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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

Louise O´Brien,[1¶] Steven W. Kerrigan,[2†¶] Gideon Kaw,[2] Michael Hogan,[2] José Penadés,[1‡] David Litt,[3§] Desmond J. Fitzgerald,[2] Timothy J. Foster[1] and Dermot Cox[2*]

[1]*Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College, Dublin 2, Ireland.*
[2]*Department of Clinical Pharmacology, Royal College of Surgeons in Ireland, Dublin 2, Ireland.*
[3]*Department of Pathology, University of Cambridge, Cambridge, UK.*

Summary

The ability of *Staphylococcus aureus* cells to induce platelet aggregation has long been recognized. However, despite several attempts to identify the mechanisms involved in this interaction, the nature of the bacterial receptors required remains poorly understood. Using genetic manipulation, this study for the first time provides clear evidence that several *S. aureus* surface proteins participate in the interaction with platelets. Mutants of *S. aureus* strain Newman lacking one or more surface proteins were tested for their ability to stimulate platelet aggregation. This approach was complemented by the expression of a number of candidate proteins in the non-aggregating Gram-positive bacterium *Lactococcus lactis*. *S. aureus*-induced aggregation was monophasic and was dependent on the platelet receptor GPIIb/IIIa. The fibrinogen-binding proteins, clumping factors A and B and the serine-aspartate repeat protein SdrE could each induce aggregation when expressed in *L. lactis*. Although protein A expressed in *L. lactis* was not capable of inducing aggregation independently, it enhanced the aggregation response when expressed on the surface of *S. aureus*. Thus, *S. aureus* has multiple mechanisms for stimulating platelet aggregation. Such functional redundancy suggests that this phenomenon may be important in the pathogenesis of invasive diseases such as infective endocarditis.

Accepted 8 February, 2002. *For correspondence. E-mail dcox@rcsi.ie; Tel. (+353) 1 402 2152; Fax (+353) 1 402 2543. Present addresses: [†]Department of Laboratory Medicine, University of California San Francisco, Room S5-77: Box 0100, 513 Parnassus Avenue, San Francisco, CA 94143, USA. [‡]Unit of Biochemistry, Department of Basic Biomedical Sciences, Cardenal Herrera-CEU University, 46113 Moncada, Valencia, Spain. [§]Department of Academic Paediatrics, Imperial College of Science, Technology and Medicine, Norfolk Place, London W2 1PG, UK. [¶]These authors contributed equally to the work in this paper.

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Introduction

One complication of *Staphylococcus aureus* bacteraemia is the development of infective endocarditis (IE) (Chang, 2000). This is characterized by the formation of a vegetative growth on the surface of a heart valve. If untreated, this is often fatal and, even with aggressive therapy, mortality can be as high as 20–40% (Wilson *et al*., 1995). *S. aureus* endocarditis is unusual because a history of prior valvular damage is not required, and it is becoming more common on account of its association with intravenous drug use. The emergence of *S. aureus* strains that are resistant to antibiotics (methicillin-resistant *S. aureus*, MRSA) has increased the mortality from infection (Gentry *et al*., 1997).

An important factor in the development of IE is the formation of platelet–bacteria thrombi on the valve surface. The interaction of platelets and bacteria is essential for the development of IE (Sullam *et al*., 1996). When *S. aureus* cells are added to platelet-rich plasma (PRP), platelet aggregation can be observed (Hawiger *et al*., 1979). Induction of platelet aggregation by *S. aureus* is likely to be mediated by bacterial cell wall-associated proteins known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (Patti *et al*., 1994), as trypsin-treated *S. aureus* cells no longer promote aggregation (Hawiger *et al*., 1979). *S. aureus* is also known to adhere to platelets via a fibrinogen/fibrin-dependent mechanism (Herrmann *et al*., 1993).

The *S. aureus* proteins ClfA and ClfB (clumping factors A and B) are two structurally related fibrinogen-binding MSCRAMMs (McDevitt *et al*., 1994; Ní Eidhin *et al*., 1998). Both ClfA and ClfB contain all the classical features associated with *S. aureus* cell wall-associated proteins, including an N-terminal secretory signal sequence, a C-terminal LPXTG motif, a hydrophobic wall- and membrane-spanning domain and a positively charged tail. ClfA

and ClfB are characterized by the presence of a region known as region R, which consists of repeating serine and aspartic acid dipeptides (SD repeats). Region R connects the cell wall-spanning domain to the unique ligand-binding region (region A). ClfA and ClfB are members of a larger family of *S. aureus* proteins known as the serine-aspartate repeat (Sdr) protein family (Josefsson *et al*., 1998a). The Sdr proteins are predicted to have a similar structural organization to ClfA and ClfB. In addition, they contain two (SdrC), three (SdrE) and five (SdrD) B repeats of 110–113 residues, which separate the A region from the R domain.

A previous study suggested that protein A (Spa) was required for *S. aureus*-induced platelet aggregation (Hawiger *et al*., 1979). Protein A is a surface-displayed, cell wall-associated protein that contains varying numbers of repeat domains (Sjödahl, 1977; Moks *et al*., 1986). It is primarily known for its ability to bind to the Fc region of mammalian IgG. However, recently, Spa was shown to bind to von Willebrand factor (Hartleib *et al*., 2000), which is recognized by the platelet glycoprotein GPIb, and to the complement receptor gC1qR/p33 (Nguyen *et al*., 2000), which is also on the platelet surface. Thus, there are several possible mechanisms by which Spa-expressing *S. aureus* cells may interact with platelets.

One of the problems encountered in comparing the ability of different *S. aureus* strains to interact with platelets or indeed to other host components is the fact that they often express a different profile of surface proteins (Peacock *et al*., 2000; Rice *et al*., 2001), and the level of expression can also vary from strain to strain (McAleese *et al*., 2001). Therefore, when comparing strains to identify novel interactions, it is essential to use site-specific isogenic mutants. A report claiming that the failure of the protein A-deficient *S. aureus* strain Wood 46 to bind platelets compared with the positive interaction by the protein A-expressing *S. aureus* strain Cowan I, implying that protein A was responsible, did not account for the possibility that these strains most probably differed in the expression of other surface proteins (Nguyen *et al*., 2000).

To identify the *S. aureus* surface proteins involved in the activation of human platelets, we tested the ability of specific mutants of *S. aureus* strain Newman that were defective in the expression of various cell wall-associated proteins to activate platelet aggregation. This approach was complemented by the expression of *S. aureus* surface proteins individually in the non-aggregating Gram-positive bacterium *Lactococcus lactis*.

Results

Expression of surface proteins by S. aureus *and* L. lactis

Staphylococcus aureus mutants defective in the expression of specific surface proteins were prepared by site-specific mutagenesis, and *L. lactis* strains that expressed ClfA, ClfB, SdrC, SdrD, SdrE, protein A (Spa) and Map were constructed using the expression vector pKS80. The expression of these proteins was studied by Western immunoblotting using specific antibodies, except for protein A, which was recognized by its ability to bind IgG in a non-immune reaction (Fig. 1).

