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Platelet activation by *Staphylococcus aureus*

A thesis submitted for the degree of Doctor in Philosophy

by

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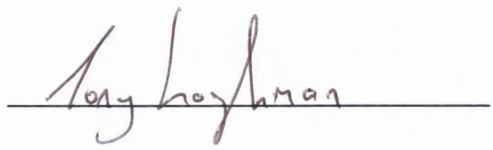

Anthony J. Loughman

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Summary

Staphylococcus aureus is the leading cause of infective endocarditis (IE). Platelet activation promoted by *S. aureus* resulting in aggregation and thrombus formation is thought to be an important step in the pathogenesis of IE. A detailed understanding of the molecular interactions between *S. aureus* and human platelets may identify targets for the development of novel therapeutic strategies to combat this often fatal infection. The fibrinogen-binding surface protein clumping factor (Clf) A is the dominant surface protein responsible for platelet aggregation by *S. aureus* cells in the stationary phase of growth. This study used genetically manipulated *S. aureus* and *Lactococcus lactis* strains engineered to express ClfA and a site-directed ClfA mutant defective in fibrinogen-binding (ClfA PY). Expression of ClfA or ClfA PY from a nisin-inducible promoter in *L. lactis* demonstrated that a minimum level of surface-expressed ClfA was required for aggregation, which is similar to the expression levels of ClfA observed in *S. aureus* Newman cells grown to stationary phase. A less-efficient fibrinogen-independent mechanism of platelet activation was promoted by bacteria expressing the non-fibrinogen-binding ClfA PY mutant. Using platelets that were separated from plasma, the requirement for both bound fibrinogen and immunoglobulin (Ig) G was demonstrated. Fibrinogen promoted adhesion of platelets to bacteria expressing the wild-type ClfA protein, but not to bacteria expressing the ClfA PY mutant. This adhesion was dependent on the GPIIb/IIIa integrin on resting platelets. The IgG requirement is consistent with the potent inhibition of ClfA-mediated platelet activation by a monoclonal antibody specific for the platelet low-affinity Fc receptor FcγRIIa. Furthermore the IgG had to contain antibodies specific for ClfA. A model is proposed whereby bacterial cells armed with a sufficient number of surface-bound fibrinogen molecules mediate bacterial adhesion to the low affinity platelet glycoprotein GPIIb/IIIa receptor, aided by bound IgG molecules which encourages the clustering of FcγRIIa receptors. This can trigger activation leading to up-regulation of GPIIb/IIIa and fibrinogen-dependent platelet aggregation. Bacteria expressing the ClfA PY mutant protein required IgG and complement assembly on the bacterial surface for interactions with platelets leading to activation. The lag times before aggregation were significantly prolonged compared to aggregation caused by bacteria expressing the wild-type protein. This likely reflects the time taken for complement assembly on the bacterial cell surface. The classical pathway of complement activation was crucial, which is consistent with the absolute requirement of ClfA-specific IgG.

The fibronectin-binding proteins FnBPA and FnBPB were recently identified as the major mediators of platelet activation expressed by exponentially growing *S. aureus* cells. FnBPA and truncates comprising either the fibrinogen-binding A domain or the fibronectin-binding BCD domains were expressed on the surface of *L. lactis*. Two independent mechanisms of platelet activation promoted by *L. lactis* expressing FnBPA were identified. Platelet activation stimulated by bacteria expressing the FnBPA A domain required a fibrinogen-bridge to platelet GPIIb/IIIa and bound IgG interacting with FcγRIIa. Fibronectin acted as a bridging molecule linking bacteria expressing the FnBPA BCD domains to GPIIb/IIIa on platelets, and bound IgG was crucial for an interaction with FcγRIIa leading to activation and aggregation.

A general mechanism of platelet activation by *S. aureus* is proposed. The expression of surface proteins capable of binding a platelet-reactive factor such as fibrinogen or fibronectin mediate high-affinity adhesion to platelets via a plasma protein bridge. This allows the interaction of bacterial-bound IgG with FcγRIIa causing rapid activation and aggregation. *S. aureus* mutants lacking ClfA and FnBPs caused activation with prolonged lag times, which is proposed to be dependent on complement assembly and IgG. These data have broad implications for our understanding of the pathogenesis of infective endocarditis and for the development of novel therapeutics against vascular infections.

Publications

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Arrecubieta, C., Asai, T., Bayern, M., Loughman, A., Shelton, C.E., Baron, H.M., Dang, N.C., Deng, M.C., Naka, Y., Foster, T.J., and Lowy, F.D. (2006) The role of *Staphylococcus aureus* adhesins in the pathogenesis of ventricular assist device-related infections. *J Inf Dis* **193:** 1109-1119.

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Key to abbreviations

Single letter amino acid code

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

Nucleotides

A	Adenine
T	Thymine
C	Cytosine
G	Guanine

Key to abbreviations

Antibiotics	
Amp	Ampicillin
Cm	Chloramphenicol
Em	Erythromycin
Tet	Tetracycline
Kan	Kanamycin
aa	amino acid
bp	base pair(s)
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
dNTP	deoxy nucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
Fg	fibrinogen
Fn	fibronectin
GFP	gel filtered platelets
h	hour(s)
Ig	immunoglobulin
kb	kilobase pair
kDa	kilodalton
min	minute(s)
nt	nucleotides
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PRP	platelet rich plasma
PPP	platelet poor plasma
rpm	revolutions per minute
SDS	sodium dodecyl sulfate

Key to abbreviations	
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris	trishydroxymethylaminomethane
TSA	trypticase soy agar
TSB	trypticase soy broth
v/v	volume per volume
w/v	weight per volume
wt	wild-type

Chapter 1

Introduction

1.1 Biology of the staphylococci

1.1.1 Classification and identification

Bacteria of the genus *Staphylococcus* are Gram-positive organisms that characteristically divide in more than one plane to form grape-like clusters. Molecular taxonomic studies have placed the staphylococci in the *Bacillus-Lactobacillus-Streptococcus* cluster of the *Micrococcaceae* (Ludwig *et al.*, 1985; Stackebrandt and Teuber, 1988). Staphylococci are most closely related to *Enterococcus*, *Bacillus*, or *Listeria*, and their genomes contain DNA of a low G + C content (30 – 39 %). The staphylococci are extremely halotolerant (growing at up to 3.5 M NaCl) and are resistant to dessication.

