *L. lactis* containing the expression vector pNZ8037.
E. Stimulation of calcium flux by *L. lactis* expressing FnBPA variants. The *L. lactis* host strain carrying the empty pNZ8037 expression vector did not stimulate activation. The traces shown are representative of three independent experiments.

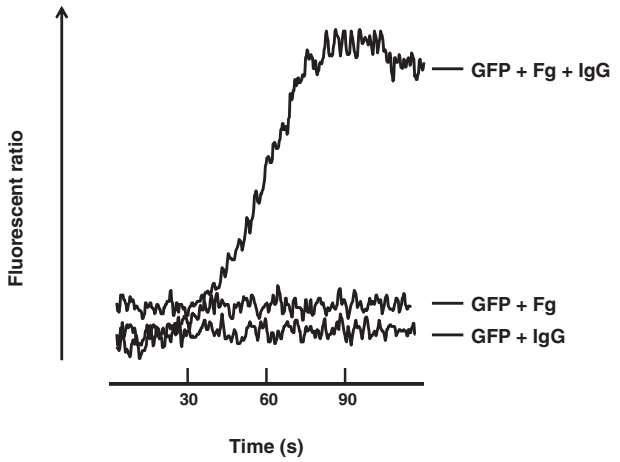


Fig. 7. FnBPA A domain-mediated platelet activation. Stimulation of calcium flux by *L. lactis* expressing the FnBPA A domain truncate. FURA-2-AM loaded gel-filtered platelets (GFP; 8×10^7 platelets) were suspended in 0.4 ml of JNL buffer containing fibrinogen (Fg; 1 mg ml^{-1}) and/or pooled human IgG (4 mg ml^{-1}) as indicated. FURA-2 fluorescence was measured after addition of washed *L. lactis* (pNZ8037*fnbA*-A) cells (10^8 cells in 50 μl of PBS) to GFP samples.

required for activation or if antibodies recognizing other surface components such as peptidoglycan or teichoic acid might be sufficient, antibodies recognizing the FnBPA A domain and/or the BCD domains in complex with fibronectin were absorbed from pooled IgG. It should be noted that the immune response to FnBPs is predominantly against epitopes created when the unfolded BCD domain binds the N-terminal F1 modules of fibronectin (Casolini *et al*., 1998).

IgG depleted of antibodies recognizing both the A domain of FnBPA and the BCD domain/fibronectin complex supported much reduced aggregation of GFPs by either *S. aureus* (Fig. 8A) or *L. lactis* (Fig. 8B) expressing FnBPA. The pooled IgG that was depleted of antibodies recognizing only the A domain of FnBPA failed to support aggregation of GFP by *L. lactis* (pNZ8037*fnbA*-A), but retained the ability to support aggregation by *L. lactis* (pNZ8037*fnbA*-BCD) (Fig. 8C). Conversely, IgG depleted of antibodies recognizing the BCD domain in complex with fibronectin failed to support aggregation by *L. lactis* (pNZ8037 *fnbA*-BCD) but retained the ability to support aggregation by *L. lactis* (pNZ8037*fnbA*-A) (Fig. 8). This demonstrates that antibodies specific to the particular bacterial surface component that engage the quiescent platelet are required to stimulate activation.

A monoclonal antibody specific for the FcγRIIa platelet receptor inhibits platelet activation by bacteria expressing FnBPA, and by S. aureus *clinical isolates*

The importance of IgG in FnBPA-mediated platelet activation suggests a possible role for the FcγRIIa platelet receptor. The monoclonal antibody IV-3 blocks the interaction of the FcγRIIa receptor on platelets with the Fc region of IgG. Platelet aggregation in PRP promoted by *L. lactis* expressing FnBPA, the FnBPA A domain and FnBPA BCD domains was completely inhibited by IV-3 (Table 1). This indicates a crucial role for FcγRIIa in FnBPA-mediated platelet aggregation stimulated both via the A domain and the BCD domain and supports the conclusion about the absolute requirement for IgG.

We then investigated the possibility that IV-3 could inhibit platelet activation by *S. aureus* clinical isolates. In

Table 1. The effect of FcγRIIa blockade on platelet aggregation.

	Lag time[a] in PRP		Lag time in PRP with IV-3[b]	
Strain	Exponential phase	Stationary phase	Exponential phase	Stationary phase
L. lactis (pNZ8037)	ND	25 ± 0	ND	25 ± 0
L. lactis (pNZ8037*fnbA*)	ND	0.9 ± 0.1	ND	25 ± 0
L. lactis (pNZ8037*fnbA*-A)	ND	1 ± 0.1	ND	25 ± 0
L. lactis (pNZ8037*fnbA*-BCD)	ND	1 ± 0.1	ND	25 ± 0
Newman	3.5 ± 0.3	1 ± 0.1	25 ± 0	25 ± 0
P1	1 ± 0	1.6 ± 0.1	25 ± 0	25 ± 0
079	2.25 ± 0	1.9 ± 0.3	25 ± 0	25 ± 0
080	1.8 ± 0.3	2 ± 0.3	25 ± 0	25 ± 0
126	1 ± 0	1.1 ± 0.2	25 ± 0	25 ± 0
151	1.3 ± 0.2	2.0 ± 0.3	25 ± 0	25 ± 0
152	1.5 ± 0.1	2.0 ± 0.4	25 ± 0	25 ± 0
206	0.75 ± 0	1.4 ± 0.2	25 ± 0	25 ± 0
208	1.0 ± 0.25	2.1 ± 0.2	25 ± 0	25 ± 0
209	1.0 ± 0	3.1 ± 0.4	25 ± 0	25 ± 0

Table showing the lag time to aggregation in response to *L. lactis* and *S. aureus* strains in both exponential and stationary growth phase. The numbered strains are clinical endocarditis strains.

a. Lag times (in minutes) are presented as Mean ± SEM for three separate experiments.

b. PRP samples were incubated with IV-3 (20 μg ml^{-1}) for 20 min before addition of bacterial cells. 25 ± 0 indicates no aggregation after 25 min. ND, not determined.