When tested with anti-ClfA antibodies, *S. aureus* Newman showed a single immunoreactive band of 170 kDa, which was missing in the *clfA* mutant, whereas *L. lactis* (pKS80:*clfA*) expressed two immunoreactive bands of 170 kDa and 130 kDa (Fig. 1A). Both Newman and *L. lactis* (pKS80:*clfB*), but not Newman *clfB*, expressed two immunoreactive bands of 150 kDa and 120 kDa when reacted with anti-ClfB antibodies (Fig. 1B). The 170 kDa ClfA molecule and the 150 kDa ClfB molecule are the full-length intact forms of the proteins, whereas the 130 kDa ClfA and the 120 kDa ClfB moieties have been cleaved at the motif SLAAVA (ClfA) or SLAVA (ClfB) by the staphylococcal metalloprotease (McAleese *et al*., 2001; unpublished data). The ClfA truncate is functional, whereas the ClfB truncate does not bind fibrinogen (O'Connell *et al*., 1998; McAleese *et al*., 2001).

A protein of 180 kDa reacted with anti-SdrE antibodies in Newman and *L. lactis* (pKS80:*sdrE*), but not in Newman *sdrE* (Fig. 1C). N-terminal sequencing of the *L. lactis*-expressed 180 kDa protein revealed the sequence AENTST, which corresponds to that predicted to be at the N-terminus after cleavage of the signal peptide. We conclude that the full-length form of SdrE is anchored to the surface of *L. lactis.*

Staphylococcus aureus Newman wild-type cells and *L. lactis* (pKS80:*spa*) cells both expressed an IgG-binding protein of 55 kDa. This protein was not detected in either the *L. lactis* host strain or a Newman *spa* mutant. *L. lactis* (pKS80:*sdrC*) expressed a 160 kDa protein that reacted with anti-SdrC antibodies, *L. lactis* (pKS80:*sdrD*) expressed a 205 kDa protein that reacted with anti-SdrD antibodies, and *L. lactis* (pKS80:*map*) expressed a 70 kDa protein that reacted with anti-map antibodies (data not shown).

Comparative analysis by flow cytometry of *S. aureus* Newman and *L. lactis* cells expressing either ClfA or protein A showed that *L. lactis* cells expressed similar levels of protein on their surface compared with *S. aureus*. The relative fluorescence of ClfA expressed on the surface of *S. aureus* was 179 ± 70, whereas the relative fluorescence of ClfA expressed on the surface of *L. lactis* was 169 ± 56 ($P > 0.05$). Similarly, the relative fluorescence of protein A expressed on the surface of *S. aureus* was 29 ± 16, whereas the relative fluorescence given by the protein A molecules expressed on the surface of *L. lactis* was 27 ± 11 ($P > 0.05$). These data show that the

heterologous proteins are expressed on the surface of *L. lactis* at levels similar to *S. aureus*. Furthermore, the ability of protein A to recognize and bind non-immune IgG indicates that this protein is functional when expressed on the surface of *L. lactis*.

Functional analysis of S. aureus *protein expressed in* L. lactis

In order to determine whether *S. aureus* cell wall-associated proteins were expressed in a functional form on the surface of *L. lactis*, the ClfA- and ClfB-expressing strains were tested for their ability to adhere to immobilized fibrinogen. Both strains adhered in a dose-dependent and saturable fashion (Fig. 2) similar to the ClfA- or ClfB-dependent adhesion of *S. aureus* Newman (ClfA$^+$ ClfB$^-$) and Newman (ClfA$^-$ ClfB$^+$). *L. lactis* cells expressing either ClfA or ClfB adhered to fibrinogen with a slightly higher affinity than the *S. aureus*-expressed protein (Fig. 2). It should be noted that ClfB-mediated fibrinogen binding by *S. aureus* (Newman) can only be studied with cells from the exponential phase of growth (Ní Eidhin *et al*., 1998; McAleese *et al*., 2001). In contrast, ClfA and SdrE were expressed on cells from both exponential and stationary phases. These data showed that both ClfA and ClfB were expressed in a fully functional form on the surface of *L. lactis*.

Platelet aggregation

The ability of *S. aureus* strain Newman cells to induce platelet aggregation in PRP was tested. *S. aureus* Newman wild-type cells, which were grown to exponential phase, induced platelet aggregation with a lag time of