Coagulase is a secreted protein that binds fibrinogen and also activates prothrombin to initiate the host blood coagulation pathway. The production of coagulase has long been used to divide the staphylococci into two major groups, coagulase-positive staphylococci (*Staphylococcus aureus*) and coagulase-negative staphylococci (CoNS), which are less virulent. Coagulase-positive staphylococci are defined by the ability of cell culture supernatants to clot rabbit plasma (Phonimdaeng *et al.*, 1990).

1.1.2 Colonization and disease

The moist squamous epithelium of the anterior nares acts as the primary ecological habitiat of *S. aureus* in humans. Approximately 20 % of healthy adults are persistently colonized by *S. aureus*, and another 20 % never carry *S. aureus* in the nasopharynx (Lowy, 1998; Foster, 2004). The remainder may or may not carry *S. aureus* at any point in time. It is not known why some individuals are persistently colonized and others not. Healthy individuals have a small but finite risk of contracting an invasive *S. aureus* infection, and this risk is increased among carriers both in the hospital and the community (von Eiff *et al.*, 2001; Wertheim *et al.*, 2004). Infections are initiated when a breach of the skin or mucosal barrier allows staphylococci access to adjoining tissues or the bloodstream. Whether an infection is contained or spreads depends on a complex interplay between *S. aureus* virulence determinants and host defense mechanisms. The most common types of *S. aureus* infection are superficial skin lesions such as boils, impetigo and abscesses. If the organism gains access to the bloodstream (bacteremia), it can infect internal tissues such as bone (osteomyelitis), joints (septic arthritis), lungs (pneumonia), and heart valves (endocarditis) (Lowy, 1998). Many instances of nosocomial *S. aureus* bacteremia are attributable to an endogenous source

(von Eiff *et al.*, 2001), although it has been noted that bacteremia-related death was significantly higher in infected non-carriers compared to infected carriers, suggesting that carriers could be immunologically adapted to the strain of *S. aureus* that they carry (Wertheim *et al.*, 2004). Treatment of invasive *S. aureus* infections relies heavily on the use of antimicrobial agents, to which the organism is increasingly developing resistance.

1.2 Genome structure of *Staphylococcus aureus*

The circular *S. aureus* genome contains approximately 2.8 Mb AT-rich DNA (average G + C content of 33 %) (Kuroda *et al.*, 2001). To date, the entire genome of seven *S. aureus* isolates have been sequenced. Strains N315 and Mu50 are closely related, hospital-acquired methicillin-resistant *S. aureus* (MRSA) isolates from Japan (Kuroda *et al.*, 2001). The genome sequences of strain MW2 (a community-acquired MRSA isolate from the USA; Baba *et al.*, 2002) and a closely related, methicillin-sensitive isolate (MSSA476; Holden *et al.*, 2004) have been determined. Recently, the genome sequence of an early MRSA isolate (strain COL) was published (Gill *et al.*, 2005), and the sequence of a related methicillin-sensitive isolate (strain NCTC 8325) is in the public domain (http://www.genome.ou.edu/staph). Finally, the genome sequence of an MRSA strain (MRSA252), which is representative of the highly successful, epidemic EMRSA-16 clone, has been determined (Holden *et al.*, 2004).

1.2.1 Core genome

DNA microarray analysis of diverse *S. aureus* isolates from different geographical locations indicates that approximately 75 % of the *S. aureus* genome is highly conserved between isolates (Fitzgerald *et al.*, 2001). The core genome contains genes involved in metabolism and other house-keeping functions, and also contains some genes not essential for growth, such as surface expressed proteins and exoenzymes (Lindsay and Holden, 2004). Subtle differences in the conserved core genome have been exploited as a means of understanding the structure of the *S. aureus* population. Multilocus sequence typing (MLST) is the main method for analyzing the clonality of *S. aureus* isolates (Feil and Enright, 2004). Other methods for analyzing the population dynamics of *S. aureus* have been utilized. The "gold standard" for epidemiological analysis is pulsed-field gel electrophoresis (PFGE) where

genomic DNA is cleaved with endonucleases that recognize a small number of sites on the chromosome, yielding a distinct pattern of bands upon agarose gel electrophoresis (Trindade *et al.*, 2003). Amplified fragment length polymorphism (AFLP) is a PCR-based method that scans for polymorphisms in selected restriction sites and the nucleotides bordering these sites (Melles *et al.*, 2004). Other typing methods based upon the sequence of selected surface protein genes have been used in some studies (Shopsin *et al.*, 1999; Robinson and Enright, 2003).

1.2.1.1 MLST typing

Typing of *S. aureus* strains by MLST involves accurately sequencing DNA fragments (approximately 450 bp) of seven housekeeping genes: *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*. These slowly evolving metabolic genes were selected to represent the stable core genome. The sequences obtained are compared to known alleles at each locus (http://www.mlst.net). A single nucleotide change in a sequence is sufficient to define a new allele. The resulting seven-integer allelic profile defines a sequence type (ST) for each strain. Therefore, *S. aureus* strains that share the same ST share identical alleles at all seven MLST loci. Strains that differ at only one allele out of seven are known as single-locus variants (SLV), and this indicates a close genetic relatedness. Isolates can be grouped based on their MLST sequence types into clonal complexes to give an overall picture of the population structure of *S. aureus*. Clonal complexes are defined as groups of STs in which every ST shares at least five out of seven alleles with at least one other ST in the group (Feil *et al.*, 2003; Robinson and Enright, 2004).

1.2.1.2 Population structure of *S. aureus*

MLST analysis of a large strain collection (n = 334), obtained from the Oxfordshire area in the U.K., revealed that the population structure of *S. aureus* is highly clonal (Feil *et al.*, 2003). Grouping of isolates into clonal complexes based on their MLST genotype showed that 77 % of all tested isolates fall into eight major clonal complexes (Feil *et al.*, 2003). Phylogenetic analysis revealed that the different clonal complexes within this population show significant divergence from each other. Analysis of clonal diversification within individual clonal complexes suggests that alleles are at least 15-fold more likely to change by point mutation rather than by recombination (Feil *et al.*, 2003). This is in contrast to naturally transformable bacteria such as *Neisseria meningitidis* and *Streptococcus pneumoniae*, in

which alleles change between 5- to 10-fold more frequently by recombination than by mutation (Feil and Spratt, 2001). For, *S. aureus*, carriage and invasive isolates were evenly distributed among the clonal complexes, suggesting there is no link between MLST genotype and the propensity to cause disease (Feil *et al.*, 2003).

