Molecular Microbiology (2005) **57**(3), 804–818 doi:10.1111/j.1365-2958.2005.04731.x

Roles for fibrinogen, immunoglobulin and complement in platelet activation promoted by *Staphylococcus aureus* clumping factor A

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Summary

Staphylococcus aureus **is an important cause of infective endocarditis (IE) in patients without a history of prior heart valve damage. The ability to stimulate the activation of resting platelets and their subsequent aggregation is regarded as an important virulence factor of bacteria that cause IE. Clumping factor A is the dominant surface protein responsible for platelet activation by *S. aureus* cells in the stationary phase of growth. This study used *Lactococcus lactis* as a surrogate host to study the mechanism of ClfA-promoted platelet activation. Expression of ClfA from a nisin-inducible promoter demonstrated that a minimum level of surface-expressed ClfA was required. Using platelets that were purified from plasma, the requirement for both bound fibrinogen and immunoglobulin was demonstrated. The immunoglobulin G (IgG) requirement is consistent with the potent inhibition of platelet activation by a monoclonal antibody specific for the platelet FcγRIIa receptor. Furthermore the IgG must contain antibodies specific for the ClfA A domain. A model is proposed whereby bacterial cells armed with a sufficient number of surface-bound fibrinogen molecules can engage resting platelet glycoprotein GPIIb/IIIa, aided by bound IgG molecules, which encourages the clustering of FcγRIIa receptors. This can trigger activation of signal transduction leading to activation of GPIIb/IIIa and aggregation of platelets. In addition, analysis of a mutant of ClfA totally lacking the ability to bind fibrinogen revealed a second, although less efficient, mechanism of platelet activation. The fibrinogen-independent pathway required IgG and complement deposition to trigger platelet aggregation**

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Introduction

In humans, *Staphylococcus aureus* is predominantly a harmless commensal of the moist squamous epithelium but occasionally the organism can cause serious invasive disease. Indeed, *S. aureus* is the predominant cause of infective endocarditis (IE) whereby bacteria colonize and invade previously undamaged heart valves. This is a serious condition with a high mortality rate even if treated aggressively with antibiotics (Moreillon and Que, 2004). It is widely recognized that the ability of *S. aureus* to cause activation of platelets and their consequent aggregation is an important contributory factor in the pathogenesis of IE (Sullam *et al*., 1996; Moreillon and Que, 2004).

Until recently virtually nothing was known either about the surface components of *S. aureus* that promote interactions with the platelet surface or about the platelet receptors involved in recognizing bacteria. By studying mutants of *S. aureus* Newman that lacked one or more surface proteins and by expressing surface proteins individually on the surface of the surrogate Gram-positive host bacterium *Lactococcus lactis*, clumping factors (Clf) A and B as well as the serine-aspartate repeat protein (Sdr) E were each shown to cause the activation of human platelets in plasma (O'Brien *et al*., 2002). In addition, protein A, which binds von Willebrand factor (Hartleib *et al*., 2000), was shown to have an accessory role (O'Brien *et al*., 2002). It was noted that ClfA had more potent pro-aggregatory activity than ClfB or SdrE. However, the underlying mechanisms involved were not characterized.

Clumping factor A is the archetype of a family of surface proteins expressed by staphylococci (Foster and Höök, 1998; Roche *et al*., 2003). It is expressed on the surface of nearly all *S. aureus* strains (Peacock *et al*., 2002; Patti, 2004) and mediates bacterial adherence to immobilized fibrinogen and cell clumping in soluble fibrinogen (McDevitt *et al*., 1994). The structural organization of ClfA is outlined in Fig. 1. ClfA has a large N-terminal A domain

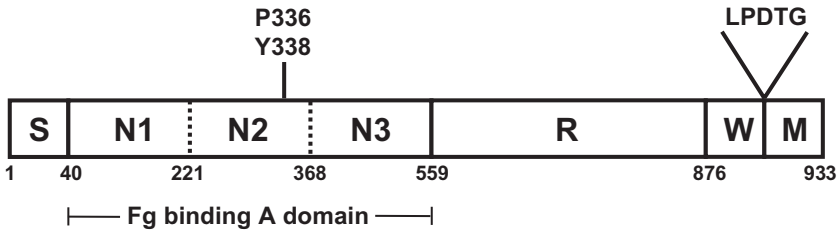

Fig. 1. Schematic organization of ClfA. The N-terminus of ClfA contains a signal sequence of 40 residues that target the protein for secretion. The fibrinogen (Fg)-binding domain (A domain) comprises of subdomains N1, N2 and N3. The minimum ligand binding truncate of ClfA comprises domain N2 and N3. Amino acids P336 and Y338 within N2 have been identified as being essential for fibrinogen binding by ClfA. C-terminal to the A domain is a repeat region (R) consisting of Ser–Asp dipeptide repeats which are required for functional expression of the A domain on the bacterial surface. At the C-terminus of ClfA are wall (W) and membrane (M) spanning domains and the LPDTG motif required for anchoring of ClfA to the cell wall by sortase.

of 519 residues that is exposed on the cell surface and binds to fibrinogen (McDevitt *et al*., 1995) at the extreme C-terminal residues of the γ-chain (McDevitt *et al*., 1997) which protrude from each end of the two E domains of the dumbbell-shaped protein. This is the same site to which the platelet integrin glycoprotein (GP) IIb/IIIa binds (Farrell *et al*., 1992; Hettasch *et al*., 1992). Covalent anchoring via the LPDTG motif at the C-terminus of ClfA to cell wall peptidoglycan is catalysed by sortase (Mazmanian *et al*., 2001). Linking the A domain to the sorting signal is a repeated region comprising the dipeptide Ser–Asp. Structural analysis of ClfA and ClfB from *S. aureus* and SdrG from *Staphylococcus epidermidis* showed that the A domains comprise three independently folded regions (Deivanayagam *et al*., 2002; Ponnuraj *et al*., 2003). Domains N2 and N3 are each composed of a novel form of the immunoglobulin G (IgG) fold. The peptide ligands of SdrG and ClfA bind in a hydrophobic trench lying between domains N2 and N3. Residues lining the trench (Pro336 and Tyr338) were shown to be essential for ligand binding by purified recombinant ClfA (Deivanayagam *et al*., 2002).

Clumping factor A has been shown to be an important virulence factor in *S. aureus*-promoted experimental rat endocarditis (Moreillon *et al*., 1995) and murine septic arthritis (Josefsson *et al*., 2001). In addition, *L. lactis* cells heterologously expressing ClfA were 100-fold more infective than the *L. lactis* parent strain in the rat model of IE (Que *et al*., 2001). Furthermore, active immunization with the recombinant A domain of ClfA and passive immunization with IgG containing high titres of anti-ClfA antibodies afforded significant protection in experimental infections (Josefsson *et al*., 2001; Vernachio *et al*., 2003). Recently, a humanized monoclonal antibody recognizing ClfA was shown to protect against challenge with an MRSA strain in murine septicaemia and rabbit IE models (Patti, 2004).

Platelet aggregation occurs when the bivalent fibrinogen binds to its receptor, GPIIb/IIIa, and cross-links platelets. GPIIb/IIIa requires prior activation to bind to soluble fibrinogen. This occurs when platelet agonists such as ADP bind to their receptor generating an activation signal resulting in a conformational change in GPIIb/IIIa (Xiao *et al*., 2004). However, resting GPIIb/IIIa can bind to immobilized fibrinogen which is thought to be mediated by conformational changes that occur in fibrinogen when it is immobilized.

Several distinct mechanisms of activation of platelets by bacteria have been described. Some strains of *Streptococcus sanguis* can interact directly with the platelet von Willebrand factor receptor GPIb (Kerrigan *et al*., 2002) while others require complement and IgG to bind to the bacterial surface allowing interaction with the platelet via a complement receptor and FcγRIIa (Ford *et al*., 1996; 1997). In contrast, *Helicobacter pylori* binds indirectly to GPIb via a von Willebrand factor bridge but activation of platelets also requires an IgG bridge to the platelet FcγRIIa receptor (Byrne *et al*., 2003). FcγRIIa and IgG is also involved in platelet aggregation by viridans group streptococci (Sullam *et al*., 1988). Thrombus formation under flow conditions by *S. aureus* Newman, likely involving ClfA as the cell surface-associated agonist, was mediated through FcγRIIa (Sjöbring *et al*., 2002).

Soluble agonists such as ADP generate an immediate response in platelet aggregation. Collagen as an insoluble suspension has a short lag before aggregation occurs. Platelet aggregation stimulated by bacteria is preceded by a variable lag phase (1–25 min), 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 to aggregation observed 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). Lag time is dependent to some extent on the concentration of bacterial cells. At a final OD of 0.16 the shortest lag time is usually achieved. Diluting cells two- to fourfold results in a longer lag time but this is never more than twofold. However, unlike solu-

ble agonists, the response is always maximal with only the lag time changing.