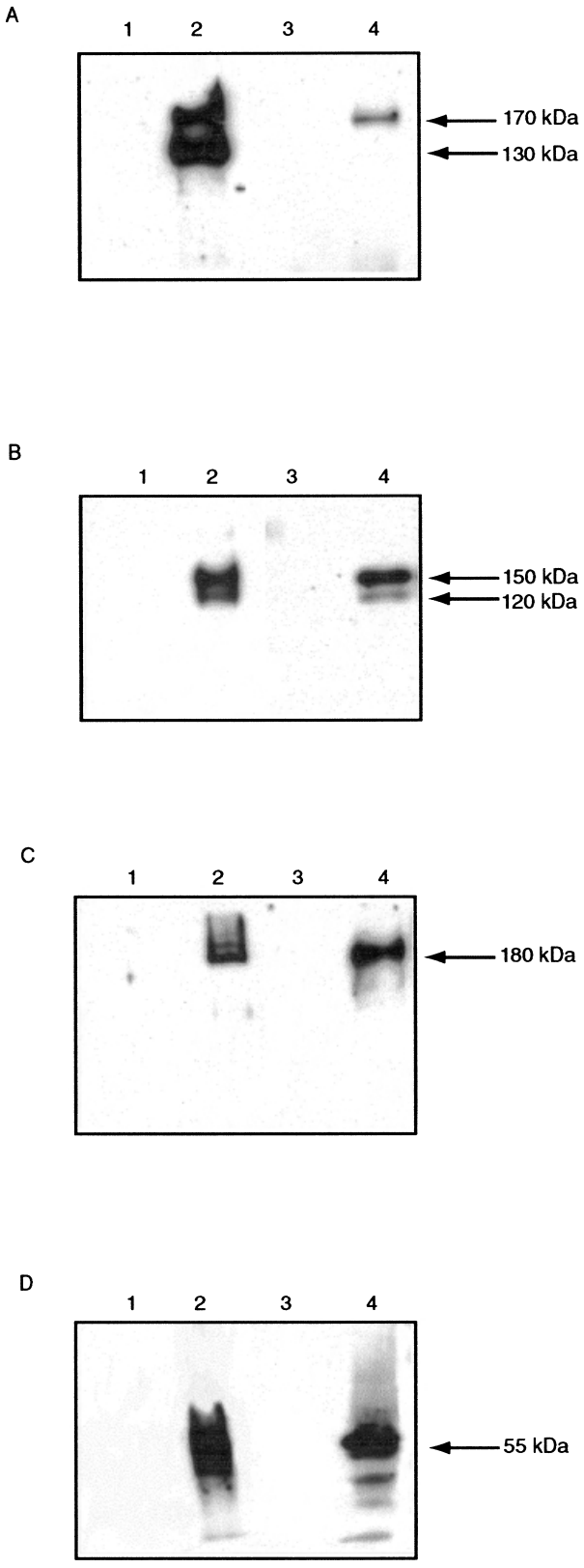

Fig. 1. Western immunoblot analysis of ClfA, ClfB, SdrE and Spa expression in *L. lactis* MG1363 and *S. aureus* Newman.
A. Cell wall-associated proteins were released from stabilized protoplasts of *L. lactis* MG1363 (lane 1) and *L. lactis* (pKS80:*clfA*) (lane 2) by mutanolysin/lysozyme digestion. Cell wall-associated proteins were released from stabilized protoplasts of *S. aureus* Newman *clfA* (lane 3) and *S. aureus* Newman wild-type cells (lane 4) by lysostaphin digestion.
B. Cell wall-associated proteins were isolated from *L. lactis* MG1363 (lane 1), *L. lactis* (pKS80:*clfB*) (lane 2B), *S. aureus* Newman *clfB* (lane 3) and *S. aureus* Newman wild type (lane 4).
C. Cell wall-associated proteins were isolated from *L. lactis* MG1363 (lane 1), *L. lactis* (pKS80:*sdrE*) (lane 2), *S. aureus* Newman *sdrE* (lane 3) and *S. aureus* Newman wild type (lane 4).
D. Cell wall-associated proteins were isolated from *L. lactis* MG1363 wild type (lane 1), *L. lactis* (pKS80:*spa*) (lane 2), *S. aureus* Newman *spa* (lane 3) and *S. aureus* Newman wild type (lane 4).
In each blot, proteins were separated by SDS–PAGE, transferred to polyvinylidene difluoride (PVDF) membranes and probed with specific rabbit antibodies to ClfA (A), antibodies to ClfB (B) and antibodies to SdrE (C) followed by protein A peroxidase. In (D), membranes were probed with non-immune rabbit IgG followed by peroxidase-conjugated goat anti-rabbit IgG.

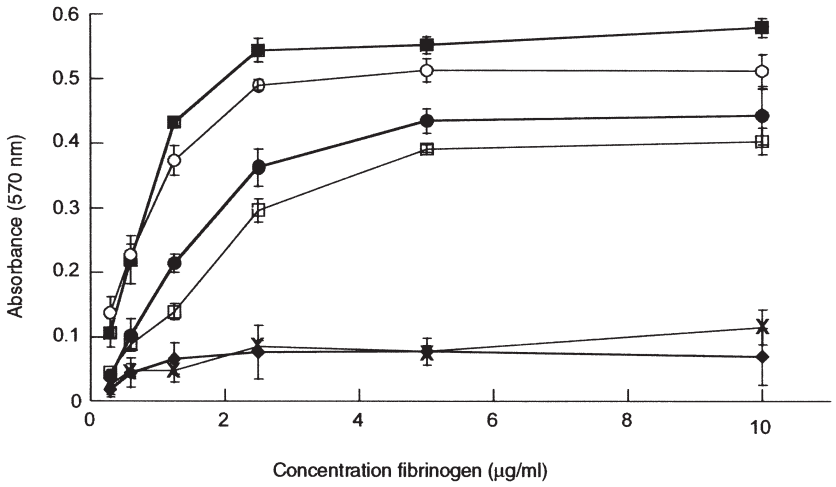

Fig. 2. Adherence of *L. lactis* and *S. aureus* cells expressing ClfA and ClfB to immobilized fibrinogen. ELISA plates (96-well) were coated with different concentrations of fibrinogen ranging from 0.3 to 10 μg ml^{-1}. Adherence of *L. lactis* MG1363 wild type (closed diamonds), *L. lactis* (pKS80:*clfA*; closed squares), *L. lactis* (pKS80:*clfB*; closed circles) and *S. aureus* Newman ClfA$^-$ (open squares), Newman ClfB$^-$ (open circles) and Newman ClfA$^-$ ClfB$^-$ (crosses) to immobilized fibrinogen was tested. Adherent cells were stained with crystal violet and measured in an ELISA plate reader at 570 nm.

7 ± 1 min (n = 6) with a monophasic response. *S. aureus* Newman wild-type cells, which had been grown to stationary phase, induced platelet aggregation with a lag time of 1.5 ± 0.1 min (n = 3), also with a monophasic response. This is in contrast to arachidonic acid-induced platelet aggregation, which has a lag time of 0.12 ± 0.01 min (n = 3) in PRP. These results showed that *S. aureus* strain Newman could activate platelet aggregation and that this phenomenon was dependent on the growth phase of the bacterium.

Effects of platelet inhibitors

A previous report (Bayer *et al.*, 1995) suggested that *S. aureus*-induced platelet aggregation was not dependent on the platelet receptor GPIIb/IIIa, so we decided to investigate this. Human platelets (PRP) were incubated with the GPIIb/IIIa antibody inhibitors abciximab, eptifibatide, tirofiban or the GPIIb/IIIa peptide inhibitor, RGDS (Arg-Gly-Asp-Ser). Aggregation experiments were then performed using either *S. aureus* strain Newman or adenosine diphosphate (ADP) as agonists. The results showed that each of these inhibitors prevented platelet aggregation by both *S. aureus* cells and ADP (Fig. 3). *S. aureus* strain Newman-induced platelet aggregation was also completely inhibited by 1 μM aspirin (a cyclooxygenase inhibitor; 47 ± 1% control and 0% with aspirin, n = 4) and by 1 μM prostaglandin E1 (PGE_1; 48 ± 9% control and 1 ± 0.3% with PGE_1, n = 3). Although 10 U ml^{-1} apyrase (ADPase) totally inhibited ADP-induced aggregation, it had no effect on *S. aureus* Newman-induced aggregation (43 ± 5% control and 36 ± 4% with apyrase, n = 3). (Some agonists depend on ADP secretion during activation.) These results indicate that *S. aureus*-induced platelet aggregation is indeed true aggregation that occurs in a GPIIb/IIIa-dependent fashion, but that it is not secretion dependent. It should be noted that the comparison between *S. aureus*-induced platelet aggregation and ADP-induced platelet aggregation is expressed here as percentage aggregation rather than as lag time. When comparing bacterial variants (see below), data are expressed as the lag time to aggregation.

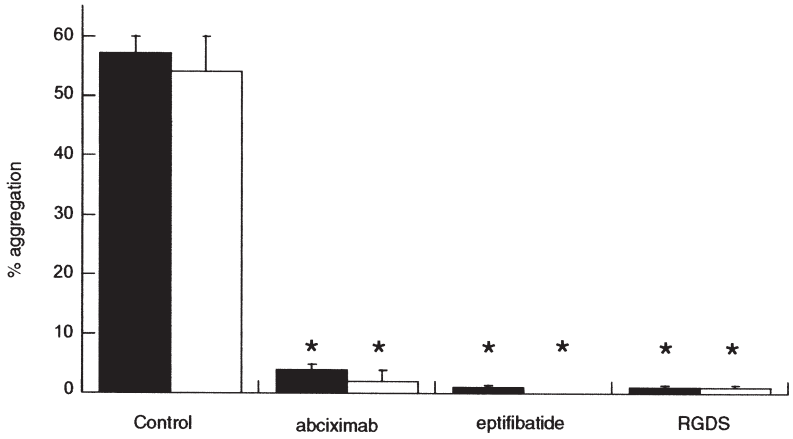

Fig. 3. Inhibition of *S. aureus*-induced platelet aggregation by GPIIb/IIIa inhibitors. Platelet-rich plasma was pretreated with abciximab (10 μg ml^{-1}), eptifibatide (1 μM), RGDS (500 μM) or no inhibitor (control) for 10 min before the addition of the agonists (ADP, 20 μM, black columns; or *S. aureus* Newman cells from exponential phase, OD 1.6, blank columns). * indicates $P < 0.01$. Results are expressed as percentage aggregation.