The population structure of a large number of *Staphylococcus aureus* strains (n = 993), isolated either from healthy carriers or from patients with invasive disease in the Netherlands, was recently studied using AFLP analysis (Melles *et al.*, 2004). A clonal population structure was identified, comprising five major AFLP clusters, which match the major clonal complexes identified by MLST (Feil *et al.*, 2003; Robinson and Enright, 2004). This suggests that the same clonal clusters have spread successfully in both the U.K and the Netherlands, and probably worldwide. It was found that carriage strains fell into the same main clusters as isolates from invasive disease, and that MRSA strains from international sources grouped in these same clusters (Melles *et al.*, 2004). Some AFLP sub-clusters contained proportionately more invasive than carriage isolates (Melles *et al.*, 2004). This confirms the suggestion that essentially any *S. aureus* strain has the capacity to cause invasive disease (Feil *et al.*, 2003), but strains from some clonal lineages are more virulent than others (Melles *et al.*, 2004). Invasive disease encompasses a very wide range of disease symptoms, which is associated with the wide variety of virulence factors expressed by *S. aureus* (see section 1.3). It is suggested that closely related isolates of the same ST may differ in their content of virulence genes and therefore differ in their capacity to cause disease. The presence of seven virulence factors in the strain collection from the U.K., including surface proteins and exotoxins, was associated with invasive disease (Peacock *et al.*, 2002). Some virulence factors or antibiotic-resistance determinants may be carried on mobile accessory genetic elements. This is discussed in section 1.2.2.

The percentage identity of the core genomes of the hospital-acquired isolates N315 and Mu50 suggest that these isolates are closely related (Kuroda *et al.*, 2001). These strains share identical MLST genotypes (ST-5; Figure 1.1). The closely related community acquired strains MW2 and MSSA476 both belong to ST-1. This sequence type represented the ancestral strain of clonal complex 1 in the Oxfordshire study (Feil *et al.*, 2003). Strains NCTC 8325 and COL (both isolated from the same geographical location in the U.K.) belong to different, but closely related, sequence types (ST-8 and ST-250 respectively; Lindsay and Holden, 2004; Figure 1.1). Strain MRSA252 is the most divergent of the sequenced *S. aureus* strains (Holden *et al.*, 2004). This is reflected in its MLST profile (ST36) which clearly

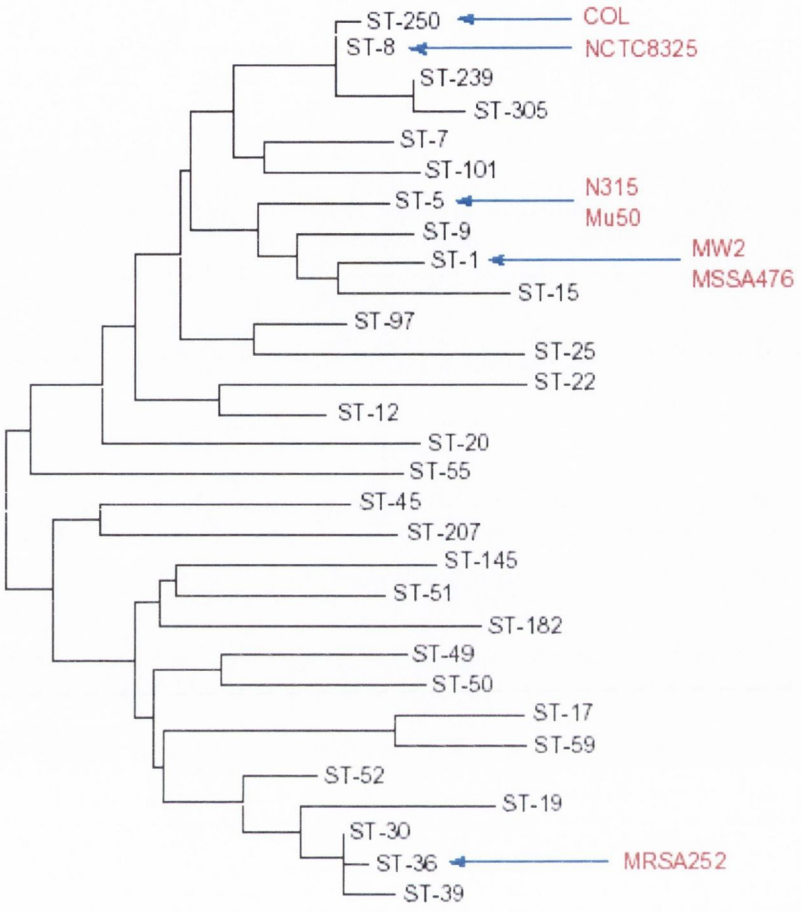


Figure 1.1 Phylogenetic relatedness of *S. aureus* MLST genotypes

A neighbour-joining tree of a representative sample of 30 sequence types (STs) from the *Staphylococcus aureus* MLST database (http://www.mlst.net). The positions of the seven sequenced *S. aureus* strains are indicated. The sequences of each allele were concatenated to produce 3198-bp of sequence for each ST for construction of the neighbour joining tree.

Adapted from Lindsay and Holden, 2004.

separates this strain from the other sequenced isolates based on phylogenetic analysis (Figure 1.1). This ST is well represented in the major CC30/39 clonal complex found in the U.K. (Feil *et al.*, 2003). The levels of relatedness inferred by MLST correlates well with the overall genomic divergence in the core genomes of the sequenced strains (Lindsay and Holden, 2004). This highlights the suitability of MLST as a means of understanding the evolution and population biology of *S. aureus*.