Our previous study identified ClfA as an important surface component of *S. aureus* for promoting activation of platelets in plasma (O'Brien *et al*., 2002). However, the precise role of fibrinogen in the process was not investigated. This article describes experiments designed (i) to determine whether fibrinogen binding by ClfA is required for initial activation, (ii) to determine whether other plasma proteins are required and (iii) to identify the platelet receptors involved. We expressed on the surface of *L. lactis* under the control of a regulatable promoter both wild-type ClfA and a ClfA mutant that could not bind fibrinogen. By studying aggregation of platelets that had been separated from plasma we were able to investigate the requirement for individual plasma proteins. We showed that ClfA-promoted activation of platelets involved fibrinogen-dependent and fibrinogen-independent processes and that both mechanisms required the presence of IgG molecules bound to the A domain of ClfA while the fibrinogen-independent mechanism also required complement assembly.

Results

Platelet activation by a ClfA mutant defective in fibrinogen binding

Previously we showed that ClfA caused the stimulation of platelet aggregation when it was expressed on *S. aureus* or constitutively on the surface of the non-aggregating surrogate host *L. lactis* (O'Brien *et al*., 2002). In order to investigate the role for fibrinogen binding by ClfA in the initial process of platelet activation a mutant of ClfA with the substitutions P336S Y338A (ClfA PY) was constructed in plasmid pCF77. These substitutions have been shown to eliminate binding to fibrinogen by the recombinant form of the ClfA A domain protein expressed in *Escherichia coli* (Deivanayagam *et al*., 2002). *S. aureus* Newman DU5944 (*clfAclfB*) was used as the host for expression of ClfA PY. The double *clfA-clfB* mutant was used as the fibrinogen-binding surface protein ClfB was previously shown to participate in platelet activation by *S. aureus* Newman (O'Brien *et al*., 2002). DU5944 and its derivatives expressing ClfA or ClfA PY from the multicopy pCUI-derived plasmids were grown to stationary phase, where ClfA is maximally expressed (O'Brien *et al*., 2002; J. Higgins, unpubl. data). DU5944 (pCF77) adhered to immobilized fibrinogen and clumped strongly in soluble fibrinogen, whereas DU5944 (pCF77 PY) had no detectable fibrinogen binding activity (Fig. 2A and B).

The level of ClfA or ClfA PY on the bacterial surface was assessed by whole cell dot immunoblotting. A mutation in the *spa* gene (*spa*::Ka^r) was transduced from DU5971 into DU5944 derivatives to prevent non-specific binding of antibodies to protein A. The level of expression of ClfA and ClfA PY from pCF77 and pCF77 PY was the same as ClfA expression by DU5971 (Fig. 2C). Newman *clfAclfBspa* (DU6014) did not react with anti-ClfA antibodies. The lag time to aggregation in platelet-rich plasma (PRP) was measured. Lag time was defined as the interval between addition of bacterial cells to PRP samples and the earliest detectable signs of rapid aggregation. A representative aggregation trace by DU5944 expressing ClfA and ClfA PY is shown in Fig. 2D. DU5944 (pCF77 PY) caused platelet activation with a lag time of 9.6 ± 4.2 min ($n = 3$) compared with DU5944 (pCF77) (0.9 ± 0.1 min; $n = 3$; $P < 0.05$) demonstrating that the ability of ClfA to bind fibrinogen results in rapid activation but that the ClfA PY mutant could still cause activation, although more slowly. DU5944 cells stimulated activation with a lag time of 23 ± 4.5 min ($n = 3$).

Nisin-inducible expression of ClfA in L. lactis

We then cloned *clfA* in the nisin-regulated expression vector pNZ8037 in *L. lactis* allowing the study of ClfA-promoted platelet activation under conditions where the level of surface expression could be precisely controlled in order to investigate the relationship between expression level and lag time to activation. We compared wild-type ClfA with the ClfA PY mutant expressed by *L. lactis* grown in concentrations of nisin ranging from 0.025 to 3.2 ng ml^{-1}. Doubling dilutions of cells were spotted onto a nitrocellulose membrane and probed with anti-ClfA antibodies. The data in Fig. 3A and B show that low levels of ClfA were expressed when no inducer was present indicating that repression is leaky. Antibodies did not bind detectably to cell wall extracts of the *L. lactis* host strain carrying the empty expression vector (Fig. 3C). The level of expression of ClfA and ClfA PY was increased by about 64-fold in fully induced cells compared with uninduced cells. There was no difference in the level of expression of ClfA PY compared with ClfA at the same inducer concentration. The level of expression of ClfA or ClfA PY at 0.8 ng ml^{-1} coincided with that expressed by stationary-phase *S. aureus* Newman cells (Fig. 2C).

Proteins were solubilized by mutanolysin and analysed by SDS-PAGE and Western immunoblotting. Both wild-type ClfA and the ClfA PY mutant proteins migrated as single immunoreactive bands with apparent molecular weights of 175 kDa (Fig. 3C), indicating that ClfA and ClfA PY were expressed in their full-length forms (O'Brien *et al*., 2002).

Cells from uninduced cultures of *L. lactis* pNZ8037 *clfA* (*L. lactis* ClfA$^+$) adhered detectably to immobilized