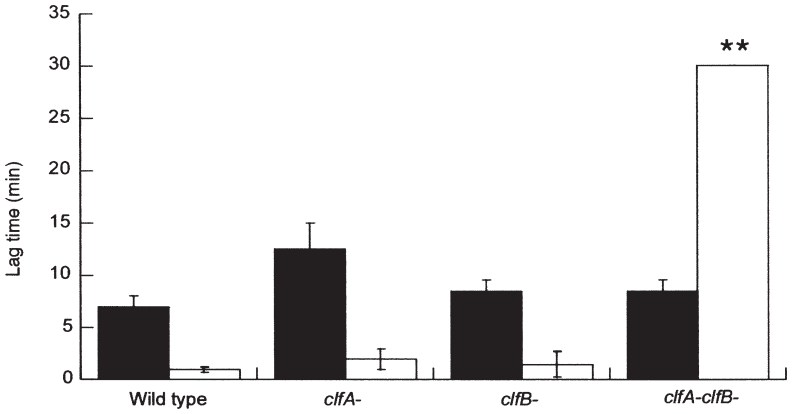

Fig. 4. Activation of platelet aggregation by *S. aureus* Newman in platelet-rich plasma and with gel-filtered platelets. The ability of Newman wild-type and Newman ClfA$^-$, Newman ClfB$^-$ and Newman ClfA$^-$ ClfB$^-$ mutant cells, grown to exponential phase, to activate platelet aggregation in PRP (black columns) or GFP (blank columns) was tested. ** indicates that no aggregation occurred after 30 min incubation. Results are presented as a comparison between the time taken to aggregation for each strain. The degree of aggregation observed (percentage aggregation) was comparable for each of the strains in which aggregation occurred.

Ability of S. aureus *mutants to induce human platelet aggregation*

In order to identify factors responsible for the activation of platelet aggregation by *S. aureus* Newman, a series of mutants defective in surface factors was tested. Strains deficient in the clumping factors ClfA or ClfB, protein A, the elastin-binding protein EbpS, the fibronectin-binding proteins FnBPA and FnBPB, the SD repeat proteins SdrC or SdrD, the major histocompatibility complex (MHC) class II analogous protein, coagulase and capsular polysaccharide 5 induced platelet aggregation with lag times similar to that of the wild-type strain (5 ± 2 min). Exponential phase cells were used because work in our laboratory and elsewhere has demonstrated that *S. aureus* Newman proteins ClfB, FnBPA and FnBPB are expressed predominantly in that phase (Greene *et al*., 1995; Saravia-Otten *et al*., 1997; McAleese *et al*., 2001). However, it should be noted that other proteins are expressed on the surface of *S. aureus* cells throughout the growth cycle. We compared, by flow cytometry, the level of ClfA on the surface of Newman *spa* cells from exponential phase and from stationary phase, in which the mean fluorescence was 2283 ± 66 and 426 ± 122 respectively ($P < 0.01$). The approximately fivefold higher ClfA on stationary phase cells could explain the faster activation time of cells from this phase of growth described above.

Role of ClfA and ClfB in S. aureus*-induced platelet aggregation*

As ClfA is known to interact directly with platelets and fibrinogen binding plays a crucial role in platelet aggregation, it was decided to investigate the possible roles for the fibrinogen-binding *S. aureus* proteins ClfA and ClfB in this process. *S. aureus* strain Newman wild type and Newman *clfA*, *clfB* and *clfA clfB* mutants were grown to exponential phase and tested for their ability to induce platelet aggregation. In gel-filtered platelets (GFPs) supplemented with fibrinogen, both *clfA* and *clfB* single mutants induced platelet aggregation with lag times similar to that of the wild-type strain (Fig. 4). Supplementing the GFPs with fibrinogen was essential, as *S. aureus* wild-type cells failed to induce aggregation of GFPs in the absence of fibrinogen (47 ± 4% with fibrinogen and 0% without). In contrast, the *clfA clfB* double mutant failed to induce aggregation. However, when the experiment was performed with PRP, platelet aggregation was detected with both *clfA* and *clfB* single mutants and with the *clfA clfB* double mutant (Fig. 4). The lag times observed with each of the mutant strains in PRP were comparable with that of the wild-type strain. These results indicated that both ClfA and ClfB stimulated *S. aureus*-induced platelet aggregation in a fibrinogen-dependent fashion and that at least one further fibrinogen-independent mechanism was identified. The third mechanism was capable of stimulating platelet aggregation independently of ClfA and ClfB only in PRP. Thus, there is considerable functional redundancy in factors used by *S. aureus* Newman to activate platelets.

Effect of S. aureus *proteins expressed in* L. lactis *on human platelet aggregation*

As the mechanism by which *S. aureus* activated platelet aggregation appeared to be multifactorial and compensatory, an alternative approach to identifying the protein(s) involved in the process was used. Candidate *S. aureus* surface proteins ClfA, ClfB, SdrC, SdrD, SdrE, protein A, EbpS and Map were expressed in the non-aggregating surrogate Gram-positive host *L. lactis*. *L. lactis* cells expressing SdrC, SdrD, protein A, Map or EbpS did not induce platelet aggregation in PRP, whereas *L. lactis* expressing ClfA, ClfB or SdrE did induce platelet aggregation (Fig. 5). *L. lactis* cells expressing ClfA induced platelet aggregation with the shortest lag times (1.5 ± 0.5 min, $n = 3$). In contrast, SdrE-expressing *L. lactis* cells activated platelet aggregation with relatively long lag times (13.5 ± 3.5 min, $n = 8$), whereas ClfB-expressing *L.*

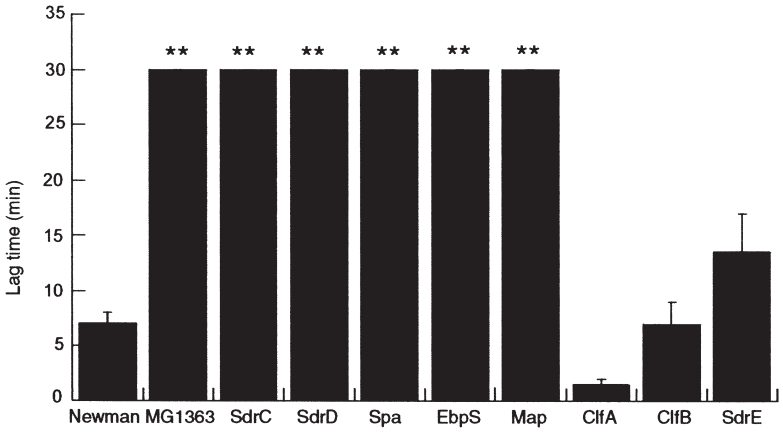

Fig. 5. Activation of platelet aggregation by *L. lactis* cells expressing *S. aureus* surface proteins. *L. lactis* (MG1363) and *L. lactis* MG1363 cells expressing SdrC, SdrD, Spa, EbpS, Map, ClfA, ClfB or SdrE were tested for their ability to activate platelet aggregation. ** indicates that aggregation did not occur after 30 min incubation. Results are expressed as a comparison of the time taken to aggregation for each strain. *S. aureus* Newman wild-type cells (grown to exponential phase) and *L. lactis* cells expressing ClfA, ClfB or SdrE all induced a similar degree (percentage) of aggregation.

lactis cells activated platelets with a lag time of 7 ± 2 min ($n = 3$).