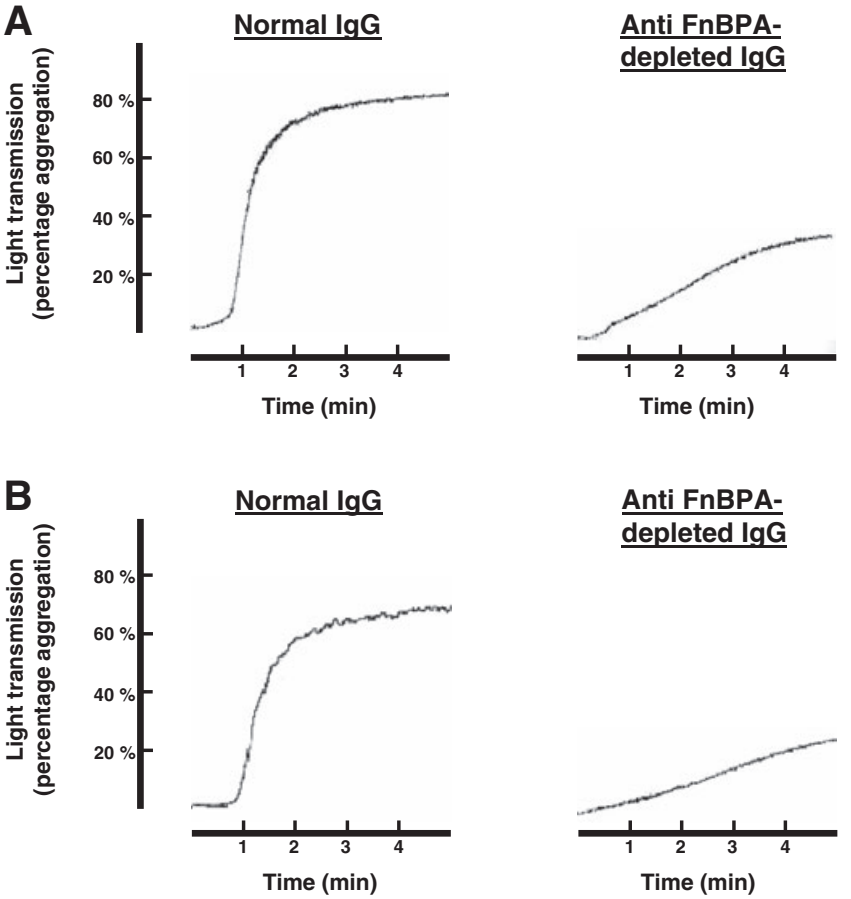


Fig. 8. Role of specific IgG in FnBPA-mediated platelet activation. GFPs were supplemented with pure fibrinogen and IgG samples as described in Fig. 6.
A and B. Representative aggregation traces stimulated by DU5998 (pFnBA4) (A) and *L. lactis* (pNZ8037*fnbA*) (B) in the presence of normal IgG or IgG depleted of antibodies recognizing FnBPA.
C. Showing percentage aggregation by *L. lactis* expressing the FnBPA A domain (pNZ8037*fnbA*-A; closed columns) or the FnBPA BCD domain (pNZ8037*fnbA*-BCD; open columns) in GFP and pure fibrinogen after addition of normal pooled human IgG, normal pooled human IgG depleted of A domain-specific antibodies or normal pooled human IgG depleted of BCD domain-specific antibodies.

addition to strains Newman and P1, we tested eight *S. aureus* strains isolated from patients with IE. Bacteria were grown to the exponential and stationary phases of growth where different repertoires of surface proteins are present (Saravia-Otten *et al*., 1997; McAleese *et al*., 2001; O'Brien *et al*., 2002) and the lag time to platelet aggregation in PRP determined. The lag times ranged from 1 min to 3.5 min (Table 1). Antibody IV-3 completely inhibited platelet activation by each strain from both phases of growth. This suggests that the interaction between antibody bound to the bacterial surface and the FcγRIIa receptor is of fundamental importance to *S. aureus*-induced platelet activation.

Adhesion of resting platelets to FnBPA-expressing bacterial cells is mediated by fibrinogen and fibronectin bridging to platelet GPIIb/IIIa

The above data suggest that an important event in FnBP-expressing *S. aureus* or *L. lactis* initiating the stimulation of resting platelets is binding of bacteria by a fibronectin or fibrinogen bridge to platelet receptors for the plasma proteins. In order to test this directly, and to identify the platelet receptor involved, adhesion of washed resting platelets to immobilized bacteria was measured. Platelets adhered weakly to immobilized *S. aureus* P1 and its derivatives (Fig. 9A). Adhesion increased three- to fourfold for *S. aureus* P1 and DU5998 (pFNBA4) in the presence of plasma, fibrinogen, or fibronectin. Adhesion of platelets to DU5998 was promoted in the presence of plasma and fibrinogen, but not to the same extent as for FnBPA-expressing strains. Fibronectin did not support platelet adhesion to DU5998 (Fig. 9A). Fibrinogen-dependent adhesion to DU5998 is likely mediated by clumping factors A and B. Fibrinogen and fibronectin-dependent adhesion of platelets to *S. aureus* was completely inhibited by the GPIIb/IIIa-specific blocking monoclonal antibody abciximab (Fig. 9A). Monoclonal antibody 7C5 and recombinant FnBPA D1-D4 protein blocked *L. lactis* (FnBPA-A)- and *L. lactis* (FnBPA-BCD)-promoted platelet adhesion, respectively, but not that of *L. lactis* expressing full length FnBPA (data not shown).

Similarly, plasma, fibrinogen and fibronectin increased platelet adhesion to *L. lactis* expressing FnBPA approximately threefold compared with GFP alone (Fig. 9B). Plasma proteins did not support platelet adhesion to the *L. lactis* host strain. Adhesion was dependent on GPIIb/IIIa, as it was blocked by abciximab (Fig. 9B). These data demonstrate that FnBPA-dependent platelet binding is mediated through fibrinogen-bridging (involving the A domain) or fibronectin-bridging (involving the BCD domain) to GPIIb/IIIa on resting platelets, even though it is in the low affinity conformation.

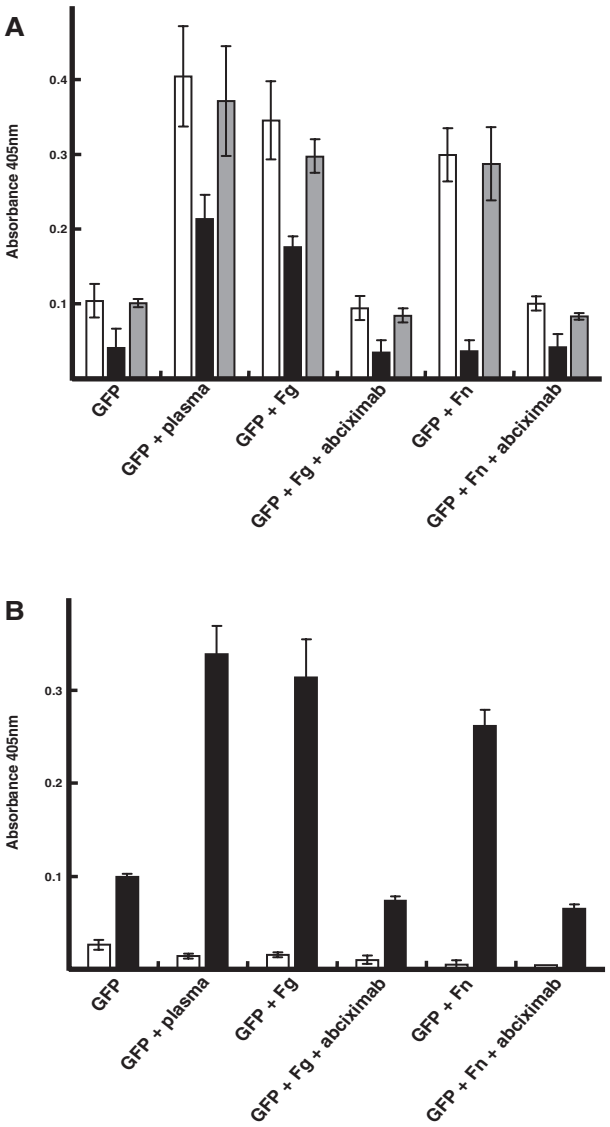


Fig. 9. FnBPA-mediated bacterial adherence to platelets. The ability of GFPs to adhere to immobilized bacterial cells was measured. GFPs were supplemented with plasma (10%), fibrinogen (Fg; 1 mg ml^{-1}) or fibronectin (Fn; 300 μg ml^{-1}) with or without the anti-GPIIb/IIIa antibody abciximab (20 μg ml^{-1}) where indicated.
A. Adhesion of GFPs to exponential phase cells of *S. aureus* P1 (open columns), DU5998 (closed columns) and DU5998 (pFNBA4) (grey columns).
B. Adhesion of GFPs to *L. lactis* (pNZ8037) (blank columns) or *L. lactis* expressing FnBPA (black columns).
Adherent platelets were detected using a substrate for acid phosphatase and measuring the absorbance of the wells at 405 nm. This experiment was performed using platelets from three different donors.