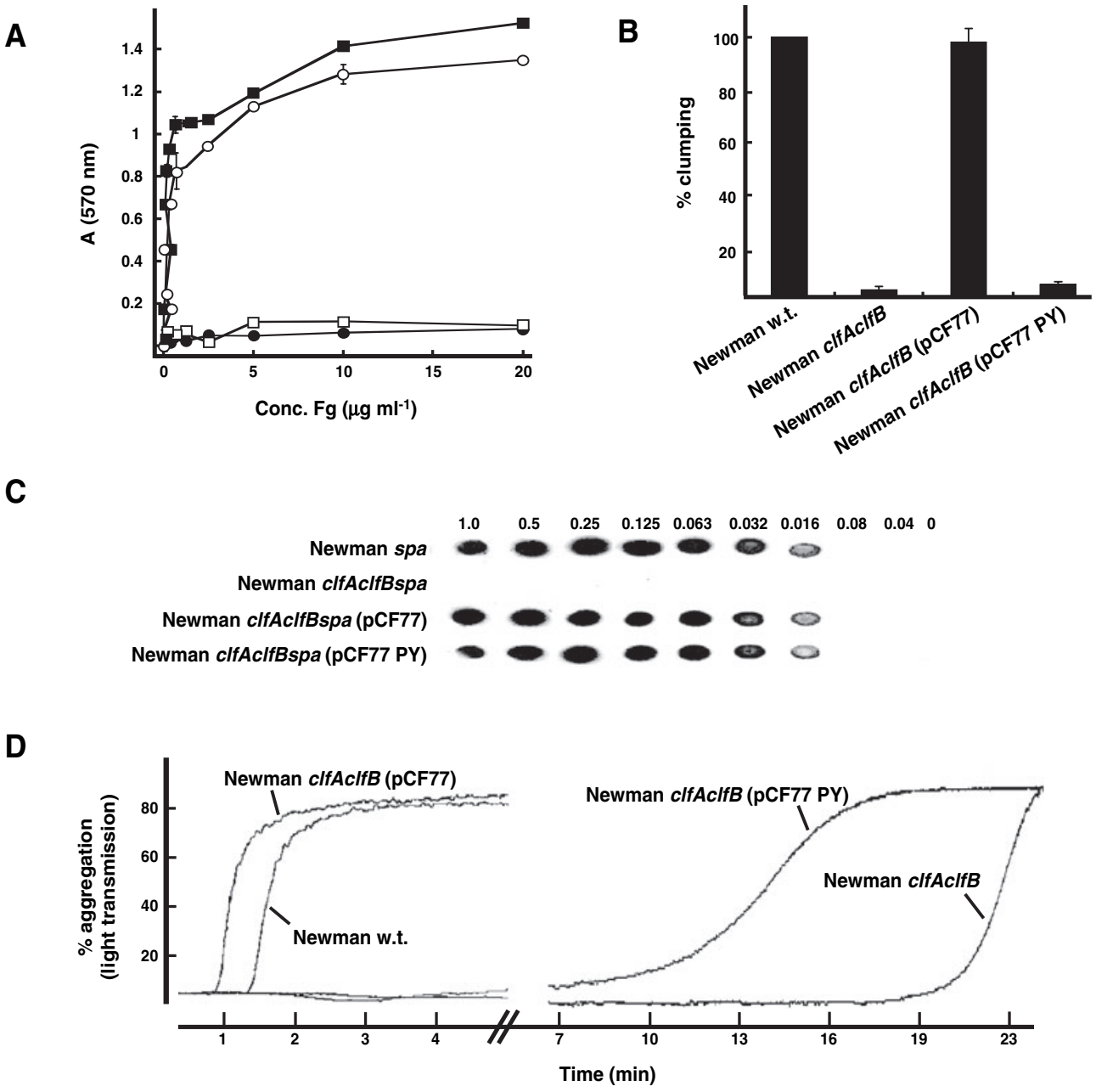

Fig. 2. Activation of human platelet aggregation by *S. aureus* cells expressing ClfA and ClfA PY.
A. Adherence of *S. aureus* Newman (closed squares), Newman deficient in ClfA and ClfB (DU5944) (closed circles), DU5944 (pCF77) (open circles) and DU5944 (pCF77 PY) (open squares) to immobilized fibrinogen (0.08–20 μg ml^{-1}). Adherent cells were stained with crystal violet and the absorbance at 570 nm was determined.
B. Measurement of cell clumping in a solution of fibrinogen (3 mg ml^{-1}). Results are expressed as percentage clumping compared with Newman wild type (w.t.).
C. Expression of ClfA and ClfA PY on the surface of *S. aureus* Newman. Serial dilutions of washed cells (OD_{600} 1.0–0.04) were applied to a nitrocellulose membrane and probed with rabbit anti-ClfA A domain antibodies and goat anti-rabbit antibodies conjugated to horseradish peroxidase and developed by chemiluminescence.
D. Activation of platelet aggregation by *S. aureus* Newman strains: (i) wild type, lag time 1.5 min, (ii) DU5944 (pCF77), lag time 0.9 min, (iii) DU5944 (pCF77 PY), lag time 11.7 min, and (iv) DU5944, lag time 21.5 min. All strains stimulated similar levels of aggregation (> 80% light transmission). The aggregation trace shown is a representative of three different experiments.

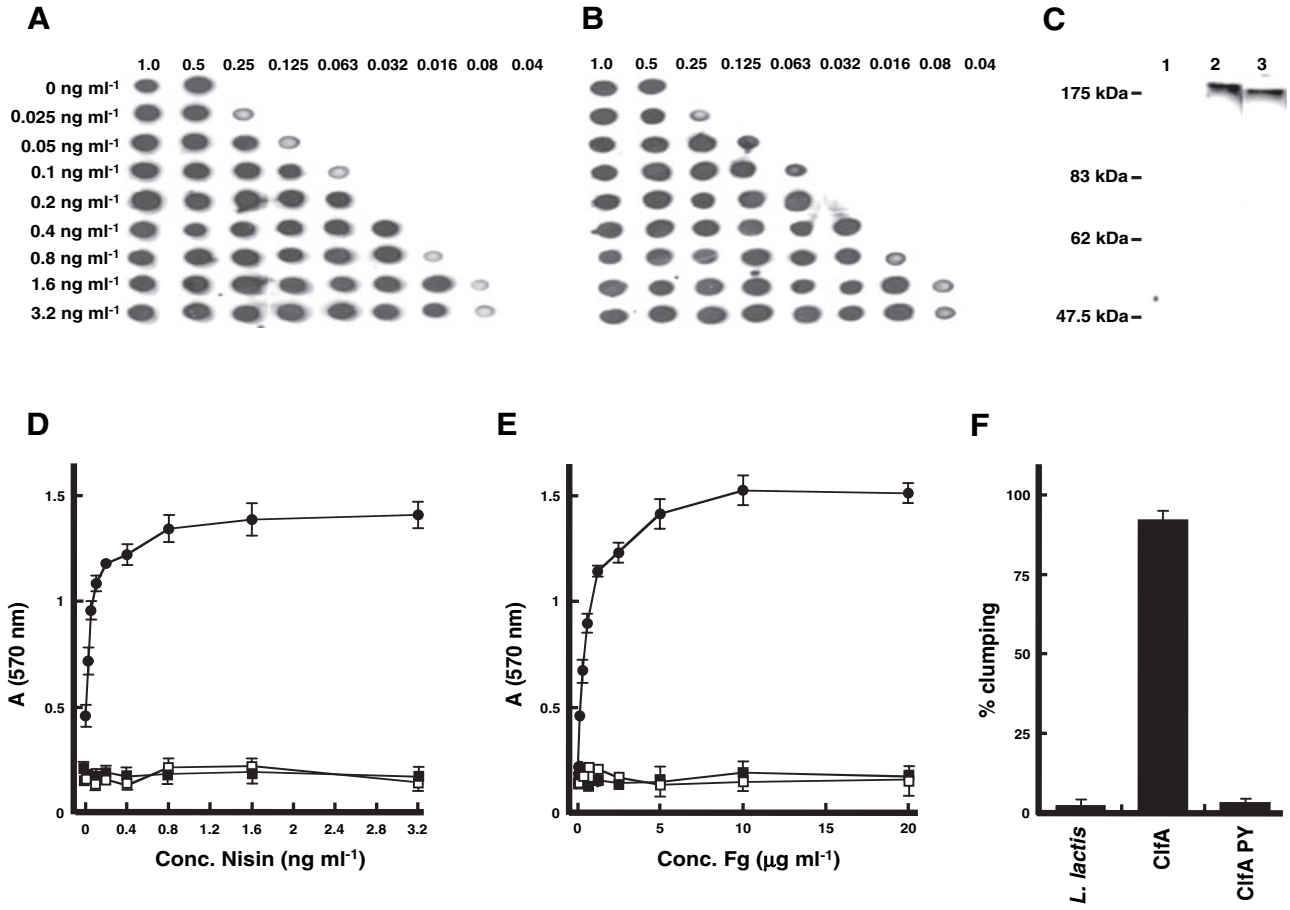

Fig. 3. Controlled expression of ClfA and ClfA PY by *L. lactis*.
A and B. (A) *L. lactis* pNZ8037*clfA* cells and (B) *L. lactis* pNZ8037*clfA* PY cells were grown to stationary phase in concentrations of nisin ranging from 0.025 to 3.2 ng ml^{-1}. Serial dilutions of washed cells (OD_{600} 1.0–0.04) were applied to a nitrocellulose membrane and probed with rabbit anti-ClfA A domain antibodies and goat anti-rabbit antibodies conjugated to horseradish peroxidase and developed by chemiluminescence.
C. Cell wall-associated proteins of *L. lactis* pNZ8037 (lane 1), *L. lactis* pNZ8037*clfA* (lane 2) and *L. lactis* pNZ8037*clfA* PY (lane 3) were separated on 7.5% SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membranes and probed with rabbit anti-ClfA A domain antibodies and goat anti-rabbit antibodies conjugated to horseradish peroxidase and developed by chemiluminescence.
D. *L. lactis* pNZ8037 (closed squares), *L. lactis* pNZ8037*clfA* (closed circles) and *L. lactis* pNZ8037*clfA* PY (open squares) were grown to stationary phase in increasing concentrations of nisin. The ability to adhere to immobilized fibrinogen (5 μg ml^{-1}) coated onto 96-well ELISA dishes was tested.
E. *L. lactis* pNZ8037 (open squares), *L. lactis* pNZ8037*clfA* (closed circles) and *L. lactis* pNZ8037*clfA* PY (closed squares) were grown to stationary phase in the presence of 1.6 ng ml^{-1} nisin. The ability to bind to immobilized fibrinogen (Fg) (0.08–20 μg ml^{-1}) coated onto 96-well ELISA dishes was tested.
F. *L. lactis* pNZ8037, *L. lactis* pNZ8037*clfA* and *L. lactis* pNZ8037*clfA* PY were grown to stationary phase in the presence of 1.6 ng ml^{-1} nisin. The ability of cells to form clumps in soluble fibrinogen (3 mg ml^{-1} solution) was tested. Results are expressed as percentage clumping.

fibrinogen (Fig. 3D). The number of adherent cells increased concomitantly with increasing concentrations of nisin. This correlates with protein expression seen in Fig. 3A. The number of fully induced *L. lactis* ClfA$^+$ cells that adhered increased in proportion to the concentration of fibrinogen used to coat ELISA wells, reaching saturation at 10 μg ml^{-1} (Fig. 3E). Even at the highest fibrinogen concentration used or at maximal expression levels, no adherence of *L. lactis* pNZ8037*clfA* PY (*L. lactis* ClfA PY$^+$) could be detected. *L. lactis* ClfA$^+$ cells clumped strongly in physiological concentrations of soluble fibrinogen, whereas *L. lactis* ClfA PY$^+$ cells were completely defective in clumping (Fig. 3F). This concurs with the previous report that the P336S and Y338A substitutions completely eliminated fibrinogen binding by the recombinant ClfA A domain protein (Deivanayagam *et al.*, 2002).