The ability of *L. lactis* cells expressing ClfA, ClfB or SdrE to activate platelet aggregation in GFPs was also tested. *L. lactis* cells expressing ClfA or ClfB activated platelet aggregation in GFPs with similar lag times to that of PRP, whereas no aggregation in GFPs was observed for *L. lactis* cells expressing SdrE (data not shown). In addition, the recombinant A region of SdrE (rSdrEA) inhibited SdrE-expressing *L. lactis*-mediated platelet aggregation in a dose-dependent manner (data not shown) after preincubation of the protein with the platelets. This indicates that activation by SdrE results from a specific receptor–ligand interaction.

These data confirmed observations with *S. aureus* mutants that ClfA and ClfB are capable of activating platelet aggregation and show that they can do so independently of other *S. aureus* surface molecules. A third mechanism involving the SD repeat-containing protein SdrE was also identified.

The role of ClfA, SdrE and protein A in the activation of platelet aggregation identified using S. aureus *mutants*

In order to show that ClfA and SdrE could promote the activation of platelet aggregation when expressed on the surface of *S. aureus* cells and to investigate the role of protein A further, experiments were performed with mutants lacking different combinations of surface proteins. It should be pointed out that cells from the stationary phase of growth were used where ClfA, SdrE and Spa are known to be expressed, but where ClfB is lacking (McAleese *et al*., 2001).

Cells of Newman wild type, Newman *sdrE*, Newman *spa*, Newman *clfA*, Newman *clfA spa*, Newman *clfA sdrE* and Newman *clfA spa sdrE* from the stationary phase of growth (which were phenotypically ClfB$^-$) were tested. Newman wild-type, Newman *spa* and Newman *sdrE* cells all induced platelet aggregation with very similar lag times (1.8 ± 0.1 min, $n = 3$). In contrast, a significant delay in lag time to aggregation was observed with the Newman *clfA* mutant cells (9 ± 1 min, $n = 3$), suggesting that ClfA is the dominant activating factor in the absence of ClfB. A further increase in lag time was observed with the Newman *clfA spa* mutant cells (21 ± 9 min, $n = 3$) and with Newman *clfA sdrE* mutant cells (17.8 ± 3.5 min, $n = 3$), whereas the Newman *clfA spa sdrE* mutant did not promote platelet aggregation (Fig. 6). Aggregations by the mutants were inhibited by GPIIb/IIIa antagonists (data not shown),

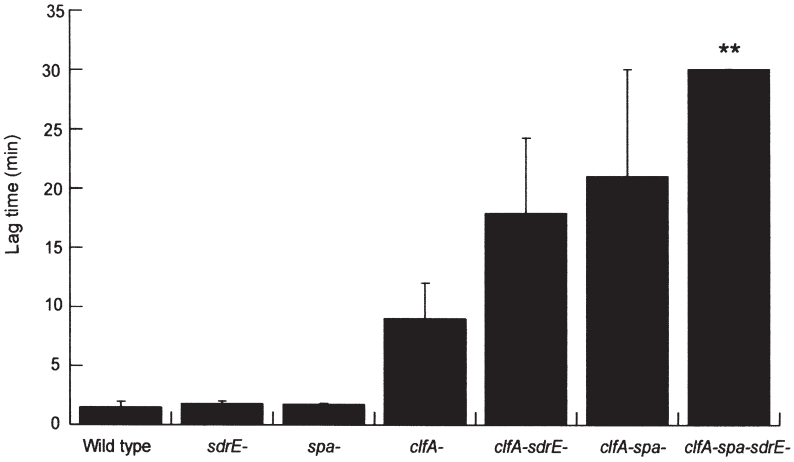

Fig. 6. Activation of platelet aggregation by *S. aureus* strain Newman *clfA*, *spa* and *sdrE* mutants. *S. aureus* strain Newman wild type and *S. aureus* Newman *spa* (DU5971), Newman *sdrC sdrD sdrE* (DU5973), Newman *clfA* (DU5876), Newman *clfA spa* (DU5976) and Newman *sdrC sdrD sdrE spa clfA* (DU5975) were grown to stationary phase and tested for their ability to activate platelet aggregation in PRP. ** indicates that aggregation did not occur after 30 min incubation. Results are expressed as a comparison of the time taken to aggregation for each strain.

indicating that they were true aggregation events and not agglutination. However, the aggregation profile of Newman *clfA sdrE* with PRP from two of the three donors presented as a slow drift rather than a normal aggregation trace.

These data showed that both ClfA and SdrE could stimulate platelet aggregation when expressed on the surface of *S. aureus* strain Newman, thus confirming the results obtained with *L. lactis.* Protein A was not capable of activating platelet aggregation independently, but we showed that it does play a role when expressed on the surface of *S. aureus* cells.

Discussion

In this paper, we have exploited our ability to manipulate genetically the expression of the surface proteins of *S. aureus* strain Newman in order to dissect the multifactorial process by which *S. aureus* cells induce platelet aggregation. *S. aureus* mutants expressing different combinations of surface proteins were constructed. This approach was complemented by the expression of each protein separately on the surface of the surrogate Gram-positive bacterium *L. lactis*.

We validated this strategy by showing that each of the *S. aureus* surface proteins implicated in the process of platelet activation was expressed in the full-length, functional form on the surface of *L. lactis*. Each protein was analysed by Western immunoblotting after enzymatic release from the cell wall, and the level of expression of ClfA and protein A on *S. aureus* and *L. lactis* was compared by flow cytometry.

Analysis of the function of staphylococcal surface proteins in the Clf–Sdr family is confounded by the fact that they are cleaved by a metalloprotease. In the case of ClfB, cleavage destroys the ligand-binding activity of the protein and is partly responsible for the loss of ClfB-promoted binding by cells in the stationary phase of growth (McAleese *et al*., 2001). In contrast, the population of ClfA molecules on *S. aureus* cells from the stationary phase is only partially degraded and, in any case, the cleaved form of ClfA still binds fibrinogen (O'Connell *et al*., 1998). When staphylococcal Clf–Sdr proteins were expressed on the surface of *L. lactis* and analysed by Western immunoblotting, we observed that the proteins were degraded in a time-dependent fashion during mutanolysin/lysozyme solubilization of cell wall peptidoglycan, presumably as a result of exposure to membrane-associated proteases (data not shown). It is our contention that the majority of molecules of each protein are intact and fully functional on the surface of *L. lactis* cells. Indeed, the ClfA and ClfB proteins expressed on the surface of *L. lactis* promoted more avid adherence to fibrinogen than *S. aureus* Newman, despite being expressed at similar levels. This may result from there being more full-length intact molecules on the surface of *L. lactis* compared with *S. aureus*. Alternatively, it is possible that the fibrinogen-binding capacity of both ClfA and ClfB is masked slightly by other surface proteins that are expressed by *S. aureus*.

Platelet aggregation that followed the addition of *S. aureus* cells to PRP or GFPs was monophasic and inhibited by several GPIIb/IIIa antagonists, by the cyclooxygenase inhibitor aspirin and by prostaglandin E_1, which elevates intracellular cAMP, thereby inhibiting platelet aggregation. This indicates that the response was true aggregation, that was dependent on cyclooxygenase followed by activation of GPIIb/IIIa and fibrinogen binding. Activation was not dependent on ADP secretion because it occurred normally in the presence of apyrase (ADPase). Similar results were obtained with endocarditis isolates (data not shown), indicating that this is a general property of *S. aureus*.