1.2.2 Accessory genomic elements

It is evident from the available genome sequences and DNA microarray analysis that up to 25 % of any *S. aureus* genome is comprised of dispensable genetic material (Fitzgerald *et al.*, 2001; Lindsay and Holden, 2004). This accessory genome consists mostly of mobile (or once mobile) genetic elements that can be transferred horizontally between different clonal lineages. These elements include bacteriophages, pathogenicity islands, chromosomal cassettes, genomic islands, plasmids and transposons, many of which carry genes associated with virulence or resistance (Baba *et al.*, 2002; Holden *et al.*, 2004). Many of the genes that encode secreted virulence factors, including superantigens, enterotoxins and leukocidins, are found on genomic islands, pathogenicity islands and prophages (Narita *et al.*, 2001; Gill *et al.*, 2005). Perhaps the most striking case of rapid evolution of virulent clones is the acquisition of the genes encoding the Panton-Valentine leukocidan, which is discussed below. Plasmids, transposons and chromosomal cassettes frequently contain genes encoding resistance to antimicrobials and heavy metals (Ito *et al.*, 2003). Selected mobile genetic elements that contribute to the success of *S. aureus* as a pathogen are discussed below.

1.2.2.1 Staphylococcal chromosomal cassette (SCC) *mec*

Acquisition of the staphylococcal chromosomal cassette (SCC) *mec* element in certain clonal lineages of *S. aureus* has resulted in the development of MRSA strains that are endemic in many hospitals in developed countries, and are emerging as invasive pathogens in the community. SCC*mec* elements are mobile genetic cassettes that integrate at the same site near the origin of the *S. aureus* chromosome (Ito *et al.*, 2001). They are defined by the presence of the *mecA* gene encoding a penicillin-binding-protein (PBP2') that has low affinity for methicillin and all other β-lactam antibiotics. The expression of PBP2' catalyzes the transpeptidation of cell-wall peptidoglycan in the presence of β-lactams, and therefore strains

carrying SCC*mec* (methicillin-resistant *S. aureus*: MRSA) are resistant to this major class of antibiotic (Hartman and Tomasz, 1984). Five classes of SCC*mec* have been characterized. SCC*mec* types I, II and III are large (34 – 66 kb) and principally found amongst hospital-acquired MRSA strains, whereas community-acquired strains typically contain smaller (20 -28 kb) type IV or type V SCC*mec* elements (Ito *et al.*, 2001, 2004; Baba *et al.*, 2002; Katayama *et al.*, 2005). Transposons and insertion sequences carrying resistance determinants (in addition to *mecA*) are often found inserted within SCC*mec* elements from hospital isolates (Kuroda *et al.*, 2001; Holden *et al.*, 2004), resulting in the emergence of strains that are resistant to most classes of antibiotic and, as a result, are more difficult to eradicate. The smaller type IV and type V SCC*mec* elements found in community-acquired MRSA contain *mecA* as the only resistance determinant (Baba *et al.*, 2002; Ito *et al.*, 2004).

MRSA strains were identified in 1961, less than 2 years after the introduction of methicillin into clinical use to treat penicillinase-resistant strains (Jevons, 1961). SCC*mec* elements were probably acquired horizontally from coagulase-negative staphylococci such as *Staphylococcus haemolyticus* (Katayama *et al.*, 2001). Acquisition of SCC*mec* in *S. aureus* appears to occur infrequently, and it is proposed that MRSA strains have emerged about 20 times by horizontally acquiring SCC*mec* elements (Robinson and Enright, 2003, 2004). The ability to harbour SCC*mec* and express β-lactam resistance appears to be strain dependent, with MRSA strains belonging to five major clonal lineages (Robinson and Enright, 2003; Katayama *et al.*, 2005). The reason why some strains are permissive for SCC*mec* and others are not is unclear.

While SCC*mec* does not encode any virulence factors *per se*, the expression of β-lactam resistance and other drug resistance determinants associated with SCC*mec* elements confers a survival advantage in rendering infections refractory to treatment with most classes of antimicrobials. Worryingly, isolates containing the enterococcal *vanA* gene are emerging that are fully-resistant to vancomycin (Cosgrove *et al.*, 2004), the last line of defence against multiply-resistant strains. The emergence of *S. aureus* strains that are resistant to all clinically relevant antimicrobials is a real threat.

1.2.2.2 Evolution of virulent clones

The *lukF-PV* and *lukS-PV* genes that encode the bicomponent Panton-Valentine leukocidan (PVL) are found on lysogenic phages in the chromosome of some *S. aureus* strains

(Narita *et al.*, 2001; Baba *et al.*, 2002). Approximately 2 % of *S. aureus* strains (carriage and blood-culture isolates) contain the *lukF-PV* and *lukS-PV* genes (Prevost *et al.*, 1995b; Peacock *et al.*, 2002; Melles *et al.*, 2004). Isolates causing abcesses and septic arthritis were found to be significantly enriched (39 %) for *lukF-PV* and *lukS-PV* (Melles *et al.*, 2004). PVL has a potent cytolytic activity on human leucocytes, and its expression is strongly associated with severe skin infections such as recurrent furunculosis (Prevost *et al.*, 1995a). Recently, PVL-expressing community-acquired MRSA strains (represented by MW2) have emerged that cause severe necrotizing pneumonia and contagious severe skin infections in previously healthy individuals (Gillet *et al.*, 2002). Strain MW2 was chosen for genome sequencing as it was a highly-virulent strain responsible for a community-acquired fatal case of septicaemia and septic arthritis (Baba *et al.*, 2002). MW2 contains a prophage (ΦSa2(MW2)) encoding PVL that is absent from MSSA476 (Baba *et al.*, 2002; Holden *et al.*, 2004). It was concluded that the high virulence of MW2 is at least partly due to PVL expression (Baba *et al.*, 2002). The increasing prevalence of PVL-producing *S. aureus* strains in the community is likely due to horizontal transfer of phages carrying this toxin into naïve *S. aureus* MLST types that subsequently spread and cause disease. This is well illustrated in comparison of the genomes of MW2 and MSSA476. Both strains have ST-1 MLST genotypes, a major clone associated with community-acquired disease, and their core genomes are very similar (Baba *et al.*, 2002; Holden *et al.*, 2004; Lindsay and Holden, 2004). However, their differing prophage patterns means that the *lukF-PV* and *lukS-PV* genes are only present in MW2, conferring a heightened pathogenic potential for this strain. This demonstrates the significant role that bacteriophages (and other mobile genetic elements encoding virulence factors) can play in the very short term evolution of *S. aureus*.