A threshold level of expression of ClfA and ClfA PY is required to activate platelet aggregation

We exploited our ability to control the level of ClfA protein on the surface of *L. lactis* to investigate the level of

ClfA required for aggregation to be stimulated. *L. lactis* pNZ8037, *L. lactis* pNZ8037*clfA* and *L. lactis* pNZ8037*clfA* PY were induced with varying nisin concentrations. Bacterial cells were added to PRP samples and the lag times to aggregation were measured. Expression levels of ClfA and ClfA PY achieved at 0.4 ng ml^{-1} nisin were sufficient to promote platelet aggregation, whereas expression levels at 0.2 ng ml^{-1} nisin were not (Fig. 4). Increasing inducer concentrations resulted in shorter lag times to aggregation for *L. lactis* ClfA$^+$ (2.6 ± 0.375 min at 0.4 ng ml^{-1} compared with 1.5 ± 0.1 min at 3.2 ng ml^{-1}; $P < 0.01$; $n = 4$) and *L. lactis* ClfA PY$^+$ (16.4 ± 4.5 min at 0.4 ng ml^{-1} compared with 7.25 ± 1.9 min at 1.6 ng ml^{-1}; $P < 0.01$; Fig. 4). The fastest aggregation occurred at inducer concentrations of 1.6 ng ml^{-1} and 3.2 ng ml^{-1}. The fact that the lag times at these concentrations were the same could be explained by these cells expressing the same level of ClfA protein (Fig. 3A and B). These results demonstrate that a threshold expression level of ClfA and ClfA PY is required to stimulate aggregation, and that the time to aggregation is then dependent on the number of molecules of ClfA on the cell surface. The short lag time for wild-type ClfA likely reflects a high-affinity fibrinogen-dependent interaction with resting platelets compared with a less efficient fibrinogen-independent interaction for ClfA PY.

Lactococcus lactis *ClfA$^+$ and ClfA PY$^+$ stimulate true platelet activation*

Treatment of platelets with prostaglandin E1 (PGE_1) results in an increase in intracellular cAMP which inhibits platelet activation and aggregation. Addition of PGE_1 completely inhibited aggregation by fully induced *L. lactis* ClfA$^+$ [83 ± 6% aggregation in normal PRP compared with 1.5 ± 0.5% in PRP treated with PGE_1 (2 μM); $n = 3$; $P < 0.01$] and *L. lactis* ClfA PY$^+$ (84 ± 3% control compared with 2 ± 1% with PRP treated with PGE_1; $n = 3$; $P < 0.01$). In these assays, the degree of activated platelets (% aggregation) was measured. The times to aggregation were similar to those reported in Fig. 4. The anti-GPIIb/IIIa monoclonal antibody abciximab inhibited aggregation by both *L. lactis* ClfA$^+$ (5 ± 3%; $n = 3$; $P < 0.01$) and *L. lactis* ClfA PY$^+$ (4 ± 2%; $n = 3$; $P < 0.01$) indicating that the bacteria stimulated platelet activation and aggregation mediated by the platelet integrin, and not agglutination. One of the earliest markers for platelet activation is an increase in cytosolic Ca^{2+} (Nesbitt *et al*., 2003; Pietrocola *et al*., 2004). When *L. lactis* cells expressing ClfA and ClfA PY were pre-incubated in plasma samples, washed and added to FURA-2-AM-loaded gel-filtered platelets (GFPs), an increase in cytosolic Ca^{2+} was observed, whereas no increase was observed upon addition of bacterial cells pre-incubated in PBS. Addition of *L. lactis* pNZ8037 cells pre-incubated in plasma samples to FURA-2-AM-loaded platelets did not result in any detectable Ca^{2+} flux (Fig. 5). This shows that ClfA and ClfA PY stimulate true platelet activation and that this is dependent on bound plasma proteins.

Role of IgG in ClfA-promoted platelet activation

Previous studies have suggested that antibodies are important for platelet activation by bacteria (Sullam *et al*., 1988; Ford *et al*., 1997; Sjöbring *et al*., 2002). Here experiments were performed with GFPs that allowed determination of the role of individual plasma proteins in activation promoted by *L. lactis* ClfA$^+$ and *L. lactis* ClfA PY$^+$. The

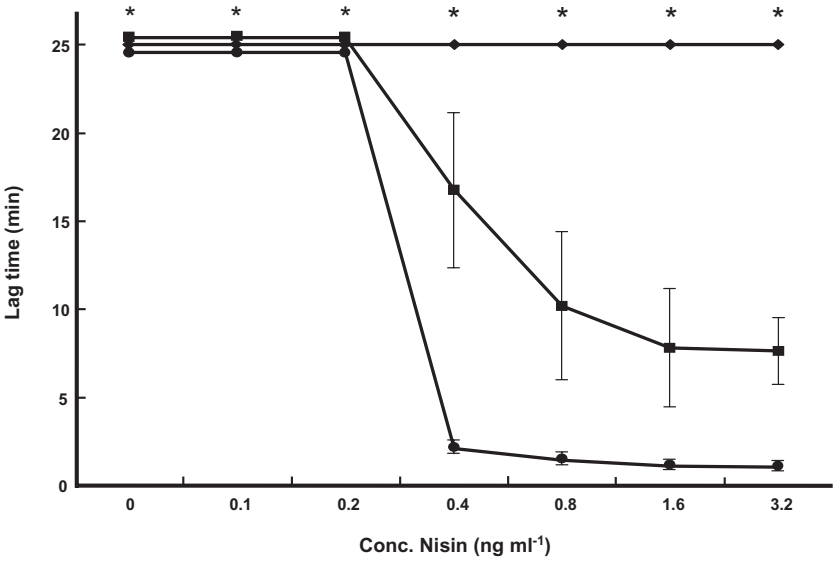

Fig. 4. A threshold level expression of ClfA and ClfA PY is required to stimulate platelet aggregation. *L. lactis* pNZ8037 (diamonds), *L. lactis* pNZ8037*clfA* (circles) and *L. lactis* pNZ8037*clfA* PY (squares) were grown to stationary phase in increasing concentrations of nisin. Cells were washed and resuspended in PBS. The ability of cells from each culture (10^8 cells) to stimulate platelet aggregation in PRP (450 μl) was tested. Asterisk (*) indicates no aggregation occurred after 25 min incubation. Results are presented as a comparison between the time taken to aggregation for each strain (lag time). All cultures that stimulated aggregation showed comparable levels of aggregation (percentage aggregation).

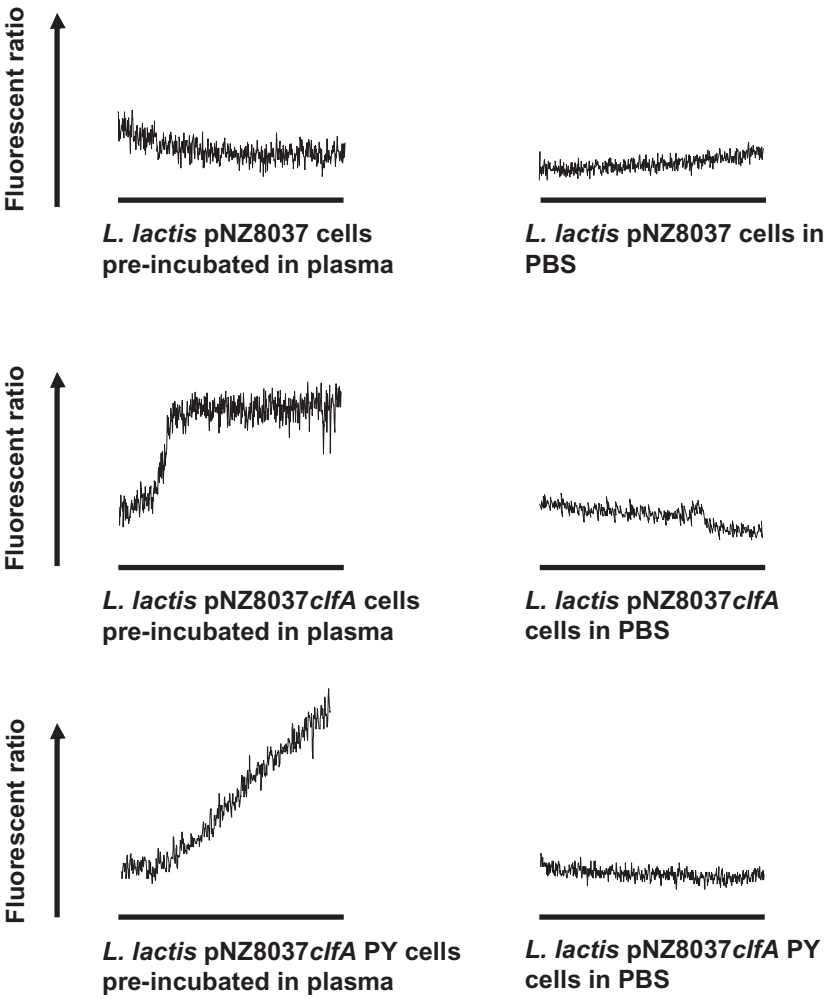

Fig. 5. ClfA stimulates cytosolic calcium increases in platelets. Platelet activation was measured by detection of intracellular calcium flux. Bacterial cells were incubated either in plasma samples or in PBS as indicated before addition to FURA-2-AM-loaded GFPs. Increases in the fluorescent ratio (ratio of the excitation of FURA-2-AM at 340 nm compared with that at 380 nm) indicate that platelet activation is occurring. The traces shown are representative of three separate experiments.