In contrast, a previous study indicated that aggregation of rabbit platelets by *S. aureus* strains Newman and Lafferty was biphasic and was not inhibited by the RGDS peptide, an antagonist of GPIIb/IIIa (Bayer *et al*., 1995). The action of GPIIb/IIIa antagonists including RGDS is highly species specific, and most have very little activity against platelets from rabbits or rodents (Cox *et al*., 1992). The aggregation response of rabbit platelets caused by Newman *clfA* was altered, in that there was no second phase in the biphasic response characteristic of the wild type (Bayer *et al*., 1995). However, in contrast to our studies, the lag time to activation with the Newman *clfA* mutant was not prolonged. This suggests that a factor other than ClfA had a more dominant role in promoting the aggregation of rabbit platelets, whereas ClfA is the dominant factor for the activation of human platelets.

With gel-filtered human platelets, we have shown that *S. aureus* Newman cells expressing either ClfA or ClfB induced aggregation whereas the Newman *clfA clfB* double mutant did not. However, in contrast, the Newman *clfA clfB* mutant did support aggregation in PRP. These results suggested that an additional mechanism, capable of inducing aggregation in plasma, must exist.

Clearly, *S. aureus*-mediated platelet aggregation is a complex process that involves at least three different surface proteins. Therefore, using isogenic mutants to identify the proteins is difficult. Hence, in order to identify the ClfA- and ClfB-independent mechanism(s), an alternative approach was adopted. The genes for candidate proteins were cloned and expressed in the Gram-positive surrogate host *L. lactis* allowing the expression of individual proteins from *S. aureus*. *L. lactis* does not induce platelet aggregation and therefore provides a suitable host for studying the proaggregatory ability of individual *S. aureus* proteins. Our results showed that ClfA, ClfB and SdrE could independently induce platelet aggregation in

PRP and thus supported data obtained with *S. aureus* Newman *clfA* and *clfB* mutants. In addition, a mechanism involving SdrE was identified.

In contrast, although protein A was shown to be intact and functional on the surface of *L. lactis* (pKS80:*spa*), these cells could not promote platelet activation. However, Spa did appear to be capable of contributing to the activation of platelet aggregation when expressed on the surface of *S. aureus*. Both Newman *clfA spa* and Newman *clfA sdrE* mutants activated platelets, whereas a Newman *clfA spa sdrE* mutant did not. In *S. aureus*, it is possible that protein A acts in conjunction with a surface protein other than ClfA, ClfB or SdrE to trigger aggregation and that this is the reason why *L. lactis* cells expressing protein A do not activate

One possible explanation for the long lag time to aggregation for Newman *clfA sdrE* could be that protein A might act by binding antibody and allowing complement formation. *Streptococcus sanguis* induces platelet aggregation by interacting with a platelet complement receptor and the Fc receptor (Ford *et al*., 1997). This aggregation has a long lag time of 18–20 min. Another possibility is that the interaction between protein A and SdrE with their platelet receptors is relatively weak and requires a longer period of time to trigger the aggregation process. We propose that the interaction between ClfA and its receptor is strong and thus triggers a rapid reaction, independently of other molecules. In fact, the level of ClfA present on the surface of *S. aureus* cells correlates directly with the length of lag time to aggregation. Stationary phase *S. aureus* Newman cells have about five times more ClfA on their surface, and their lag time is five times faster than exponential phase bacteria.

Plasma proteins play an important role in *S. aureus*-induced platelet aggregation. The SdrE platelet interaction did not occur with gel-filtered platelets but required the presence of plasma. Neither the *S. aureus* Newman *clfA clfB* double mutant nor the *L. lactis* SdrE-expressing cells induced platelet aggregation in GFPs. ClfA might interact with resting platelets by binding fibrinogen, which can then bind to GPIIb/IIIa (McDevitt *et al*., 1994). A similar interaction might also occur between ClfB and the platelet. However, ClfA has also been shown to interact directly with a platelet membrane protein (Siboo *et al*., 2001). Protein A could bind platelets via an IgG bridge to the platelet Fc receptor, or via von Willebrand factor, which binds to GPIb (Hartlieb *et al*., 2000), or by interacting directly with the complement receptor gC1qR/p33 (Nguyen *et al*., 2000). These potential interactions are summarized in Fig. 7.

In summary, three *S. aureus* proteins have been identified that are capable of activating platelet aggregation independently. Our results indicate that ClfA is the primary factor involved because it induced aggregation with the shortest lag time when expressed on the surface of *L. lactis.* ClfB appears to play a secondary role, as *L. lactis* cells expressing ClfB activated platelet aggregation with a longer lag time compared with ClfA. *L. lactis* cells expressing SdrE induced platelet aggregation with a relatively long lag time, implying that this reaction is weaker

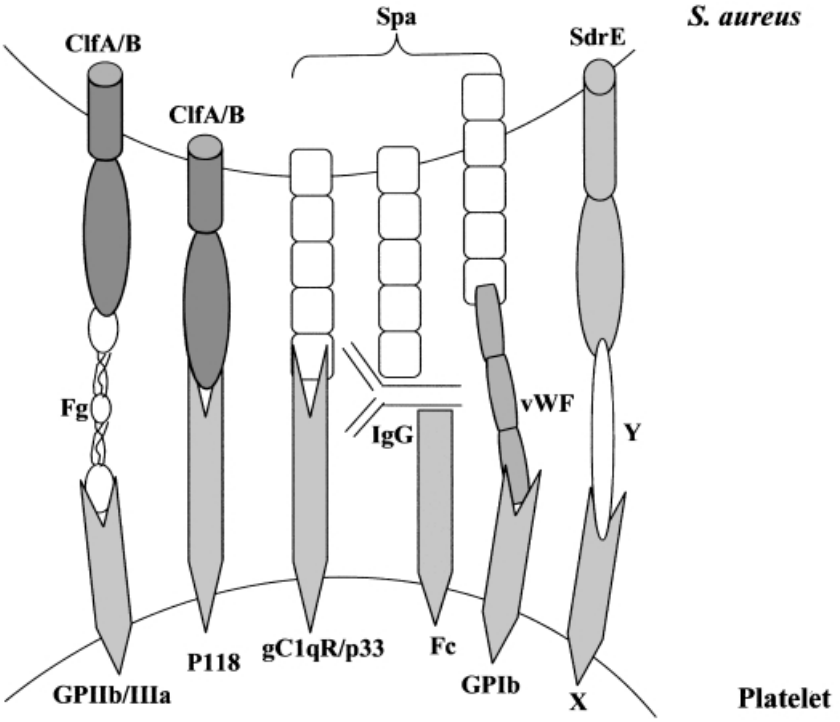

Fig. 7. Schematic representation of the known and proposed interactions between *S. aureus* surface proteins and platelet receptors. ClfA and ClfB can interact with their platelet receptor(s) directly or via a fibrinogen (Fg) bridge. Similarly, protein A (Spa) can interact with at least one platelet receptor directly. Spa also interacts with different platelet receptors indirectly via an IgG or vWF bridge. SdrE appears to interact with at least one unknown platelet receptor (X) via a plasma protein bridge (Y).

than that of ClfA and ClfB. SdrE appears to interact with the platelet indirectly via a plasma protein bridge, as no aggregation occurs with an *S. aureus clfAclfB* mutant (SdrE dependent) or with *L. lactis* cells expressing SdrE in gel-filtered platelets (from which the plasma proteins have been removed). Our data show that, although protein A plays a role in the induction of platelet aggregation, it is not capable of inducing the process independently. The next task is to identify the platelet receptors involved in the activation event and to determine the receptor and plasma protein involved in SdrE-promoted activation.