In conclusion, the *S. aureus* genome consists of a stable core and accessory elements that are strain specific. The strains that were used for genome sequencing only reflect a fraction of the diversity within the species as a whole. Further sequencing of the genomes of strains from major lineages would likely identify the full range of accessory genes and elements within the species *S. aureus*, and may provide insights on the success of certain clonal lineages.

1.3 *S. aureus* virulence factors

The diversity of infections associated with *S. aureus* reflects its ability to produce an arsenal of virulence factors, which can be classified into three main categories. An array of surface proteins are produced by *S. aureus* that recognize host proteins, such as those from the extracellular matrix (ECM), and other blood components such as fibrinogen. Expression of these so-called MSCRAMMS (microbial surface components recognizing adhesive matrix molecules) promotes adhesion to damaged tissue and the surfaces of host cells in the initiation of infection. Some of these factors have been shown to be important in animal infection models and are potential targets for novel therapeutics. Another group of virulence factors are the plethora of secreted exoenzymes, proteases and cytolytic toxins, which facilitate tissue destruction and spreading. A number of secreted toxins, such as toxic shock syndrome toxin (TSST) and Panton-Valentine leukocidin (PVL) are associated with particular conditions such as toxic shock syndrome and severe skin infections. However, most *S. aureus* infections probably occur through the coordinated expression of several virulence factors that give rise to disease symptoms. *S. aureus* has evolved numerous specific mechanisms by which it can avoid both the innate and adaptive immune responses. Virulence factors with other defined roles, such as some adhesins, may also protect *S. aureus* from the host immune response. This is likely to contribute to the ability of *S. aureus* to cause a wide range of infections.

1.3.1 Cell-wall components

The cell wall is the point of contact between *S. aureus* and its surrounding environment. It mainly consists of peptidoglycan (approximately 60 %), the remainder consisting of wall teichoic acids, lipoteichoic acids and small amounts of protein. The peptidoglycan is made up of a glycan backbone consisting of repeating disaccharide units, N-acetylglucosamine and N-acetylmuramic acid (GlcNAc - (β1→4) - MurNAc) (Ghuysen and Strominger, 1963). Short tetrapeptides (L-Ala – D-Glu – L-Lys – D-Ala) are found linked to MurNAc moieties in the glycan chains (Ghuysen *et al.*, 1965; Tipper and Strominger, 1965). Cross-linking of nascent peptidoglycan strands, a reaction known as transpeptidation, generates a rigid three-dimensional cell wall network (Tipper and Strominger, 1965). A characteristic feature of *S. aureus* peptidoglycan is the presence of pentaglycine interpeptide bridges that link the tetrapeptide units on neighbouring glycan chains, and renders *S. aureus*

peptidoglycan susceptible to cleavage by the glycyl-glycyl endopeptidase lysostaphin (Schliefer and Kandler, 1972).

Teichoic acids are another major component of the *S. aureus* cell wall. Wall teichoic acids are made up of ribitol-phosphate polymers substituted with N-acetylglucosamine and D-alanine residues (Ward, 1981; Endl *et al.*, 1983; Collins *et al.*, 2002). Wall teichoic acids are covalently linked to peptidoglycan. In contrast lipoteichoic acids (glycerol phosphate polymers substituted with D-alanine (Fischer *et al.*, 1990)) are attached to glycolipids in the plasma membrane. Peptidoglycan and teichoic acids together make up a polyanionic matrix that functions in cation homeostasis and trafficking of proteins and nutrients across the cell wall. Wall teichoic acids may be an important factor promoting *S. aureus* nasal colonization (Weidenmeier *et al.*, 2004) and may play a role in infective endocarditis (Weidenmeier *et al.*, 2005). The degree of D-alanyl esterification of teichoic acids regulates the net anionic charge of the bacterial cell surface and is thought to play a role in resistance to defensins (Peschel *et al.*, 1999; Collins *et al.*, 2002). The remainder of the cell wall consists of a variety of proteins that are displayed on the cell surface and mediate the interaction of *S. aureus* with its surroundings.

1.3.2 Surface proteins

1.3.2.1 Sorting

S. aureus surface proteins have a number of common motifs that target proteins to the cell wall, where they subsequently become covalently attached to pentaglycine cross-bridges in the peptidoglycan layer, a process known as sorting. These proteins have an N-terminal signal sequence consisting of approximately 40 amino acids that directs the protein into the secretory (Sec) pathway. The signal sequence is cleaved by membrane anchored signal peptidase enzymes (SpsA and SpsB in *S. aureus*) during translocation across the cytoplasmic membrane (Cregg *et al.*, 1996; Mazmanian *et al.*, 2001). N-terminal domains that contain the binding or catalytic activities are frequently followed by a set of repeat domains that may or may not possess ligand-binding activity. A cell-wall anchor domain is located at the C-terminus of these proteins whose function is to anchor the protein in an appropriate manner allowing subsequent covalent linkage to the cell wall. An LPXTG motif is followed by a hydrophobic membrane-spanning domain and short stretch of positively charged residues at the extreme C-terminus that remains in the cytoplasm (Fischetti *et al.*, 1990; Navarre and Schneewind, 1999; Mazmanian *et al.*, 2001).

Covalent attachment of surface proteins to the cell wall peptidoglycan is catalysed by sortase (Mazmanian *et al.*, 2001). *S. aureus* contains genes encoding two sortase enzymes (*srtA* and *srtB*) (Pallen *et al.*, 2001). SrtA anchors surface proteins bearing the LPXTG motif, whereas SrtB is required to anchor an iron-regulated surface protein (IsdC) with an NPQTN motif (Mazmanian *et al.*, 2002). SrtA is a membrane anchored protein, with the catalytic domain embedded in the peptidoglycan layer (Mazmanian *et al.*, 2001). The crystal structure of the N-terminal catalytic domain of SrtA has been solved. Proline (P) and threonine (T) residues of the LPXTG motif are held in position within the substrate binding pocket by hydrophobic contacts near the sortase active site residues C184, R197 and H120 (Zong *et al.*, 2004). Sortase cleaves the LPXTG motif between the threonine and glycine residues, and covalently attaches the threonine to glycine pentapeptide bridges in nascent peptidoglycan subunits (Ton-That *et al.*, 2000). The C-terminal portion of the surface protein (i.e membrane spanning domain and positively charged tail) is released and degraded. Sortase-mediated anchoring of surface proteins is schematically depicted in Figure 1.2. *S. aureus srtA* mutants, which fail to correctly anchor LPXTG-containing surface proteins, have been shown to be attenuated in animal infection models of septic arthritis and endocarditis (Jonsson *et al.*, 2002; 2003; Weiss *et al.*, 2004).