percentage of platelet aggregation upon exposure to *L. lactis* ClfA$^+$ or *L. lactis* ClfA PY$^+$ cells was measured, with a low percentage aggregation indicating the absence of a factor required for activation to occur. Fibrinogen that had been purified to remove contaminating IgG was added to GFP. This was not sufficient to stimulate activation by either *L. lactis* ClfA$^+$ ($14 \pm 2\%$ aggregation in GFP plus fibrinogen compared with $76 \pm 6\%$ aggregation in PRP; $n = 4$; $P < 0.01$) or by *L. lactis* ClfA PY$^+$ ($4 \pm 1\%$ in GFP plus fibrinogen compared with $74 \pm 7\%$ in PRP; $n = 4$; $P < 0.01$; Fig. 6) indicating that other plasma proteins are required. (In this assay fibrinogen is necessary for aggregation to occur once platelets have been activated). Addition of human serum and purified fibrinogen to GFP restored aggregation to the same level observed in PRP ($70 \pm 6\%$ for *L. lactis* ClfA$^+$ and $66 \pm 5\%$ for *L. lactis* ClfA PY$^+$ Fig. 6). Removal of IgG from serum by absorption with protein A eliminated the ability of *L. lactis* ClfA$^+$ and ClfA PY$^+$ to stimulate aggregation, demonstrating the requirement of IgG ($P < 0.01$; Fig. 6). Aggregation mediated by *S. aureus* Newman cells in the stationary phase was also dependent on the presence of IgG (data not shown). This is consistent with the observation that the monoclonal antibody IV-3, which blocks the IgG Fc receptor on platelets (FcγRIIa), inhibited aggregation by *L. lactis* ClfA$^+$ ($2 \pm 1\%$ aggregation; $n = 3$; $P < 0.01$), *L. lactis* ClfA PY$^+$ ($1 \pm 1\%$ aggregation; $n = 3$; $P < 0.01$) and *S. aureus* Newman stationary-phase cells ($1 \pm 0.5\%$, $n = 3$; $P < 0.01$).

ClfA-specific antibodies are required for ClfA-mediated platelet activation

To investigate further the role of IgG in ClfA-promoted platelet activation, the ability of *L. lactis* expressing ClfA wild type to stimulate aggregation of GFPs in the presence of purified fibrinogen and purified human IgG was tested. *L. lactis* ClfA$^+$ was able to stimulate aggregation to the same level as in PRP when pooled human IgG was added to GFP and fibrinogen ($72 \pm 4\%$; Fig. 7A). Pooled IgG was

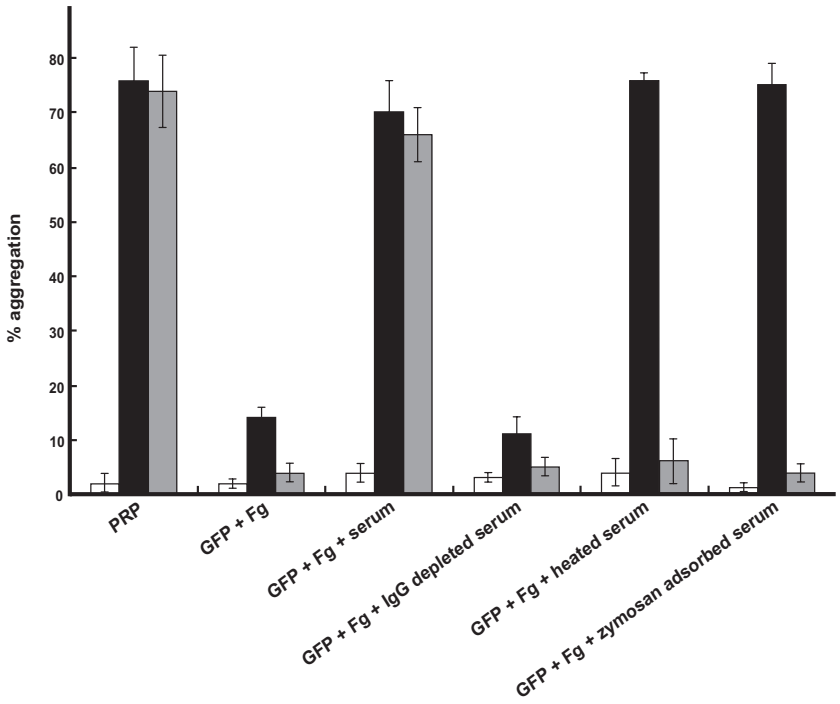

Fig. 6. Role of plasma factors in ClfA-promoted platelet activation. *L. lactis* pNZ8037 cells (white columns), *L. lactis* pNZ8037*clfA* cells (black columns) and *L. lactis* pNZ8037*clfA* PY cells (grey columns) were added to PRP or GFP samples supplemented with fibrinogen (Fg) and treated serum samples and aggregation allowed to occur. Results are presented as percentage aggregation.