Discussion

We showed previously that ClfA expression by strain Newman in the stationary phase of growth is an important bacterial surface factor in promoting platelet activation (O'Brien *et al*., 2002; Loughman *et al*., 2005). Here we investigated surface proteins on bacteria from the expo-

nential phase of growth that promoted activation and compared strain Newman with strain P1, which most likely better represents clinical isolates. There was a notable difference in the activation of platelets by strains Newman and P1. Wild-type Newman stimulated activation with a longer lag time (~3.5 min) than P1 (~1.5 min). The presence of ClfA on cells from the exponential phase was in part responsible for activation promoted by Newman. In contrast the *clfA* mutant of P1 activated platelets with the same short lag time as the wild-type strain. Systematic elimination of ClfB, SdrE and Spa, factors that had previously been shown to contribute to activation by strain Newman in stationary phase (O'Brien *et al*., 2002) along with ClfA had no effect in extending the lag time to platelet activation by P1 cells grown to exponential phase.

The fibronectin-binding proteins have been shown by several investigators to be virulence factors in experimental endocarditis (Kuypers and Proctor, 1989; Que *et al*., 2001; 2005). They are expressed predominantly in the exponential phase of growth (Saravia-Otten *et al*., 1997). Strain Newman is defective in expression of cell surface bound FnBPs and adheres poorly to the ligand (Grundmeier *et al*., 2004). We constructed mutations in the *fnbA* and *fnbB* genes in strain P1 and found that the mutants were severely attenuated for platelet aggregation. When the mutants were complemented by plasmids encoding either FnBPA or FnBPB, a lag time similar to that of the wild-type strain was restored. Furthermore, we could confer a platelet aggregation phenotype upon the non-aggregating bacterium *L. lactis* by expression of FnBPA or FnBPB. Thus, it appears that FnBPA and FnBPB are probably the major mediators of platelet aggregation by *S. aureus* during the exponential phase of growth, the *in vitro* growth phase most likely reflecting bacteria growing in the blood stream during endovascular infection.

A recent report (Heilmann *et al*., 2004) showed that FnBPA but not FnBPB could stimulate activation of platelets in PRP when expressed by *S. carnosus*. It is difficult to reconcile the failure to demonstrate FnBPB-promoted platelet activation with our data that clearly demonstrate activation promoted by FnBPB in different strains of *S. aureus,* and in *L. lactis*. Perhaps the strain of *S. carnosus* (pFnBB4) used in the experiments expressed the protein at a reduced level that was insufficient to stimulate platelet activation.

In order to characterize the regions within FnBPA that are important for its interaction with platelets, we analysed the interaction of platelets with *S. aureus* and *L. lactis* strains expressing the full length FnBPA protein, the fibrinogen-binding A domain only or the fibronectin-binding BCD region only. We found that both the A domain and the BCD region could independently stimulate platelet aggregation in PRP. By measuring calcium flux we were able to demonstrate that that the platelet aggregation induced by both the A domain and BCD region of FnBPA was the result of true platelet activation.

A function-blocking monoclonal antibody (7C5) specific for the fibrinogen binding A domain of FnBPA inhibited platelet aggregation mediated by *S. aureus* expressing the fibrinogen-binding A domain alone. This suggests that binding of fibrinogen to the FnBPA A domain is required for A domain-mediated platelet activation. Aggregation of GFPs by *L. lactis* expressing FnBPA-A required only the presence of fibrinogen and IgG, indicating that no other plasma proteins were required for activation. Direct measurement of activation by calcium flux confirmed that fibrinogen binding was absolutely required for activation by the A domain.

The involvement of fibrinogen in binding the A domain of FnBPA to a platelet receptor is consistent with our recent findings with ClfA promoted platelet activation (Loughman *et al*., 2005). By using a mutant of ClfA that could not bind fibrinogen it was shown that bacterial adhesion to resting platelets and their subsequent activation by ClfA-expressing bacteria required fibrinogen to form a bridge between the bacterial cell and the low affinity form of the platelet integrin GPIIb/IIIa. The A domains of FnBPA and ClfA bind to the same region in the γ-chain of fibrinogen so it is reasonable to assume that they interact with platelets in a similar manner.

We also demonstrated that fibronectin is required for platelet activation promoted by the BCD region of FnBPA. Recombinant fibronectin-binding D1-D4 domains from FnBPA completely inhibited *S. aureus* or *L. lactis* expressing the BCD domain of FnBPA from mediating platelet aggregation, presumably because the recombinant protein bound and sequestered fibronectin in plasma. Purified fibrinogen (commercial fibrinogen preparations contain contaminating fibronectin) did not support aggregation of purified platelets by bacteria expressing FnBPA-BCD unless fibronectin was added. It is likely that the platelet receptor for the fibronectin attached to the bacterial surface is the integrin GPIIb/IIIa which is known to bind fibronectin at multiple sites, including the RGD motif in module 10 (Bowditch *et al*., 1991; Kauf *et al*., 2001). This is supported by the observation that fibronectin-dependent adherence of resting platelets to bacteria was inhibited by a monoclonal antibody specific for GPIIb/IIIa.

A previous report has suggested a role for soluble fibrin in *S. aureus*-mediated platelet aggregation (Niemann *et al*., 2004). However, fibrin is generated by thrombin and this would only occur after platelet activation. The events studied here relate to the initial activation steps. The use of the thrombin inhibitor PPACK slightly delayed lag time suggesting that soluble fibrin may enhance the aggregation process but it is not necessary for activation.

IgG specific for FnBPA was shown to be required for *L. lactis* expressing FnBPA to promote activation along

with fibronectin (for the BCD region) or fibrinogen (for the A region). Washed platelets were only activated by BCD domain- or A domain-expressing bacteria when pooled IgG was added. When antibodies specific for the A domain or the BCD domain in complex with fibronectin were removed from pooled IgG, activation by bacteria expressing the appropriate FnBPA domain was prevented. Furthermore, both A- and BCD-promoted activation was inhibited by a monoclonal antibody that blocks FcγRIIa. This supports the requirement for IgG.

Pooled IgG depleted of antibodies to both the A and BCD regions of FnBPA failed to support activation by *L. lactis* or *S. aureus* expressing FnBPA indicating that efficient activation can only be achieved by antibodies that recognize the surface protein that initially binds the resting platelet. Antibodies to other surface proteins could not substitute.

Based on our data we propose a model for FnBPA-mediated platelet activation (Fig. 10). FnBPA binds to soluble fibrinogen and/or fibronectin molecules present in blood and binds them on the bacterial cell surface. The low affinity form of the platelet integrin GPIIb/IIIa on resting platelets can recognize these ligands and bind to them, creating a bacterial-ligand-platelet cross-bridge. Platelets thus differ from epithelial cells which use integrin $\alpha_5\beta_1$ to bind fibronectin attached to the *S. aureus* FnBPs (Sinha *et al*., 1999). Antibodies recognizing neo-epitopes formed by the interaction between FnBPs and fibronectin predominate in IgG generated by the immune response in humans to this region of the FnBPs (Casolini *et al*., 1998). Furthermore, it is likely that two molecules of fibronectin can be accommodated by the multiple fibronectin-binding domains in FnBPA (Matsuka *et al*., 2003). The N-terminal type I modules of fibronectin bind FnBPs and the RGD sequence in type III module 10 is most likely recognized by nascent GPIIb/IIIa (Potts and Campbell, 1996; Kauf *et al*., 2001). The A domain of FnBPA binds to the extreme C-terminus of the γ-chain of fibrinogen (Wann *et al*., 2000) that protrudes from the D domains at either end of the molecule. Thus, the other D domain is free to interact with GPIIb/IIIa. Although the platelet integrin is in a quiescent state in resting platelets, bacteria coated in either fibronectin or fibrinogen behave like surfaces and can attach firmly to the platelet surface via these bridges.