1.3.2.2 Staphylococcal protein A (SpA)

The first cell-wall associated surface protein of *S. aureus* to be characterized was protein A. The exposed protein on the cell surface contains five homologous repeats (EDABC) of 58 – 62 amino acids (Sjodahl, 1977; Figure 1.3). Each repeat is composed of three anti-parallel α-helices that pack together to form a compact helical bundle that is stabilized by hydrophobic interactions in the bundle interior (Gouda *et al.*, 1992; Starovasnik *et al.*, 1996). Protein A was initially defined by its ability to bind the Fc portion of mammalian IgG in a non-immune reaction, with each repeat possessing Fc-binding activity residing in helix I (Moks *et al.*, 1986; Gouda *et al.*, 1992). Each repeat of protein A can also bind the variable region of the Fab heavy chain of V_H3 class antibodies, in particular to subsets of IgM molecules (Hillson *et al.*, 1993). Fab binds to the opposite face of the protein A domain to that mediating Fc binding, which explains why binding of protein A to Fc and Fab is non-competitive (Graille *et al.*, 2000). Protein A has been shown to bind soluble von Willebrand factor (vWF) and promote adhesion to immobilized vWF (Hartlieb *et al.*, 2000) and is therefore a true adhesin. This interaction could be important for *S. aureus*-platelet

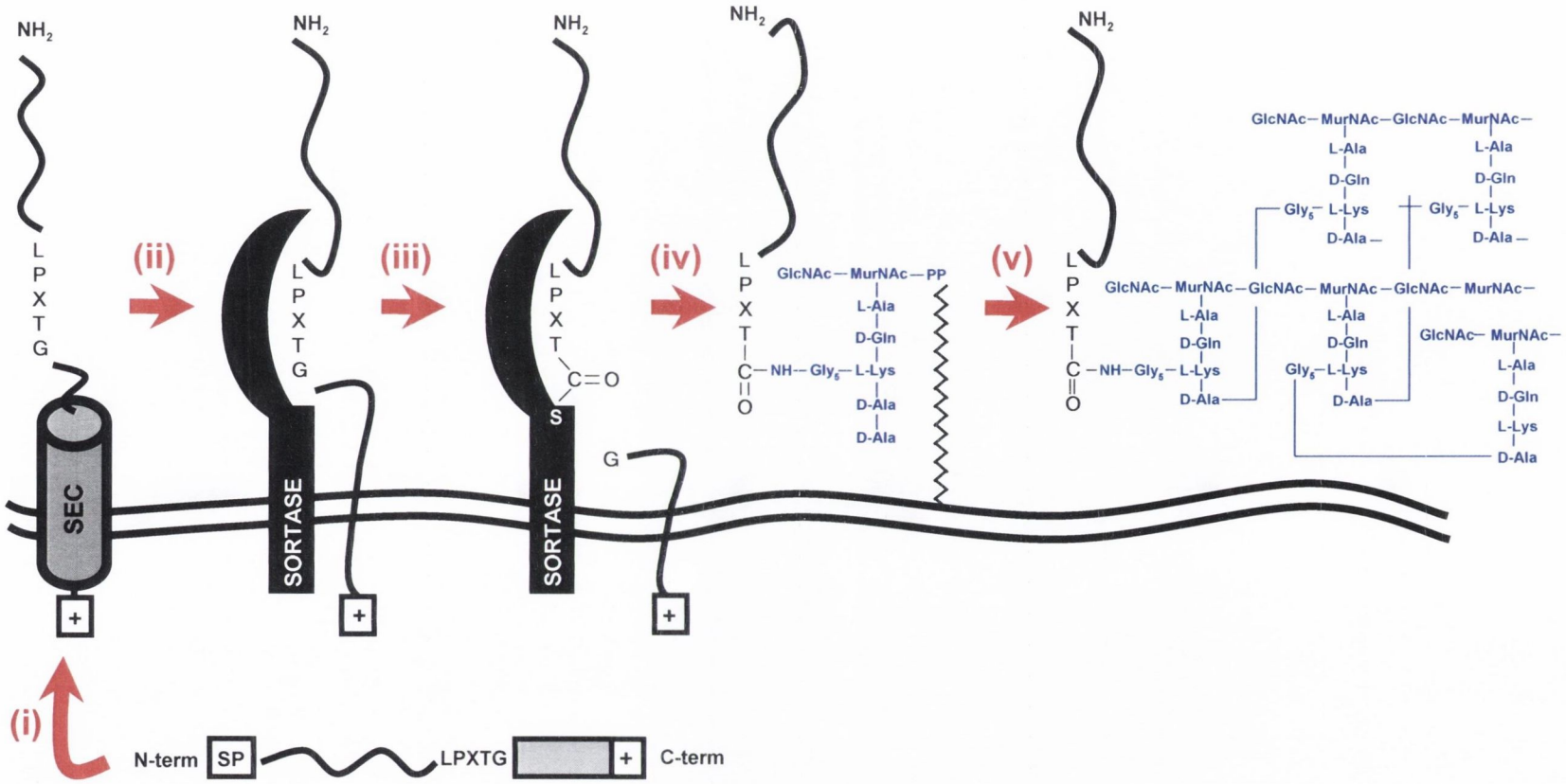


Figure 1.2. Surface protein anchoring in *Staphylococcus aureus*.

(**i**) Export. Precursor proteins with an N-terminal signal peptide (SP) are initiated into the secretory (Sec) pathway, and the signal peptide is removed. (**ii**) Retention. The C-terminal sorting signal retains polypeptides within the secretory pathway. (**iii**) Cleavage. Sortase cleaves between the threonine and the glycine of the LPXTG motif, resulting in the formation of a thioester enzyme intermediate. (**iv**) Linkage. Nucleophilic attack of the free amino group of lipid II at the thioester bond resolves the acyl-enzyme intermediate, synthesizing the amide bond between surface proteins and the pentaglycine cross-bridge and regenerating the active-site sulphydryl. (**v**) Cell wall incorporation. Lipid-linked surface protein is first incorporated into the cell wall via the transglycosylation reaction. The murein pentapeptide subunit with attached surface protein is then cross-linked to other cell wall peptides, generating the mature murein tetrapeptide.