Experimental procedures

Materials

ADP and arachidonic acid were purchased from Bio-Data. Prostaglandin E_1 was from Sigma. Human monoclonal antibody c7E3 (abciximab) recognizing GPIIb (CD41) was obtained from Eli Lilly. The GPIIb/IIIa antagonist eptifibatide was a kind gift from Dr D. Philips (COR Therapeutics, San Francisco, CA, USA). Tirofiban was from Merck. Peroxidase-labelled goat anti-rabbit polyclonal IgG and fluorescein isothiocyanate (FITC)-labelled swine anti-rabbit polyclonal IgG were purchased from Dako. Enzymes for DNA manipulation were purchased from Roche, Sigma Chemical, New England Biolabs and Stratagene and were used in accordance with the manufacturers' instructions. Human fibrinogen was obtained from Calbiochem. Bacterial growth media were from Oxoid. Recombinant lysostaphin was obtained from AMBI. Other chemicals were purchased from Sigma or BDH.

Bacterial strains and growth conditions

Escherichia coli XL1-Blue (Stratagene) were used for plasmid cloning. *E. coli* Topp3 (Qiagen) were used for the expression of *S. aureus* recombinant proteins. All *E. coli* cells were grown in L broth (Oxoid) at 37°C with shaking. *S. aureus* cells were grown in brain–heart infusion (BHI) broth (Oxoid) at 37°C with shaking. *L. lactis* strain MG1363 (Gasson, 1983) was used for plasmid cloning and heterologous expression of *S. aureus* surface proteins. *L. lactis* MG1363 was routinely grown statically on M17 agar or broth (Difco), incorporating 0.5% glucose at 30°C. The following antibiotics were incorporated into the media where appropriate: ampicillin (100 μg ml^{-1}), 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 (Newman) strains defective in protein A (*spa*::Ka^r; DU5971) (F. Roche, unpublished), clumping factor A (*clfA2*::Tn*917*; DU5876) (McDevitt *et al*., 1994), clumping factor B (*clfB*::Tc^r; DU5943) (Ní Eidhin *et al*., 1998) and the double *clfA*::Tn*917 clfB*::Tc^r mutant (DU5944) (Ní Eidhin *et al*., 1998), coagulase (*coa*::Em^r; DU5856) (Phonimdaeng *et al*., 1990), the fibronectin-binding proteins A and B (*fnbA*::Tc^r *fnbB*::Em^r; DU5886) (Greene *et al*., 1995), the elastin-binding protein (*ebpS*::Em^r; DU5981) (Downer *et al*., 2002) and capsular polysaccharide (*cap5B*1::Tn*917*; DU5912) (Wann *et al*., 1999) have been described previously. A mutant defective in the MHC class II analogous protein Map was a gift from B. Kreikemeyer and M. Höök (Texas A and M University, USA).

Insertion mutations in *sdrC* and *sdrD* were constructed by directed integration of the temperature-sensitive plasmid pG$^+$Host9 (Maguin *et al*., 1996; Hartford *et al*., 2001). A fragment internal to the region encoding the unique A domain of each protein was amplified by polymerase chain reaction (PCR; primer sequences are available on request) and cloned into the multiple cloning site of pG$^+$Host9. The plasmid was constructed in *E. coli* and transferred into *S. aureus* Newman (Oskouian and Stewart, 1990). Selection for growth at the restrictive temperature in the presence of erythromycin resulted in derivatives in which the plasmid had integrated into the *sdrC* or *sdrD* genes to form Newman *sdrC*::pG$^+$Host9 (DU5988) and Newman *sdrD*::pG$^+$Host9 (DU5989).

A similar strategy to that described above failed to generate an insertion mutation in *sdrE*. Thus, a deletion mutation in the closely linked *sdrC*, *sdrD* and *sdrE* genes of strain Newman (Δ*sdrCDE*::Tc^r) was constructed by allelic replacement and transduction in a two-step procedure. Initially, a deletion–substitution *sdrC sdrD* mutation was constructed by allele replacement in the chromosome of *S. aureus* 8325-4, a strain that lacks *sdrE*, forming strain DU5972. A fragment of 814 bp from within the unique A region of *sdrC* was amplified by PCR using primers sdrCF3 and sdrCR3, and a fragment of 916 bp from within the A region of *sdrD* was amplified by primers sdrDF3 and sdrDR3 (primer sequences available upon request). The primers placed a *Bam*HI site at the 5′ end of the *sdrC* amplimer and a *Hin*dIII site at the 3′ end to facilitate cloning into a plasmid vector. A *Hin*dIII site was placed at the 5′ end of the *sdrD* amplimer, whereas an *Eco*RI site was place at the 3′ end. The *sdrC* PCR fragment was cleaved with *Bam*HI and *Hin*dIII, and the *sdrD* PCR product was cleaved with *Hin*dIII and *Eco*RI. These products were ligated together at the *Hin*dIII site and cloned between the *Bam*HI and *Eco*RI sites of pBluescript KS+ (Short *et al*., 1988), which had been cut with *Bam*HI and *Eco*RI. A *Hin*dIII fragment encoding the *tetK* gene was cloned from pT181 (Khan and Novick, 1983) into the *Hin*dIII site separating the cloned *sdrC* and *sdrD* fragments. Subsequently, plasmid pTS2, which is temperature sensitive for replication in *S. aureus* (Greene *et al*., 1995), was cloned into the *Xba*I site of the chimeric plasmid, which was then transferred into *S. aureus* 8325-4. Allele replacement was performed by temperature shift experiments (Foster, 1998). The structure of the Δ*sdrCD*::Tc^r mutation was verified by Southern hybridization (data not shown). The mutation was transduced into *S. aureus* (Newman), where it replaced the wild-type *sdrCDE* locus forming Newman Δ*sdrCDE*::Tc^r (DU5973). The mutated locus was verified by Southern hybridization (data not shown).

The Δ*sdrCDE*::Tc^r mutation was transduced (Foster, 1998) into Newman *spa*::Ka^r to construct strain DU5974 *sdrCDE spa*. The *clfA*::Tn*917* mutation was then transduced into DU5974, forming DU5975 *sdrCDE spa clfA*, into DU5971, forming DU5976 *clfA spa*, and into DU5973, forming DU5995. The presence of the Δ*sdrCDE*::Tc^r mutation, the Newman *spa*::Ka^r and the *clfA*::Tn*917* mutations in DU5975 was verified by PCR (data not shown).