However, this bacterium–platelet interaction is not in itself sufficient for activation to occur. In addition, IgG bound to FnBPA cross-links the complex to the FcγRIIa platelet receptor causing receptor clustering which triggers platelet activation leading to aggregation and thrombus formation. This model is consistent with Sjöbring *et al*. (2002) who investigated thrombus formation by *Streptococcus pyogenes* and *S. aureus*. Antibodies specific for *S. pyogenes* M-protein were required for M protein-mediated thrombus formation and anti-FcγRIIa antibody (IV-3) prevented thrombus formation by immobilized *S. pyogenes* and *S. aureus* cells. Involvement of FcγRIIa has also been proposed for *S. sanguis* (Ford *et al*., 1997) and *H. pylori* (Byrne *et al*., 2003) as well as platelet-bound fibrinogen-promoted aggregation (Chen *et al*., 2003). Very recently, we showed that ClfA-mediated platelet activation was mediated through FcγRIIa (Loughman *et al*., 2005).

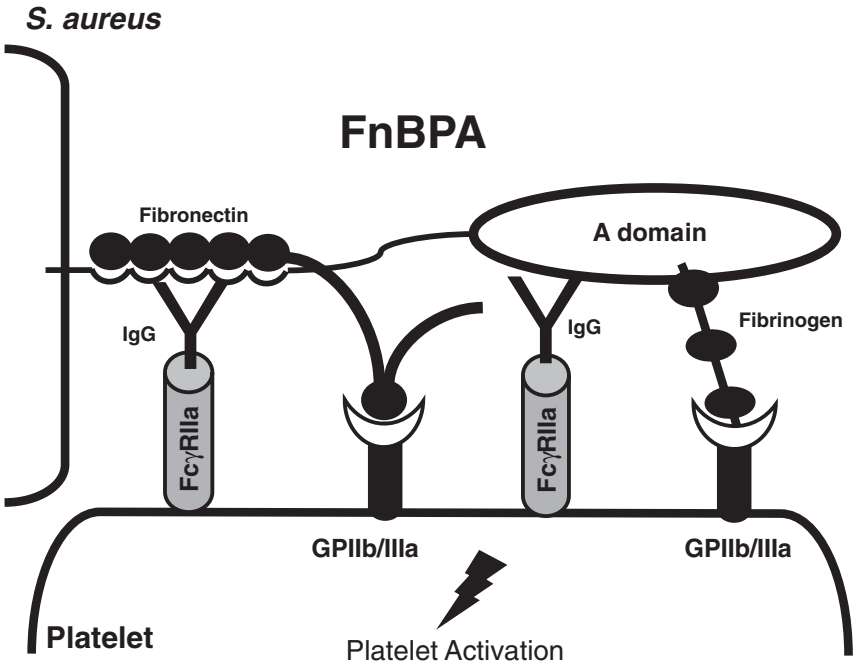


Fig. 10. Model for platelet activation by FnBPA. The diagram indicates the proposed interactions between FnBPA and platelet receptors. Part of the unfolded BCD region of FnBPA is shown in complex with the N-terminal domain of fibronectin linked to the FcγRIIa receptor by specific antibody recognizing neo-epitopes. Fibronectin is also bound to GPIIb/IIIa by its RGD motif. The A domain of FnBPA is linked to receptors by specific anti-A domain antibodies to FcγRIIa and by a fibrinogen bridge to GPIIb/IIIa.

In the study described here inhibition of platelet aggregation by clinical strains of *S. aureus* with monoclonal antibody IV-3 antibody suggests a common mechanism of platelet activation by *S. aureus* surface proteins via FcγRIIa. The majority of bacterial pathogens causing IE are commensals and most healthy individuals have antibodies that recognize their surface components. It seems that bacteria exploit these antibodies to cause platelet activation and thrombus formation. This may promote survival in the hostile environment of the host's bloodstream. Finally binding FcγRIIa may represent a common strategy employed by bacterial pathogens of the vascular system and importantly suggests a novel target to combat vascular infections.

Experimental procedures

Antibodies, chemicals and enzymes

Abciximab was from Eli Lily, Basingstoke, UK. IV-3 antibody was kindly provided by R. Klimkowski, Medarex, NJ, USA. Monoclonal antibody 7C5 was produced as previously described (Kohler and Milstein, 1975) by immunization of mice with recombinant FnBPA region A resulting in an IgGb-κ isotype antibody. Human fibrinogen and fibronectin were from Calbiochem, Nottingham, UK. Purified normal human IgG (Gammagard) was from Baxter, UK. Enzymes for DNA manipulation were from Roche, UK, New England Biolabs, MA, USA, and Stratagene, La Jolla, CA. Primers for polymerase chain reaction (PCR) amplification were from Sigma-Genosys.

Bacterial strains, plasmids and growth conditions

Strains and plasmids are summarized in Table 2. *S. aureus* cells were grown in brain-heart infusion (BHI) broth (Oxoid, Basingstoke, UK) at 37°C with shaking (200 r.p.m.). *L. lactis* NZ9800 was used for plasmid cloning and heterologous expression of *S. aureus* surface proteins. *L. lactis* NZ9800 was routinely grown statically on M17 agar or broth (Difco, BD, NJ) overnight, incorporating 0.5% glucose at 30°C and induced with nisin at a concentration of 1.6 ng ml^{-1}. The following antibiotics were incorporated into the media where appropriate: erythromycin (5 or 10 μg ml^{-1}), kanamycin (100 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}) and tetracycline (2 μg ml^{-1}).

Staphylococcus aureus *mutants*

A frameshift mutation was constructed in the *clfA* gene by replacing bases 110–114 with a HindIII site. An upstream *clfA* sequence of 890 bp was amplified by PCR with primers F1 EcoRI and R6 HindIII (Table 3) and a downstream *clfA* sequence of 1526 bp was amplified with primers and F7 HindIII and R7 BamHI (Table 3). The introduced 5′ restriction sites facilitated creating the mutation and simultaneously cloning the joined fragments between EcoRI and BamHI sites in pBluescript II SK. This construct was then ligated with pTS*erm*C using the BamHI sites in both plasmids to form pJH1. The temperature sensitive shuttle plasmid bearing the ΔclfA mutation was transferred into *S. aureus* Newman and P1 selecting for Em^r at 30°. Broth cultures were grown at 30° with Em followed by three cycles of temperature shifting alternatively growing at 43° with Em and at 30° without selection (Foster, 1998). Single colonies were tested for loss of clumping factor activity and the *clfA5* mutations validated by PCR amplification and sequencing.