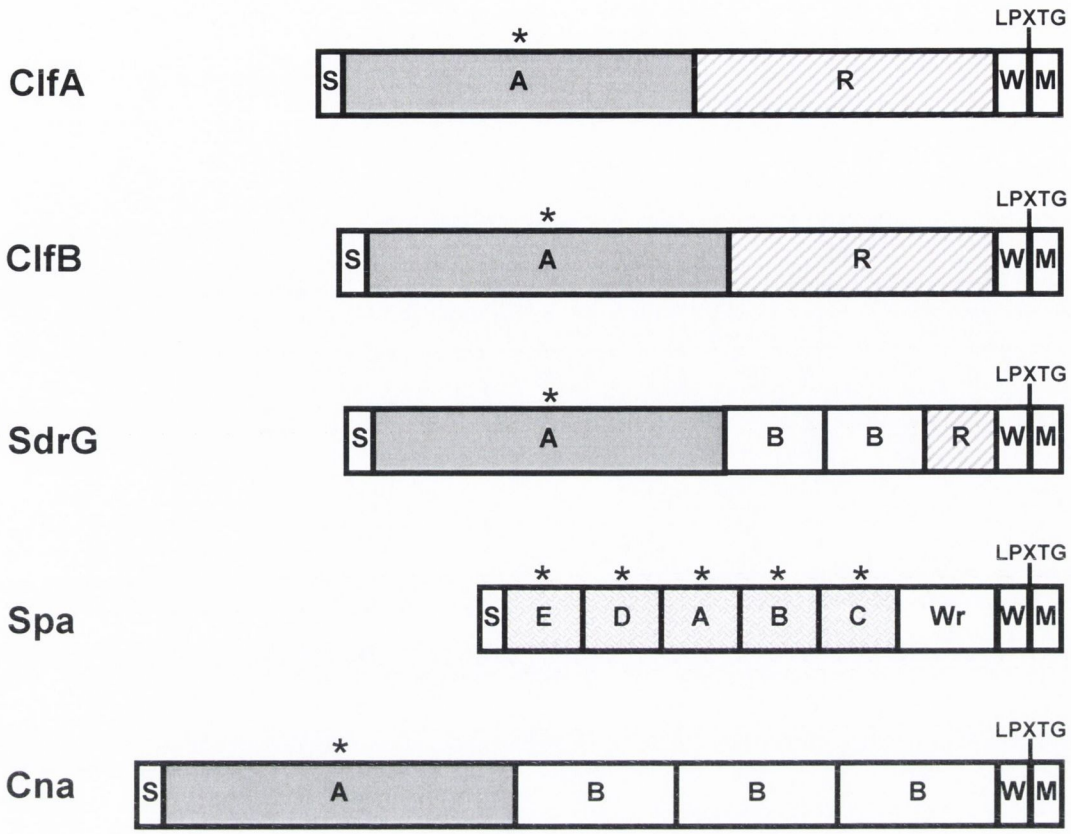


Figure 1.3 Staphylococcal surface proteins

Schematic representation demonstrating the common motif organisation found in surface proteins from *S. aureus (*ClfA, ClfB, Spa and Cna) and *S. epidermidis* (SdrG). Ligand binding domains are denoted by an asterisk. The relative sizes of the signal sequence (S), A domain (A), B-repeat region (B), SD-repeat region (R), and wall/membrane spanning regions (WM) are shown. LPXTG sortase A recognition motifs are indicated. Spa domains E, D, A, B and C are homologous ligand-binding repeats.

binding and is discussed in section 1.6.2.1. Protein A has been shown to bind and activate tumour necrosis factor-α receptor 1 ($TNFR_1$) on epithelial cells. This may be involved in the initiation of infection and the induction of inflammation during *S. aureus* pneumonia (Gomez *et al.*, 2004).

The binding of the Fc region of IgG to protein A results in the bacterium becoming coated with IgG molecules that are in the incorrect orientation to promote phagocytosis. This may facilitate immune evasion as described in section 1.3.4.4. Protein A also possesses immunosuppressive properties. The ability of protein A to bind to the Fab region on V_H3-class IgM on B-cells (Sasano *et al.*, 1993; Sasso *et al.*, 1989) causes their activation, proliferation and subsequent apoptotic destruction (Goodyear and Silverman, 2003). This could cause a significant depletion of potential antibody-secreting cells from the spleen and bone marrow (Goodyear and Silverman, 2004), and may account for the immunosuppressive activity of protein A.

1.3.2.3 Fibronectin-binding surface proteins

A characteristic feature of many *S. aureus* isolates is the ability to adhere to fibronectin immobilized on surfaces (Peacock *et al.*, 2000). Fibronectin mediates a wide variety of cellular interactions with the extracellular matrix (ECM) and plays important roles in cell adhesion, migration, growth and differentiation (Pankov and Yamada, 2002). Fibronectin exists as a dimer of approximately 250 kDa subunits that are covalently linked near their C-termini by a pair of disulfide bonds (Figure 1.4). It is an abundant constituent of blood plasma (300 μg/ml) and other bodily fluids, and also forms part of the ECM (Pankov and Yamada, 2002).

The binding of *S. aureus* to fibronectin is mediated by two closely related cell-surface proteins, fibronectin-binding proteins (FnBP) A and B (Figure 1.4). The expression of either FnBPA or FnBPB on the *S. aureus* surface is sufficient to promote bacterial adhesion to immobilized fibronectin (Greene *et al.*, 1995). These proteins are encoded by two closely linked, but separately transcribed genes (*fnbA*, Signäs *et al.*, 1989; *fnbB*, Jönsson *et al.*, 1991). Most *S. aureus* strains contain both genes, although some strains contain only one *fnb* gene (*fnbA*; Peacock *et al.*, 2000). The FnBPs are composed of a number of domains that mediate interactions with host components. FnBPA and FnBPB contain N-terminal A domains of approximately 500 amino acids that share 45 % identity (Jönsson *et al.*, 1991). These domains

have been shown to contain fibrinogen-binding and elastin-binding activity and are discussed in section 1.3.2.4.4.