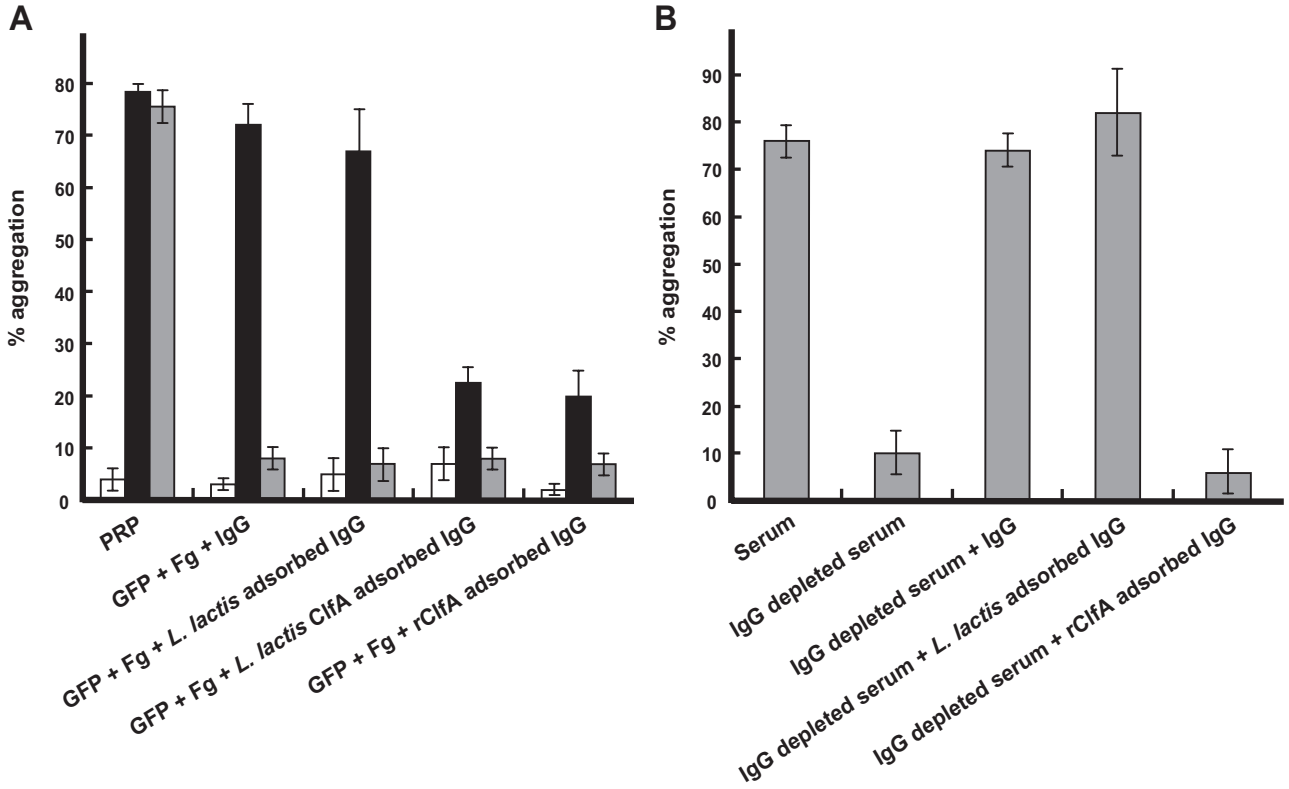

Fig. 7. Requirement for ClfA-specific IgG for ClfA-promoted platelet aggregation.
A. *L. lactis* pNZ8037 cells (white columns), *L. lactis* pNZ8037*clfA* cells (black columns) and *L. lactis* pNZ8037*clfA* PY cells (grey columns) were added to GFP samples supplemented with fibrinogen (Fg) and human IgG adsorbed against *L. lactis* pNZ8037, *L. lactis* pNZ8037*clfA*, recombinant ClfA (rClfA) A domain, or the unadsorbed control. Results are presented as percentage aggregation. The level of aggregation observed in PRP is also shown.
B. *L. lactis* pNZ8037*clfA* PY cells were added to GFP samples supplemented with fibrinogen. Serum or IgG-depleted serum was added with or without the addition of purified IgG samples. Results are presented as percentage aggregation.

adsorbed with *L. lactis* cells or *L. lactis* expressing high levels of ClfA and tested for its ability to support aggregation. *L. lactis* ClfA$^+$ stimulated aggregation of GFPs when IgG adsorbed with *L. lactis* cells was added (67 ± 8%) but when IgG adsorbed with *L. lactis* ClfA$^+$ cells was used the level of aggregation was significantly reduced (22 ± 3%; $P < 0.01$; Fig. 7A). Also IgG that was adsorbed with recombinant ClfA A domain protein supported greatly reduced aggregation (20 ± 5%; $P < 0.01$; Fig. 7A). ELISA experiments demonstrated that pooled human IgG had significant anti-ClfA antibodies (data not shown) which were completely removed by absorption with recombinant ClfA or *L. lactis* ClfA$^+$ cells. This suggests that antibodies that recognize ClfA are required, along with fibrinogen bound to ClfA, to engage low-affinity platelet receptors and stimulate rapid activation.

Both antibody and complement are required for L. lactis *ClfA PY$^+$ to promote platelet activation*

Lactococcus lactis ClfA PY$^+$ cannot bind fibrinogen. It aggregated platelets with a longer lag time than *L. lactis* ClfA$^+$ wild type in PRP. In order to promote activation of GFP *L. lactis* ClfA PY$^+$ required fibrinogen (for subsequent aggregation) and serum. Activation did not occur when IgG was adsorbed from the serum ($P < 0.01$; Fig. 6) or when anti-ClfA antibodies were removed ($P < 0.01$; Fig. 7B). This demonstrates a requirement for ClfA-specific IgG and is consistent with inhibition by the anti-FcγRIIa antibody IV-3 of *L. lactis* ClfA PY$^+$ aggregation in PRP. However, aggregation of GFP did not occur in the presence of purified fibrinogen and pooled human IgG (8 ± 2%; $P < 0.01$; Fig. 7A) which indicates that an additional factor in serum was required. In order to determine whether slow activation by *L. lactis* ClfA PY$^+$ required assembly of complement on the bacterial cell surface, complement was either inactivated by heating serum at 56°C or depleted from serum by adsorption with zymosan before adding to GFP and fibrinogen. The inability of the heated serum (7 ± 5%; $P < 0.01$) or zymosan-adsorbed serum (4 ± 2%; $P < 0.01$) to support platelet aggregation (Fig. 6) suggests that complement assembly was required. Complement-depleted serum still supported full aggregation by *L. lactis* ClfA$^+$ wild type (77 ± 2%; Fig. 6).

Adhesion of ClfA-expressing cells to platelets

The findings reported above suggest that *L. lactis* ClfA$^+$ but not *L. lactis* ClfA PY$^+$ bound to the surface of resting platelets in a fibrinogen-dependent manner. In order to demonstrate this directly, adhesion of resting platelets to immobilized ClfA-expressing bacterial cells was tested. Platelets did not adhere detectably to *L. lactis* expressing ClfA in the absence of fibrinogen (A_{405} 0.037 ± 0.011, $n = 4$), but in physiological concentrations of fibrinogen strong adhesion was observed (A_{405} 0.703 ± 0.074, $n = 4$, $P < 0.01$). The GPIIb/IIIa inhibitors abciximab and tirofiban blocked adhesion (A_{405} 0.02 ± 0.005, $n = 4$, $P < 0.01$) showing that binding occurred via GPIIb/IIIa. Platelets did not adhere to *L. lactis* ClfA PY$^+$ (A_{405} 0.015 ± 0.005, $n = 4$) or to the *L. lactis* host strain (A_{405} 0.012 ± 0.009, $n = 4$) in the presence of fibrinogen.

Discussion

The experiments described in this article were designed to investigate the mechanism by which bacterial cells displaying ClfA on their surface could trigger the activation and subsequent aggregation of platelets. For most experiments we measured aggregation (either percentage platelets aggregated or the time to aggregation) as the outcome of activation. To ensure that true activation was indeed occurring it was shown that both *L. lactis* ClfA$^+$-and *L. lactis* ClfA PY$^+$-promoted aggregation was inhibited by prostaglandin PGE_1 which blocks an enzyme in the intracellular signalling pathway. Furthermore, direct measurement of platelet activation by calcium flux confirmed that heterologously expressed ClfA promoted true platelet activation.

It has previously been shown that growth of *S. aureus* in peritoneal dialysate (which contains human plasma proteins) results in the coating of bacterial cells with proteins such as fibronectin and fibrinogen, recognized by the *S. aureus* adhesins (Massey *et al*., 2002). In plasma, bacterial cells expressing wild-type ClfA are presumably coated with fibrinogen which they bind at one end of the E domain via the protruding γ-chain (McDevitt *et al*., 1997). Resting platelets cannot bind fibrinogen in solution because of the low affinity of GPIIb/IIIa but they can adhere to fibrinogen-coated surfaces. Bacterial cells with bound fibrinogen act like a coated surface and can engage GPIIb/IIIa on resting platelets. Thus, adhesion of platelets to the bacterium occurs through GPIIb/IIIa recognizing fibrinogen immobilized on the surface of the bacterial cell (Sjöbring *et al*., 2002).

However, fibrinogen-dependent adhesion of ClfA-expressing cells to GPIIb/IIIa is itself insufficient to stimulate platelet activation. Here we show that the presence of ClfA-specific antibodies in normal human serum samples and in pooled IgG are also required to bind ClfA and link it to the FcγRIIa receptor. This is consistent with the anti-FcγRIIa antibody IV-3 blocking activation and aggregation (Sjöbring *et al*., 2002 and this study). The combination of fibrinogen- and IgG-dependent binding to platelets stimulates rapid activation. IgG has previously been shown to stimulate platelet activation. Heat-aggluti-

nated antibody binding to FcγRIIa receptors is sufficient to induce clustering and activation of intracellular signalling (Henson and Spiege, 1973). However, when IgG is bound to a bacterial cell clustering of FcγRIIa requires an additional bacterial-platelet interaction. In the case of *H. pylori* a direct interaction between the bacterial surface and GPIb occurs (Byrne *et al*., 2003). Here we show that cross-linking between ClfA and GPIIb/IIIa is required, or complement binding to a complement receptor. Circulating complexes of antibody and von Willebrand factor induced aggregation by cross-linking FcγRIIa and GPIb (Hoylaerts *et al*., 1998). Platelet factor 4 when complexed with heparin on the platelet surface creates a neo-epitope which when recognized by antibody engages FcγRIIa to cause activation (Newman and Chong, 2000). Finally a variety of antibodies that bind to platelet surface components can stimulate activation by simultaneously cross-linking platelets and clustering FcγRIIa (Tomiyama *et al*., 1992).

We have shown that a threshold number of ClfA molecules are required on the bacterial cell before activation can occur. Presumably a large number of molecules are required to coat the bacterium with sufficient amounts of fibrinogen and specific antibody to promote productive interactions with GPIIb/IIIa and FcγRIIa, stimulating receptor clustering and platelet activation. After the threshold number of ClfA molecules has been exceeded the lag time to aggregation shortened as the number of ClfA molecules increased.