The *clfB::lacZ*[Em^r] (McAleese *et al*., 2001), Δ*sdrCDE*::Tc^r (O'Brien *et al*., 2002) and the *spa*::Ka^r (O'Brien *et al*., 2002) mutations were transduced successively into the *clfA5* mutants using phage 85 (Asheshov, 1966). The linked *fnbA*::Tc^r *fnbB*::Em^r mutations (Greene *et al*., 1995) were cotransduced into strain P1. Each transductant was validated by PCR and Southern hybridization.

Strain DU5883 (8325-4 *fnbA*::Tc^r *fnbB*::Em^r) carrying the FnBPA-expressing plasmid pFNBA4 or the FnBPB-expressing plasmid pFNBB4 were described previously (Greene *et al*., 1995). Two mutants of pFNBA4 were constructed previously (Massey *et al*., 2001). pRM1 lacked the A domain and expressed only the BCD domains while pRM8 expressed only the A domain as originally defined (Signas *et al*., 1989) and lacked the BCD domains. The A domain expressed by pRM8 potentially expresses a single fibronectin-binding domain (Schwarz-Linek *et al*., 2003).

Expression of FnBPA and FnBPB by L. lactis

The *fnbA* and *fnbB* genes were amplified from strain 8325-4 by PCR using primer pairs *fnbA* F1/*fnbA* R1 and *fnbB* F1/*fnbB* R1 respectively. PCR products were cloned into pNZ8037 (De Ruyter *et al*., 1996) in *L. lactis* NZ9800 forming *L. lactis* (pNZ8037*fnbA*) and *L. lactis* (pNZ8037*fnbB*) respectively. Transformant colonies were screened for expression of surface proteins by fibronectin adhesion assays.

Derivatives of pNZ8037*fnbA* expressing truncated variants were constructed by inverse PCR (Massey *et al*., 2001) forming pNZ8037*fnbA*-A and pNZ8037*fnbA*-BCD. Positions of primers (Table 3) used for amplification are shown in Fig. 1. The primers designed to delete the BCD fibronectin-binding domains ensured that a shorter region A comprising residues 37–511 and lacking the putative fibronectin-binding motif was expressed. In contrast, pRM8 in *S. aureus* expressed the A domain as originally defined (Signas *et al*., 1989), comprising residues 37–544 including what is now known to be a fibronectin-binding domain (Schwarz-Linek *et al*., 2003).

Recombinant protein expression and purification

Nucleotides encoding FnBPA region A (residues 37–544), FnBPA region D1-D4 (residues 745–878) and FnBPA region BCD (residues 476–838) (Fig. 1) were amplified by PCR and cloned into pQE-30 (Roche *et al*., 2004). Recombinant proteins were expressed and purified by Ni^{2+} chelate chromatography followed by Q Sepharose chromatography, as described previously (O'Connell *et al*., 1998). SDS-PAGE analysis showed single major bands of > 95% purity.

Table 2. Bacterial strains and plasmids.

Strain	Genotype[a]	Phenotype	Source or reference
S. aureus			
Newman		Wild type. Expresses high levels of ClfA	Duthie and Lorenz (1952)
DU5999	Newman *clfA5*	ClfA$^-$	This work
DU6000	Newman *clfA5 clfB::lacZ*[Em^r]	ClfA$^-$ ClfB$^-$	This work
DU6001	Newman *clfA5 clfB::lacZ*[Em^r] Δ*sdrCDE*::Tc^r	ClfA$^-$ ClfB$^-$ SdrC$^-$SdrD$^-$ SdrE$^-$	This work
DU6002	Newman *clfA5*, *clfB::lacZ*[Em^r] Δ*sdrCDE*::Tc^r, *spa*::Ka^r	ClfA$^-$ ClfB$^-$ SdrC$^-$SdrD$^-$ SdrE$^-$ Spa$^-$	This work
P1		Wild type. Rabbit virulent strain	Sherertz *et al*. (1993).
DU6008	P1 *clfA5*	ClfA$^-$	This work
DU6009	P1 *clfA5*, *clfB::lacZ*[Em^r]	ClfA$^-$ ClfB$^-$	This work
DU6010	P1 *clfA5, clfB::lacZ*[Em^r], Δ*sdrCDE*::Tc^r	ClfA$^-$ ClfB$^-$ SdrC$^-$SdrD$^-$ SdrE$^-$	This work
DU6011	P1 *clfA5*, *clfB::lacZ*[Em^r] Δ*sdrCDE*::Tc^r *spa*::Ka^r	ClfA$^-$ ClfB$^-$ SdrC$^-$SdrD$^-$ SdrE$^-$ Spa$^-$	This work
DU5998	P1 *fnbA*::Tc^r, *fnbB*::Em^r	FnBPA$^-$ FnBPB$^-$	Roche *et al*. (2004).
DU5883	8325-4 *fnbA*::Tc^r, *fnbB*::Em^r	FnBPA$^-$ FnBPB$^-$	Greene *et al*. (1995).
079, 080, 126, 151, 152, 206, 208, 209		clinical infective endocarditis isolates	Peacock *et al*. 2000.
L. lactis			
NZ9800	Δ*nisA*	Host for nisin-inducible expression plasmids	Kuipers *et al*. (1993).

Plasmid	Description	Phenotype in *S. aureus*/*L. lactis*	Reference
pFNBA4	*S. aureus-Escherichia coli* shuttle plasmid expressing FnBPA.	FnBPA$^+$ Cm^r	Greene *et al*. (1995).
pFNBB4	*S. aureus-E. coli* shuttle plasmid expressing FnBPB	FnBPB$^+$ Cm^r	Greene *et al*. (1995).
pRM1	Deletion mutant of pFNBA4. Expresses truncated FnBPA lacking A domain	FnBPA-BCD$^+$ Cm^r	Massey *et al*. (2001).
pRM8	Deletion mutant of pFNBA4. Expresses truncated FnBPA lacking BCD domains	FnBPA-A$^+$ Cm^r	Massey *et al*. (2001).
pJH1	Temperature sensitive plasmid for *S. aureus*. Frameshift mutation in *clfA*	Em^r	This work
pNZ8037	*L. lactis* plasmid with *nisA* nisin-inducible promoter	Cm^r	de Ruyter *et al*. (1996).
pNZ8037*fnbA*	pNZ8037 expressing full-length FnBPA	FnBPA$^+$ Cm^r	This work
pNZ8037*fnbA*-A	pNZ8037 expressing FnBPA-A domain and lacking the BCD domains	FnBPA A$^+$ Cm^r	This work
pNZ8037*fnbA*-BCD	pNZ8037 expressing FnBPA-BCD domains and lacking the A domain	FnBPA-BCD$^+$ Cm^r	This work
pNZ8037*fnbB*	pNZ8037 expressing full-length FnBPB	FnBPB$^+$ Cm^r	This work

a. *clfA* and *clfB* are the genes encoding clumping factors A (ClfA) and B (ClfB) respectively. *sdrC*, *sdrD* and *sdrE* are the genes encoding serine-aspartate repeat proteins SdrC SdrD and SdrE respectively. *spa* is the gene encoding protein A (Spa). *fnbA* and *fnbB* are the genes encoding fibronectin-binding proteins A (FnBPA) and B (FnBPB) respectively.

Coupling of biotin to human fibronectin

Human fibronectin (1 mg in 2 ml of PBS) was incubated with 2 mg biotin for 15 min at room temperature. The reaction was stopped by addition of 10 mM NH_4Cl. Excess biotin was removed by dialysis against PBS for 16 h at 4°C.