Expression of S. aureus *proteins by* L. lactis *MG1363*

The *clfA*, *clfB*, *sdrC*, *sdrD sdrE*, *map* and *spa* genes were

amplified by PCR and cloned into the expression vector pKS80 (Hartford *et al.*, 2001) in *L. lactis* MG1363. Each primer carries a *Bam*HI site to facilitate in frame fusion of the start codon of the *S. aureus* gene with the ATG codon located within the *Bcl*I site downstream from a ribosome binding site in the vector. The PCR products were cleaved with *Bam*HI, ligated with *Bcl*I-cleaved pKS80 and electrotransformed into *L. lactis* MG1363 cells made competent as described previously (Wells *et al.*, 1993). Transformants were selected on M17 agar containing 5 μg ml^{-1} erythromycin and 0.5% glucose. Transformants were tested for protein expression by whole-cell dot immunoblotting (Hartford *et al.*, 1997) using specific antibodies recognizing the A domains of each protein (McDevitt *et al.*, 1994; Josefsson *et al.*, 1998b; Ní Eidhin *et al.*, 1998), with the exception of protein A, in which non-immune rabbit IgG was used. Bound antibodies were detected with protein A coupled to horseradish peroxidase (Sigma) and chemiluminescence. *L. lactis* MG1363 expressing EbpS was a kind gift from R. Downer, Trinity College, Dublin.

Recombinant SdrE A domain

The unique A domain of SdrE was expressed as a recombinant protein with a 6× His affinity tag at the N-terminus. The coding sequence of the A domain was amplified by PCR (primer sequences available upon request) and cloned between the *Bam*HI–*Hin*dIII sites of the expression vector pQE30 (Qiagen). The plasmids were transformed into *E. coli* Topp3, and proteins were purified by Ni^{2+}-chelate chromatography (O'Connell *et al.*, 1998). Anti-SdrE A domain antibodies were generated by immunization of a young New Zealand white rabbit (McAleese *et al.*, 2001).

Western immunoblot analysis of surface proteins

Staphylococcus aureus cells were grown to exponential phase (OD_{600} of 0.6–0.8) or stationary phase (16 h) in BHI with shaking at 37°C, whereas *L. lactis* cells were grown to stationary phase (16 h) in M17 broth containing 0.5% glucose at 30°C without shaking. Cells were harvested, washed in PBS and resuspended at an OD_{600} of 40 in 20 mM Tris (pH 8.0), 10 mM $MgCl_2$ containing 30% raffinose and protease inhibitor cocktail (Complete Mini; Roche). Cell wall-associated proteins of *S. aureus* were solubilized by lysostaphin (200 μg ml^{-1}), whereas those of *L. lactis* were released by digestion with mutanolysin (500 U ml^{-1}) and lysozyme (200 μg ml^{-1}) (Hartford *et al.*, 2001). A 20 μl sample was boiled in final sample buffer (Laemmli, 1970), electrophoresed in a 7.5% SDS–PAGE gel and transferred to nitrocellulose membranes. Specific rabbit antibodies recognizing the A domains of each protein were used to probe the filters. Bound antibodies were recognized with protein A coupled to horseradish peroxidase and chemiluminescence with LumiGlo (New England Biolabs).

Analysis of surface protein expression in L. lactis *and* S. aureus *by flow cytometry*

Staphylococcus aureus Newman, Newman *spa* and Newman *clfA spa* cells were grown to stationary phase. Newman *spa* cells were also grown to exponential phase. *L. lactis* cells expressing either ClfA or protein A were grown to stationary phase. Cells were harvested by centrifugation, washed in PBS and adjusted to an OD_{600} of 1.0. Newman *spa*, Newman *clfA spa* and *L. lactis* cells expressing ClfA were incubated for 1 h at room temperature with polyclonal anti-ClfA antibodies, whereas Newman wild-type, Newman *spa* and *L. lactis* cells expressing Spa were incubated for 1 h at room temperature with non-immune rabbit polyclonal IgG. Cells were harvested by centrifugation and washed twice with PBS. Cell pellets were resuspended in 20 μl of FITC-labelled swine anti-rabbit IgG and incubated for 1 h at room temperature. PBS (1 ml) was added to each tube, and the relative fluorescence was measured by flow cytometry on a FACSCalibur flow cytometer (Becton Dickinson) using the CELLQUEST software. The expression of ClfA in *S. aureus* compared with that in *L. lactis* was measured by comparing the fluorescence of the Newman *spa* mutant cells with the *L. lactis* ClfA$^+$ cells. (A *spa* mutant was used to eliminate non-immune binding of IgG to protein A.) Newman *clfA spa* cells were used to determine the background fluorescence. The levels of protein A expression in *S. aureus* compared with *L. lactis* were measured by comparing the fluorescence of labelled Newman wild-type cells with that of the *L. lactis* Spa$^+$ cells. Newman *spa* cells were used to determine the background fluorescence. The level of ClfA expression on the surface of exponential phase Newman *spa* cells was also compared with that of stationary phase Newman *spa* cells using rabbit anti-ClfA antibodies and FITC-labelled swine anti-rabbit IgG.

Platelet preparation

Blood was drawn from healthy human volunteers who had abstained from taking any form of non-steroidal anti-inflammatory drugs during the previous 10 days. Nine volumes of blood were collected by clean venepuncture and minimum stasis, using a 19G needle, into one volume of 3.8% sodium citrate or acid–citrate–dextrose (ACD). Platelet-rich plasma (PRP) was prepared by centrifugation of anticoagulated whole blood at room temperature at 150 *g* for 10 min. The blood remaining after removing the PRP was centrifuged at room temperature at 630 *g* for 10 min to yield platelet-poor plasma (PPP). For the preparation of gel-filtered platelets (GFPs), 2 μM PGE_1 and apyrase (1 U ml^{-1}) were added to PRP and centrifuged at 630 *g* for 10 min. The plasma was removed, and the resultant pellet was suspended in modified HEPES Tyrodes buffer (JNL: 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 $MgCl_2$). This was then layered on a Sepharose 2B column, and fractions containing platelets were pooled.

Platelet aggregation

Staphylococcus aureus and *L. lactis* cells were grown to exponential phase (OD_{600} of 0.8) or stationary phase (16 h), harvested, washed in PBS and resuspended to an OD_{600} of 1.6 in PBS. Bacterial suspension (50 μl) was added to 450 μl

of PRP or GFPs. Platelet aggregation was assayed by light transmission at 37°C using a PAP-4 aggregometer (BioData). Platelets were tested for normal responses to arachidonic acid (0.5 mg ml^{-1}) and/or ADP (20 μM). The GFPs were adjusted to a final concentration of 2×10^8 platelets ml^{-1}. Physiological concentrations of $CaCl_2$ (1.8 mM) and fibrinogen (1 mg ml^{-1}) were added to GFPs before aggregation studies. Aspirin was preincubated with PRP for 45 min at 37°C before the addition of agonist or *S. aureus*. Apyrase, PGE_1 and inhibitors of GPIIb/IIIa were incubated with PRP for 10 min at 37°C before the addition of *S. aureus* or specific agonist.

Adherence to immobilized fibrinogen

Adherence of bacteria to immobilized fibrinogen was measured as described previously (Hartford *et al.*, 1997). Briefly, flat-bottomed microtitre plates were coated with a solution of human fibrinogen (5 μg ml^{-1} in PBS) and blocked with 2 mg ml^{-1} bovine serum albumin in PBS. Bacterial cells were grown to stationary phase and resuspended at an OD_{600} of 1.0 in PBS. A sample of 100 μl of cells were added to each well and incubated at 37°C for 2 h. Adherent cells were fixed with 25% (v/v) formaldehyde, stained with 0.5% (w/v) crystal violet and read in an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm.

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