We were somewhat surprised when we found that *L. lactis* expressing the non-fibrinogen-binding mutant ClfA PY still caused aggregation, although with an extended lag time. This unexpected finding indicated that a less efficient fibrinogen-independent mechanism of activation could also occur. The data reported above clearly show that ClfA-specific antibody in human sera is required, as in the fibrinogen-dependent mechanism, along with another plasma factor. The other plasma component(s) was shown to be complement proteins. Inactivation of complement by heating sera or removing complement with zymosan prevented activation and aggregation by ClfA PY but not by the wild type. Assembly of complement is probably mediated by the classical pathway through specific IgG bound to ClfA and perhaps also by assembly on the cell surface through the alternative pathway. This is the subject of current investigations in our laboratories.

Complement-dependent platelet aggregation by *S. sanguis* NCTC 7863 occurred with slower and more variable lag times (7–20 min; Ford *et al*., 1996) similar to that observed for *L. lactis* expressing ClfA PY (4.5–20.5 min depending on expression levels). The assembly of complement on the bacterium is time-dependent which explains the longer lag time. Indeed, when *L. lactis* cells expressing ClfA PY were incubated in serum and subsequently washed they stimulated potent activation with lag times of approximately 1 min (data not shown) indicating that the extended lag time for ClfA PY results from time taken for assembly of complement on the cell surface. We speculate that slow activation of platelets by bacterial cells in plasma is a hall mark of complement-dependent activation and thus might be a more general mechanism than previously thought. Indeed we propose that any *S. aureus* surface protein has the potential to stimulate activation of platelets provided it is expressed at a high enough level and that there are antibodies to the protein present in plasma. A schematic representation of the interactions involved in both the fibrinogen-dependent and fibrinogen-independent mechanisms of activation is given in Fig. 8.

The ability of ClfA to bind to fibrinogen in blood is likely to be inhibited somewhat by the physiological concentrations of Ca^{2+} (approximately 1.8 mM). Binding of Ca^{2+} to ClfA inhibits binding to fibrinogen (IC_{50} of 2–3 mM; O'Connell *et al*., 1998). Therefore, only about 50% of ClfA molecules will be occupied by fibrinogen *in vivo*. The ability of ClfA to activate platelets by both fibrinogen-dependent and fibrinogen-independent mechanisms may maximize the interaction between ClfA-expressing cells and platelets in the bloodstream.

There have been reports of the role of IgG in platelet aggregation by various bacterial species mediated through FcγRIIa (Sullam *et al*., 1988; Ford *et al*., 1997; Sjöbring *et al*., 2002; Byrne *et al*., 2003; Pietrocola *et al*., 2004). The requirement for FcγRIIa was also demonstrated here for platelet aggregation by stationary-phase *S. aureus* Newman cells. These findings may warrant investigation into the possibility of targeting FcγRIIa in treatment of vascular infections like IE.

In platelet activation and aggregation experiments a larger number of cells (10^8 ml^{-1}) are used than occurs in bacteraemia. This is done to ensure that the majority of platelets in the plasma sample become activated at the same time as a result of collisions with bacteria. There is no getting away from the fact that aggregation in an aggregometer is an artificial situation. Nevertheless, we contend that bacteria–platelet interactions on a smaller scale are important in the development of endovascular infections. Localized circulating thrombi composed of activated platelets with bacteria attached might contribute to the initiation of endocarditis. Also, immobilized bacteria in damaged endothelium might capture circulating platelets, activate them, and thus contribute to the development of the infected thrombus.

In conclusion we have shown that ClfA expressed on the surface of *S. aureus* or the surrogate host *L. lactis* can promote rapid platelet activation by engaging the resting platelet integrin GPIIb/IIIa through a fibrinogen bridge and the IgG FcγRIIa receptor through an IgG bridge requiring

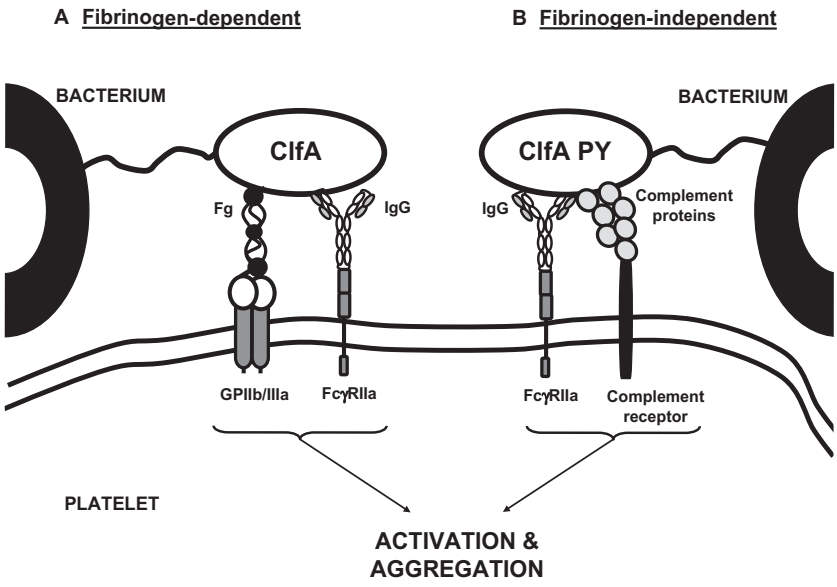

Fig. 8. Summary of the interactions between ClfA and the platelet that lead to activation.
A. Fibrinogen-dependent platelet aggregation by ClfA. Adhesion of ClfA-expressing bacteria to resting platelets is mediated by a fibrinogen bridge to GPIIb/IIIa. IgG bound to ClfA interacts with the Fc receptor FcγRIIa. These interactions generate signals resulting in activation of platelet aggregation.
B. Fibrinogen-independent platelet activation by ClfA. Binding of IgG to ClfA PY and assembly of complement proteins is required to interact with FcγRIIa and a complement receptor, respectively, on platelets resulting in platelet activation and aggregation.

specific antibodies. ClfA lacking the ability to bind fibrinogen stimulates a slower activation process dependent on specific antibodies and complement assembly.

Experimental procedures

Materials

ADP was purchased from BioData, PA, USA. Abciximab was from Eli Lily, Basingstoke, UK. IV-3 was kindly provided by R. Klimkowski, Medarex, NJ, USA. Tirofiban was from Merck. Goat anti-rabbit antibodies conjugated to horseradish peroxidase were from Dako, Denmark. Human fibrinogen and FURA-2-AM were from Calbiochem, Nottingham, UK. Purified pooled human IgG (Gammagard) was obtained from Baxter, UK. Zymosan and nisin were purchased from Sigma. Enzymes for DNA manipulation were purchased from Roche and Promega and used in accordance with the manufacturers' instructions.

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* was routinely grown on M17 agar or statically in M17 broth incorporating 0.5% (w/v) glucose at 30°C. *S. aureus* was grown in brain–heart infusion (BHI) broth at 37°C with shaking (200 r.p.m.) for 18 h. *E. coli* Topp3 (Qiagen) was used for the expression of recombinant proteins. Antibiotics were incorporated into the growth media where appropriate: ampicillin (100 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}), erythromycin (5 μg ml^{-1}) and kanamycin (50 μg ml^{-1}).

Site-directed mutagenesis

The mutations P336S and Y338A were generated in the *clfA* gene by overlap primer polymerase chain reaction (PCR) (O'Connell *et al*., 1998). A flanking forward primer (CCGG TACCATAAATTACACATC) and a reverse primer incorporating the desired nucleotide mismatches (GGTCAATA**GC**AGCG G**A**CATGGAC; mutagenic nucleotides in bold) were used in a PCR reaction with plasmid pCF77 as the template. In a second PCR reaction, a mutagenic forward primer incorporating the same nucleotide changes (GACCATG**T**C CGCT**GC**TATTGACC) and a flanking reverse primer (CGCG GATCCCTCTGGAATTGGTTCAATTTC) were used. Both PCR products were used as a template in a final PCR reaction using the flanking forward and reverse primers used above. The final PCR product with mutational changes in the codons for P336 and Y338 and the *Kpn*I and *Bam*HI sites at their termini were cloned in frame into the full-length *clfA* gene of pCF77 (Hartford *et al*., 1997) generating plasmid pCF77 PY.

Expression of ClfA and ClfA PY by S. aureus

Plasmids pCF77 and pCF77 PY were transformed into *S. aureus* RN4220 cells by electroporation (Oskouian and Stewart, 1990). Plasmids and the *spa*::Ka^r mutation were transduced from RN4220 into *S. aureus* DU5944 using phage 85 (Asheshov, 1966).