Ligand affinity blotting

Staphylococcus aureus strains were grown to exponential phase (OD_{600} 0.5) and washed twice in PBS. Cells were adjusted to an OD_{600} of 40 in PBS containing 1.1 M Sucrose, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and protease inhibitor cocktail (Complete Mini; Roche). Cell wall-associated proteins were solubilized by incubation with lysostaphin (200 μg ml^{-1}) for 10 min at 37°C. Protoplasts were removed by centrifugation and the supernatant containing the cell wall-associated pro-

Table 3. Primers.

Primer	Sequence[a]
F1 EcoRI	GCCGAATTCCAAATACATCAACATGACTTTGC
R6 HindIII	GGCAAGCTTCTTCTTTACTGCTGAGTAGTC
F7 HindIII	GCCAAGCTTGCAAGTGAAAATAGTGTTACG
R7 BamHI	CGCGGATTCCCTCTGGAATTGGTTCAATTTC
fnbA F1	CGTGCCATGGAAAACAATCTTAGGTACGGC
fnbA R1	TGCCTCGAGTTAAAATTCTAACTTTATCTCTC
fnbA F2	CCGAAGCTTGAAGAGGAATATGATTCATCAAC
fnbA R2	CGGAAGCTTTGCAGCTTCTTTGTCTTGTCC
fnbA F3	CGGAAGCTTATCGTGCCACCAACGCCAC
fnbA R3	CCGAAGCTTATTTTTCTCATTTCCGTTCGC
*fnb*B F1	CGTGCCATGGAAAGCAATCTTAGATACGGC
*fnb*B R1	TGCTCTAGACGCCTTCATAGTGTCATTG

a. Restriction sites underlined.

teins was removed. Samples were boiled in final sample buffer (Laemmli, 1970), electrophoresed in a 7.5% SDS-PAGE gel and electroblotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 10% (w/v) skimmed milk in TS Tween buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20) for 16 h at 4°C. Biotinylated fibronectin (30 μg ml^{-1}) in 10% (w/v) TS Tween was incubated with the membranes for 2 h. Excess fibronectin was removed by washing with TS Tween and bound fibronectin detected using peroxidase-conjugated streptavidin (Roche) and developed by chemiluminescence.

Bacterial adhesion to immobilized fibrinogen and fibronectin

Adherence of bacteria to immobilized fibrinogen and fibronectin was measured by staining with crystal violet (Hartford *et al*., 1997). Briefly, washed bacterial cells were incubated in 96-well flat bottomed dishes (Sarstedt) that were previously coated with human fibrinogen or fibronectin for 2 h at 37°C. Non-adherent cells were removed by washing the wells with PBS and adherent cells were stained with 5% (w/v) crystal violet for 1 min. Wells were washed with PBS and cell-associated stain was solubilized with 5% (v/v) acetic acid and the A_{570} was determined in an ELISA plate reader.

Depletion of FnBPA-A domain-specific and FnBPA-BCD domain-specific antibodies

The His-tagged rFnBPA-A domain protein ($rFnBPA_{37–544}$) was linked to a Ni^{2+}-sepharose resin (O'Connell *et al*., 1998). Normal pooled human IgG (50 mg in 1 ml; Gammagard, Baxter, Newbury, UK) was passed over the Ni^{2+}-Sepharose column with constant recycling at 4°C for 18 h to remove A domain-specific IgG. The His-tagged rFnBPA-BCD domain protein ($rFnBPA_{545–878}$; 100 μg) was incubated with the purified N-terminal 29 kDa fragment of human fibronectin (200 μg) (Blystone and Kaplan, 1992) and 50 mg of Baxter IgG for 30 min with gentle stirring before passing through a Ni^{2+} Sepharose column in order to remove antibodies that recognized the neo-epitope created by the BCD region binding to fibronectin (Casolini *et al*., 1998). IgG samples were tested by ELISA against the recombinant proteins as previously described (Roche *et al*., 2003) to verify depletion of the specific IgG.

Platelet preparation

Blood was drawn from healthy human volunteers who had abstained from non-steroidal anti-inflammatory drugs during the previous 10 days. For the preparation of PRP, nine volumes of blood were drawn into one volume 3.8% (w/v) Na citrate, centrifuged for 10 min at 150 *g* and the PRP layer removed. An aliquot of PRP was further centrifuged at 630 *g* to yield platelet-poor-plasma (PPP) which was used as the 100% light transmission reference in PRP aggregation assays. For selected experiments, the thrombin inhibitor D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride (PPACK; 50 μM; Calbiochem, Nottingham, UK) was used as an anticoagulant and PRP prepared as described above. GFPs were prepared as described previously (Walkowiak *et al*., 2000). Platelets isolated in this manner were obtained in a resting state, as verified by similar levels of P-selectin expression compared with platelets in whole blood (Walkowiak *et al*., 2000). In brief, blood was drawn into acid-citrate-dextrose (ACD) and PRP prepared as described above. The pH of the PRP was adjusted to 6.5 with ACD and prostaglandin E1 (Sigma) was added (1 μM) prior to centrifugation at 630 *g* for 10 min. The plasma supernatant was removed and the platelet pellet was gently resuspended in 1 ml of JNL buffer (6 mM Dextrose, 130 mM NaCl, 9 mM $NaCl_2$, 10 mM Na citrate, 10 mM Tris base, 3 mM KCl, 0.8 mM KH_2PO_4 and 0.9 mM $MgCl_2$; pH 7.4). The platelet suspension was passed through a column containing 10 ml packed Sepharose 2B-300 (Sigma) which had been extensively washed with JNL buffer and the platelet fraction (GFP) was collected.

Platelet aggregation

Staphylococcus aureus and *L. lactis* cells were grown to stationary phase (16 h) and washed twice in PBS. A total of 5×10^7 cells (in 25 μl of PBS) were added to 225 μl of PRP in siliconized glass cuvettes with stirring and platelet aggregation was assayed by light transmission at 37°C in a PAP-4 aggregometer (Bio-Data). For GFP aggregation, 5×10^7 bacterial cells (in 25 μl of PBS) were mixed with 4.5×10^7 GFPs. GFPs were suspended in JNL buffer containing 1.8 mM $CaCl_2$ and purified human fibrinogen (1 mg ml^{-1}) in a total volume of 225 μl. Normal pooled human IgG and treated IgG samples as outlined above were added (4 mg ml^{-1}) where indicated. Human fibronectin was added at a final concentration of 300 μg ml^{-1}.

Platelet-rich plasma samples were incubated with anti-FcγRIIa antibody IV-3 (20 μg ml^{-1}) or rD1-D4 protein (100 μg ml^{-1}) for 20 min before addition of bacterial cells. Bacteria expressing FnBPA derivatives were incubated with mAb 7C5 (20 μg ml^{-1}) for 20 min before addition to PRP.

Calcium fluorimetry

GFPs were isolated as described above except that the platelet suspension (in 1 ml of JNL buffer) was incubated for 30 min at 37°C with 5 μM FURA-2-AM before filtration on a Sepharose 2B column. GFPs were suspended in plasma to a concentration of 2×10^8 platelets ml^{-1}. For some experiments, GFPs were suspended in JNL buffer containing fibrinogen (1 mg ml^{-1}) and pooled human IgG (4 mg ml^{-1}). Intracellular Ca^{2+} changes were detected after addition of 10^8 bacterial suspension to 8×10^7 FURA-2-AM loaded GFPs using an LS-50 fluorimeter (Perkin Elmer, Norwalk, CT). Cells were stirred at 37°C in a quartz cuvette. FURA-2 fluorescence was measured following excitation at wavelengths 340 and 380 nm, with emission at 510 nm.

Platelet adhesion assay

Adhesion of platelets to immobilized bacteria was measured using an acid phosphatase assay (Kerrigan *et al*., 2002). In brief, 96-well microtitre plates (Nunc) were coated with

washed bacterial cells (1×10^8 cells) for 2 h at 37°C. The wells were then washed with PBS and blocked with 1% (w/v) BSA for a further 2 h at 37°C. The wells were washed three times with JNL buffer and 50 μl of GFPs (2×10^8 platelets ml^{-1}) were added to each well and allowed to adhere for 30 min at 37°C. Each well was washed gently twice with JNL to remove non-adherent platelets. Adherent platelets were detected using a lysis buffer containing a substrate for acid phosphatase (100 mM Na acetate, 0.1% (v/v) Trition-X-100, 10 mM *p*-nitrophenol phosphate). Wells were incubated with 100 μl of lysis buffer for 1 h at 37°C and the absorbance read in an ELISA plate reader at 405 nm.

Statistical analysis

All data are expressed as mean of three experiments ± SEM unless otherwise indicated. The significance of differences in aggregation between wild-type and mutant strains was tested by unpaired *t*-test, with significance defined as $P < 0.05$.

Acknowledgements

The authors thank Ruth Massey and Sharon Peacock for providing strains, R. Klimkowski for the gift of IV-3, and Deirdre Sharkey for technical assistance. The project was supported by a programme grant from the Health Research Board of Ireland (PRO09/2002)

References

Asheshov, E.H. (1966) Loss of antibiotic resistance in *Staphylococcus aureus* resulting from growth at high temperature. *J Gen Microbiol* **42:** 403–410.

Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., *et al.* (2004) Microarray-based analysis of the *Staphylococcus aureus* sigma B regulon. *J Bacteriol* **186:** 4085–4099.

Blystone, S.D., and Kaplan, J.E. (1992) Isolation of an amino-terminal fibronectin-binding protein on human U937 cells and rat peritoneal macrophages. *J Biol Chem* **267:** 3968–3975.

Bowditch, R.D., Halloran, C.E., Aota, S., Obara, M., Plow, E.F., Yamada, K.M., and Ginsberg, M.H. (1991) Integrin alpha IIb beta 3 (platelet GPIIb-IIIa) recognizes multiple sites in fibronectin. *J Biol Chem* **266:** 23323–23328.

Byrne, M.F., Kerrigan, S.W., Corcoran, P.A., Atherton, J.C., Murray, F.E., Fitzgerald, D.J., and Cox, D.M. (2003) *Helicobacter pylori* binds von Willebrand factor and interacts with GPIb to induce platelet aggregation. *Gastroenterology* **124:** 1846–1854.

Casolini, F., Visai, L., Joh, D., Conaldi, P.G., Toniolo, A., Höök, M., and Speziale, P. (1998) Antibody response to fibronectin-binding adhesion FnBPA in patients with *Staphylococcus aureus* infections. *Infect Immun* **66:** 5433–5442.

Chen, J., Dong, J.F., Sun, C., Bergeron, A., McBride, L., Pillai, M., *et al.* (2003) Platelet FcγRIIa His131Arg polymorphism and platelet function: antibodies to platelet-bound fibrinogen induce platelet activation. *J Thromb Haemost* **1:** 355–362.

De Ruyter, P., Kupiers, O.P., and De Vos, W.M. (1996) Controlled gene expression systems for *Lactococcus lactis* with the food grade inducer nisin. *Appl Environ Microbiol* **62:** 3662–3667.

Duthie, E.S., and Lorenz, L.L. (1952) Staphylococcal coagulase: mode of action and antigenicity. *J Gen Microbiol* **6:** 95–107.

Ford, I., Douglas, C.W.I., Heath, J., Rees, C., and Preston, F.E. (1996) Evidence for the involvement of complement proteins in platelet aggregation by *Streptococcus sanguis* NCTC 7863. *Br J Haematol* **94:** 729–739.

Ford, I., Douglas, C.W.I., Cox, D., Rees, D.G.C., Heath, J., and Preston, F.E. (1997) The role of immunoglobulin G and fibrinogen in platelet aggregation by *Streptococcus sanguis*. *Br J Haematol* **97:** 737–746.

Foster, T.J. (1998) Molecular genetic analysis of staphylococcal virulence. *Methods Microbiol* **27:** 433–454.

Fowler, V.J., Miro, J.M., Hoen, B., Cabell, C.H., Abrutyn, E., Rubinstein, E., *et al.* (2005) *Staphylococcus aureus* endocarditis: a consequence of medical progress. *JAMA* **293:** 3012–3021.

Greene, C., McDevitt, D., Francois, P., Vaudaux, P.E., Lew, D.P., and Foster, T.J. (1995) Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of *fnb* genes. *Mol Microbiol* **17:** 1143–1152.

Grundmeier, M., Hussain, M., Becker, P., Heilmann, C., Peters, G., and Sinha, B. (2004) Truncation of fibronectin-binding proteins in *Staphylococcus aureus* strain Newman leads to deficient adherence and host cell invasion due to loss of the cell wall anchor function. *Infect Immun* **72:** 7155–7163.

Hartford, O., Francois, P., Vaudaux, P., and Foster, T.J. (1997) The dipeptide repeat region of fibrinogen-binding protein (clumping factor) is required for functional expression of the fibrinogen-binding domain on the *Staphylococcus aureus* cell surface. *Mol Microbiol* **25:** 1065–1076.

Heilmann, C., Niemann, S., Sinha, B., Herrmann, M., Kehrel, B.E., and Peters, G. (2004) *Staphylococcus aureus* fibronectin-binding protein (FnBP)-mediated adherence to platelets, and aggregation of platelets induced by FnBPA but not by FnBPB. *J Infect Dis* **190:** 321–329.

Herzberg, M.C., Gong, K., MacFarlane, G.D., Erickson, P.R., Soberay, A.H., Krebsbach, P.H., *et al.* (1990) Phenotypic characterization of *Streptococcus sanguis* virulence factors associated with bacterial endocarditis. *Infect Immun* **58:** 515–512.

Herzberg, M.C., MacFarlane, G.D., Gong, K., Armstrong, N.N., Witt, A.R., Erickson, P.R., and Meyer, M.W. (1992) The platelet interactivity phenotype of *Streptococcus sanguis* influences the course of experimental endocarditis. *Infect Immun* **60:** 4809–4818.

Jönsson, K., Signäs, C., Müller, H.P., and Lindberg, M. (1991) Two different genes encode fibronectin binding proteins in *Staphylococcus aureus*: the complete nucleotide sequence and characterization of the second gene. *Eur J Biochem* **202:** 1041–1048.

Kauf, A.C., Hough, S.M., and Bowditch, R.D. (2001) Recog-

nition of fibronectin by the platelet integrin $\alpha_{IIb}\beta_3$ involves an extended interface with multiple electrostatic interactions. *Biochemistry* **40:** 9159–9166.

Kerrigan, S.W., Douglas, I., Wray, A., Heath, J., Byrne, M.F., Fitzgerald, D., and Cox, D. (2002) A role for glycoprotein Ib in *Streptococcus sanguis*-induced platelet aggregation. *Blood* **100:** 509–516.

Kohler, G., and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256:** 495–497.