Expression of ClfA and ClfA PY by L. lactis

The *clfA* and *clfA* PY genes were amplified by PCR using *S. aureus* Newman genomic DNA and pCF77 PY DNA as templates with the forward primer CATG*CCATGG* AGATGAAGAAAAAAGAAAAACCAC and reverse primer TGC*TCTAGA*CTCAGATACATATGGATTCTTC. Bases in italics indicate 5′ NcoI and XbaI sites introduced to facilitate cloning. PCR products were cleaved with NcoI and XbaI and ligated into pNZ8037 cleaved with the same enzymes. Ligated DNA was transformed into electrocompetent *L. lactis* NZ9800 cells as described previously (Wells *et al*., 1993) generating *L. lactis* pNZ8037*clfA* and *L. lactis* pNZ8037*clfA*

Table 1. Bacterial strains and plasmids.

Strain/plasmid	Relevant properties	Source or reference
S. aureus strain		
RN4220	Stable maintenance of shuttle plasmids	Kreiswirth *et al.* (1983)
Newman	Archetypal clumping factor producing strain	Duthie and Lorenz (1952)
DU5944	Newman *clfA*::Tn*917* Em^R, *clfB*::Tc^R. Deficient in clumping factor A and B	Ni Eidhin *et al.* (1998)
DU5971	Newman *spa*::Ka^R. Deficient in protein A	O'Brien *et al.* (2002)
DU6014	Newman *clfA*::Tn*917* Em^R, *clfB*::Tc^R, *spa*::Ka^R. Deficient in ClfA, ClfB and protein A	This study
L. lactis strain		
NZ9800	Host for heterologous expression. Δ*nisA*	Kuipers *et al.* (1993)
S. aureus plasmids		
pCUI	*E. coli–S. aureus* shuttle plasmid. Ap^R Cm^R	Augustin and Gotz (1990)
pCF77	pCU1 carrying full-length *clfA* gene with *Bam*HI site engineered between A and R domain encoding regions. Ap^R Cm^R	Hartford *et al.* (1997)
pCF77 PY	pCF77 with mutations encoding the P336S Y338A substitutions (*clfA* PY). Ap^R Cm^R	This study
L. lactis plasmids		
pNZ8037	Expression vector with nisin-inducible promoter. Cm^R	De Ruyter *et al.* (1996)
pNZ8037*clfA*	pNZ8037 expressing *clfA* from nisin-inducible promoter. Cm^R	This study
pNZ8037*clfA*PY	pNZ8037 expressing *clfA* PY from nisin-inducible promoter. Cm^R	This study

Ap^R, Cm^R, Tc^R, Em^R and Ka^R: resistance to ampicillin, chloramphenicol, tetracycline, erythromycin and kanamycin.

PY. Cells were grown to exponential phase (OD_{600} 0.5) and induced with a final concentration of 1.6 ng ml^{-1} nisin (unless otherwise stated) for 16 h before harvesting.

Whole-cell immunoblotting

Bacterial cells were washed twice in PBS and adjusted to OD_{600} of 1.0 in PBS. Doubling dilutions of cells were made in 96-well dishes and 5 μl of each dilution was applied to a nitrocellulose membrane (Protran) and allowed to air-dry. Membranes were blocked in 10% skimmed milk in TS buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl) followed by a 1 h incubation with rabbit antibodies recognizing the unique A domain of ClfA (1:5000 dilution in 10% milk in TS buffer). Membranes were washed three times with TS buffer and bound antibody detected with goat anti-rabbit antibody (1:2000) that was conjugated to horseradish peroxidase and developed by chemiluminescence.

Western immunoblotting

Cell wall-associated proteins of *L. lactis* strains were released by mutanolysin/lysozyme digestion, separated on SDS-PAGE gels and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Hartford *et al.*, 2001). Membranes were probed and developed as described above.

Recombinant ClfA

Recombinant ClfA A domain protein comprising residues 40–559 containing a 6×His tag were purified by Ni^{2+} chelate chromatography (O'Connell *et al.*, 1998).

Fibrinogen adherence and cell clumping assays

Adherence of bacteria to immobilized fibrinogen 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 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. Clumping of *S. aureus* and *L. lactis* cells in a solution of fibrinogen were carried out in Sarstedt flat-bottomed 96-well dishes. Purified human fibrinogen (50 μl of a 3 mg ml^{-1} solution) was mixed with bacterial cell suspension (20 μl of OD_{600} 6.0) in separate wells and the plate agitated briskly for 5 min. The absorbance of the wells at 600 nm was read. Results are presented as percentage clumping relative to *S. aureus* Newman (100% clumping).

Depletion of ClfA-specific antibodies from pooled human IgG

Pooled IgG was adsorbed with *L. lactis* pNZ8037 or *L. lactis* pNZ8037*clfA* cells maximally induced with 1.6 ng ml^{-1} nisin. Washed bacteria (10^{10} cells) were pelleted by centrifugation at 10 000 *g* for 5 min, resuspended in IgG (Baxter Gammagard; 50 mg in 1 ml) and incubated with rotation for 1.5 h at 4°C. Bacterial cells were pelleted by centrifugation to yield the IgG-containing supernatant, which was filtered through a 0.45 μM filter (Pall Corporation, Cornwall, UK). IgG was also adsorbed with recombinant ClfA A domain protein immobilized on a Ni^{2+}-Sepharose column. The hexahistidine ClfA A domain protein (1 mg) was applied to a HiTrap Chelating HP column (5 ml; Amersham Biosciences, Uppsala, Sweden) previously equilibrated with 150 mM Ni^{2+} in binding buffer (5 mM imadazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The column was washed until the A_{280} of the eluate was <0.001. Pooled human IgG (Baxter Gammagard; 50 mg in 5 ml) was passed over the column in a continuous flow system for 16 h at 4°C. The column was then washed with PBS to retrieve the unbound IgG, which was concentrated to 100 mg ml^{-1} using a centrifugal filtration device (Millipore) for use in platelet aggregation assays. IgG was tested for reactivity with

recombinant ClfA A domain protein to verify depletion of anti-ClfA IgG by ELISA as previously described (Roche *et al*., 2003).

Depletion of IgG and complement from human serum

Normal human serum was treated with protein A-sepharose to remove IgG. One millilitre of serum was passed through a column containing 1 ml of packed protein A-sepharose (Sigma) and the flowthrough collected and again passed through the column. Non-specifically bound plasma proteins were washed from the column with PBS and pooled with the serum fraction. The sample was concentrated to its original volume and depletion of IgG was verified by Western blotting using a goat anti-human antibody (Dako, Denmark) conjugated to horseradish peroxidase. Complement-depleted serum was prepared either by heating aliquots at 56°C for 10 min, or by treatment with zymosan (100 mg ml^{-1}) at 37°C for 30 min as previously described (Ford *et al*., 1996).

Purification of fibrinogen

Fibrinogen bought from commercial sources is usually contaminated with immunoglobulin. Fibrinogen was purified by protein A-sepharose chromatography as described above to remove contaminating IgG. Purity was assessed by Western immunoblotting using a goat anti-human Fc-specific antibody conjugated to peroxidase (Dako).

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 of 3.8% (w/v) Na citrate, centrifuged for 10 min at 150 *g* and the PRP layer was removed. An aliquot of PRP was further centrifuged at 1000 *g* to yield platelet-poor plasma (PPP) which was used as the 100% light transmission reference in PRP aggregation assays. GFPs were prepared as described previously (Walkowiak *et al*., 2000). Platelets isolated in this manner have been shown to be 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 PGE_1 (Sigma) was added (1 μM) before 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 of 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. Cells (1×10^8; in 50 μl of PBS) were added to 450 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. Normal human serum or treated serum samples were added where indicated (50 μl of serum in final volume of 225 μl of GFP mixture).

Calcium fluorimetry

Washed bacterial cells (5×10^8) were incubated in human serum samples (250 μl) supplemented with physiological fibrinogen concentrations (3 mg ml^{-1}) with stirring at 37°C for 30 min. Bacterial cells were harvested by centrifugation, washed in PBS and adjusted to 5×10^8 cells in 250 μl of PBS. 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. Intracellular Ca^{2+} changes were detected after addition of 1×10^8 bacterial cells (in 50 μl of PBS) to 8×10^7 FURA-2-AM-loaded GFPs (in 0.4 ml of JNL buffer) 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 resultant colour 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 samples was tested by unpaired *t*-test, with significance defined as $P < 0.05$.

Acknowledgements

We kindly thank R. Klimkowski for providing the monoclonal antibody IV-3. This project was supported by a programme grant from the Health Research Board of Ireland (PRO09/2002).

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