Kuipers, O.P., Beerthuysen, M.M., Siezen, R.J., and De Vos, W.M. (1993) Characterisation of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*. Requirement of expression of the *nisA and nisI* genes for the development of immunity. *Eur J Biochem* **216:** 281–291.

Kuypers, J.M., and Proctor, R.A. (1989) Reduced adherence to traumatized rat heart valves by a low-fibronectin-binding mutant of *Staphylococcus aureus*. *Infect Immun* **57:** 2306–2312.

Laemmli, U. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Loughman, A., Fitzgerald, J.R., Brennan, M., Higgins, J., Downer, R., Cox, D., and Foster, T.J. (2005) Roles for fibrinogen, immunoglobulin and complement proteins in platelet activation by *Staphylococcus aureus* clumping factor A. *Mol Microbiol* **57:** 804–818.

McAleese, F.M., Walsh, E.J., Sieprawska, M., Potempa, J., and Foster, T.J. (2001) Loss of clumping factor B fibrinogen binding activity by *Staphylococcus aureus* involves cessation of transcription, shedding and cleavage by metalloprotease. *J Biol Chem* **276:** 29969–29978.

Massey, R.C., Kantzanou, M.N., Fowler, T., Day, N.P., Schofield, K., Wann, E.R., *et al.* (2001) Fibronectin-binding protein A of *Staphylococcus aureus* has multiple, substituting, binding regions that mediate adherence to fibronectin and invasion of endothelial cells. *Cell Microbiol* **3:** 839–851.

Matsuka, Y.V., Anderson, E.T., Milner-Fish, T., Ooi, P., and Baker, S. (2003) *Staphylococcus aureus* fibronectin-binding protein serves as a substrate for coagulation factor XIIIa: evidence for factor XIIIa-catalyzed covalent cross-linking to fibronectin and fibrin. *Biochemistry* **42:** 14643–14652.

Niemann, S., Spehr, N., Van Aken, H., Morgenstern, E., Peters, G., Herrmann, M., and Kehrel, B.E. (2004) Soluble fibrin is the main mediator of *Staphylococcus aureus* adhesion to platelets. *Circulation* **110:** 193–200.

O'Brien, L., Kerrigan, S.W., Kaw, G., Hogan, M., Penadés, J., Litt, D., *et al.* (2002) Multiple mechanisms for the activation of human platelet aggregation by *Staphylococcus aureus*: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A. *Mol Microbiol* **44:** 1033–1044.

O'Connell, D.P., Nanavaty, T., McDevitt, D., Gurusiddappa, S., Höök, M., and Foster, T.J. (1998) The fibrinogen-binding MSCRAMM (clumping factor) of *Staphylococcus aureus* has a Ca^{2+}-dependent inhibitory site. *J Biol Chem* **273:** 6821–6829.

Oue, Y.A., Haefliger, J.A., Piroth, L., Francois, P., Widmer, E., Entenza, J.M., *et al.* (2005) Fibrinogen and fibronectin binding cooperate for valve infection and invasion in *Staphylococcus aureus* experimental endocarditis. *J Exp Med* **201:** 1627–1635.

Pawar, P., Shin, P.K., Mousa, S.A., Ross, J.M., and Konstantopoulos, K. (2004) Fluid shear regulates the kinetics and receptor specificity of *Staphylococcus aureus* binding to activated platelets. *J Immunol* **173:** 1258–1265.

Peacock, S.J., Day, N.P., Thomas, M.G., Berendt, A.R., and Foster, T.J. (2000) Clinical isolates of *Staphylococcus aureus* exhibit diversity in *fnb* genes and adhesion to human fibronectin. *J Infect* **41:** 23–31.

Plummer, C., Wu, H., Kerrigan, S.W., Meade, G., Cox, D., and Douglas, C.W.I. (2005) A serine-rich glycoprotein of *Streptococcus sanguis* mediates adhesion to platelets via GP1b. *Br J Haematol* **129:** 101–109.

Potts, J.R., and Campbell, I.D. (1996) Structure and function of fibronectin modules. *Matrix Biol* **15:** 313–321.

Que, Y., Francois, P., Haefliger, J.A., Entenza, J.M., Vaudaux, P., and Moreillon, P. (2001) Reassessing the role of *Staphylococcus aureus* clumping factor and fibronectin-binding protein by expression in *Lactococcus lactis*. *Infect Immun* **69:** 6296–6302.

Roche, F.M., Massey, R., Peacock, S.J., Day, N.P.J., Visai, L., Speziale, P., *et al.* (2003) Characterization of novel LPXTG-containing proteins of *Staphylococcus aureus* identified from genome sequences. *Microbiology* **149:** 649–654.

Roche, F.M., Downer, R., Keane, F., Speziale, P., Woo Park, P., and Foster, T.J. (2004) The amino-terminal A domain of fibronectin binding proteins A and B promotes adhesion of *Staphylococcus aureus* to elastin. *J Biol Chem* **277:** 243–250.

Saravia-Otten, P., Muller, H.P., and Arvidson, S. (1997) Transcription of *Staphylococcus aureus* fibronectin-binding protein genes is negatively regulated by *agr* and an *agr*-independent mechanism. *J Bacteriol* **179:** 5259–5263.

Schwarz-Linek, U., Werner, J.M., Pickford, A.R., Gurusiddappa, S., Kim, J.H., Pilka, E.S., *et al.* (2003) Pathogenic bacteria attach to human fibronectin through a tandem beta-zipper. *Nature* **423:** 177–181.

Sherertz, R.J., Carruth, W.A., Hampton, A.A., Byron, M.P., and Solomon, D.D. (1993) Efficacy of antibiotic-coated catheters in preventing subcutaneous *Staphylococcus aureus* infection in rabbits. *J Infect Dis* **167:** 98–108.

Signäs, C., Raucci, G., Jönsson, K., Lindgren, P.E., Anantharamaiah, G.M., Höök, M., and Lindberg, M. (1989) Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus*: use of this peptide sequence in the synthesis of biologically active peptides. *Proc Natl Acad Sci USA* **86:** 699–703.

Sinha, B., Francois, P.P., Nusse, O., Foti, M., Hartford, O.M., Vaudaux, P., *et al.* (1999) Fibronectin-binding protein acts as *Staphylococcus aureus* invasion via fibronectin bridging to integin alpha5beta1. *Cell Microbiol* **1:** 101–117.

Sjöbring, U., Ringdahl, U., and Ruggeri, Z.M. (2002) Induction of platelet thrombi by bacteria and antibodies. *Blood* **100:** 4470–4477.

Sullam, P.M., Jarvis, G.A., and Valone, F.H. (1988) Role of immunoglobulin G in platelet aggregation by viridans group streptococci. *Infect Immun* **56:** 2907–2911.

Sullam, P.M., Bayer, A.S., Foss, W.M., and Chenng, A.L. (1996) Diminished platelet binding in vitro by *Staphylococcus aureus* is associated with reduced virulence in a rabbit model of infective endocarditis. *Infect Immun* **64:** 4915–4921.

Walkowiak, B., Kralisz, U., Michalec, L., Majewska, E., Koziolkiewicz, W., Ligocka, A., and Cierniewski, C.S. (2000) Comparison of platelet aggregability and P-selectin surface expression on platelets isolated by different methods. *Thromb Res* **99:** 495–502.

Wann, E.R., Gurusiddappa, S., and Höök, M. (2000) The fibronectin-binding MSCRAMM FnBPA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. *J Biol Chem* **275:** 13863–13871.