C-terminal to the A domains are the CD domains that contain fibronectin-binding activity (Signäs *et al.*, 1989; Massey *et al.*, 2001), and share 95 % identity between FnBPA and FnBPB (Jönsson *et al.*, 1991; Figure 1.4). FnBPA contains two additional fibronectin-binding B-repeat domains that are absent in FnBPB (Jönsson *et al.*, 1991; Massey *et al.*, 2001; Figure 1.4). The primary recognition sequence in fibronectin for *S. aureus* FnBPs are the five type I modules in the N-terminus (Sottile *et al.*, 1991; Figure 1.4), although a second binding site has been identified in the heparin-binding type III module 14 (Bozzini *et al.*, 1992).

The structure of the B3 peptide of the Sfbl fibronectin-binding adhesin of *Strep. dysgalactiae* in complex with two of the N-terminal type I modules from fibronectin has recently been solved (Schwarz-Linek *et al.*, 2003). The fibronectin-binding activity of B3 was the result of a tandem β-zipper interaction, where binding motifs in the B3 protein form additional antiparallel β-strands on sequential type I modules of fibronectin (Schwarz-Linek *et al.*, 2003; Figure 1.5). Eleven fibronectin-binding modules were identified in *S. aureus* FnBPA that stretch from the C-terminus of the A domain through to the D-repeats, each containing type I module-binding motifs (Schwarz-Linek *et al.*, 2003). This region of FnBPA (BCD domain) lacks a discernable secondary structure, however upon binding to fibronectin type I modules this unfolded region takes on an ordered conformation (House-Pompeo *et al.*, 1996). It has also been demonstrated that one FnBPA molecule can accommodate two or more fibronectin molecules (Matsuka *et al.*, 2003). It is likely that *S. aureus* FnBPs bind to multiple fibronectin modules through a tandem β-zipper, accounting for the high affinity and specificity of this interaction (Schwarz-Linek *et al.*, 2003).

S. aureus is capable of adhering to and invading a number of different cell types, including endothelial cells and epithelial cells (Ogawa *et al.*, 1985; Dziewanowska *et al.*, 1999). FnBPs have been demonstrated to be crucial determinants in the invasion of host cells by *S. aureus*, through the formation of a fibronectin-bridge between *S. aureus* and the α5β1 integrin on host cells (Sinha *et al.*, 1999; Peacock *et al.*, 1999; Fowler *et al.*, 2000; Massey *et al.*, 2001). FnBP-mediated cell invasion may provide a means of bacterial dissemination from the bloodstream to other body tissue such as bones and joints. Cell invasion may also provide a means of evading the immune response and antibiotics.

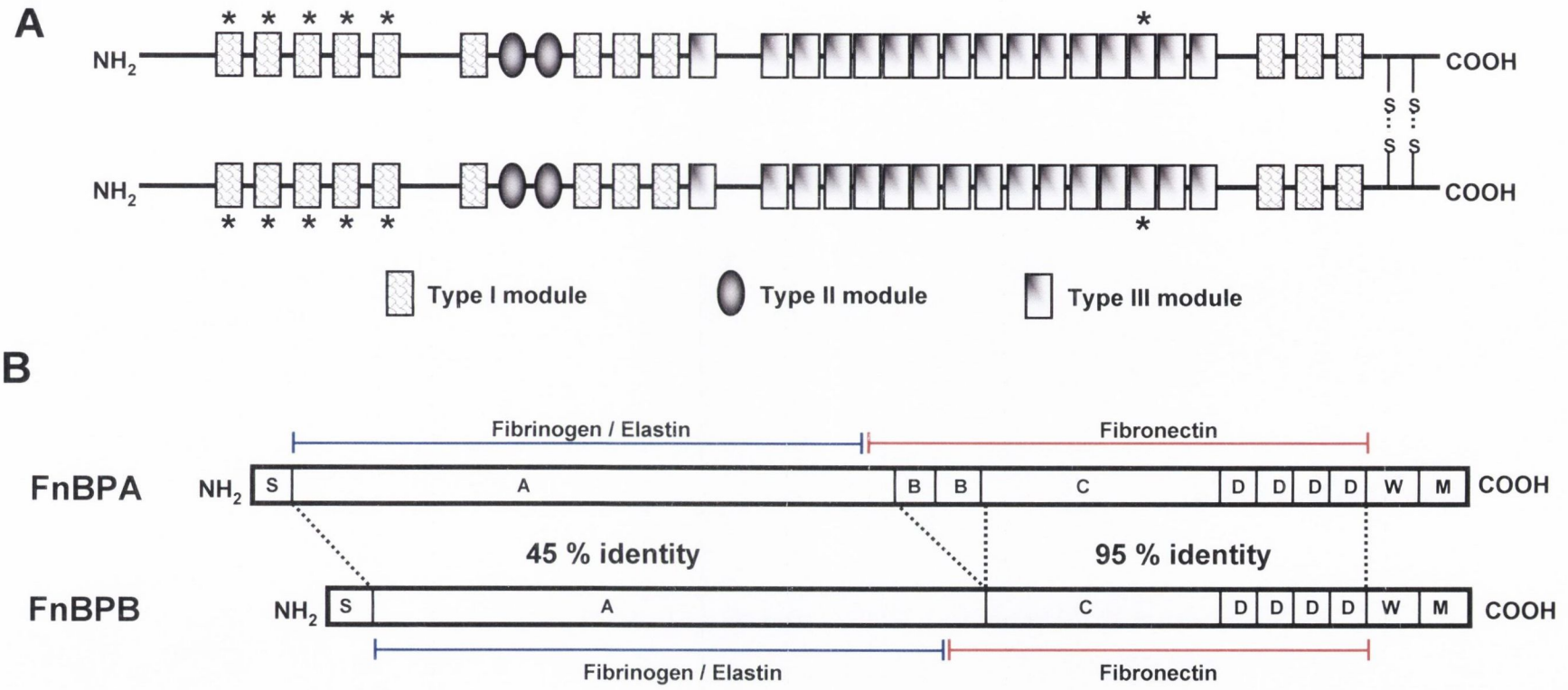


Figure 1.4 Fibronectin-binding surface proteins of *Staphylococcus aureus*.

A. Schematic diagram of human fibronectin. Fibronectin monomers are linked at their C-terminus by a disulfide bond to produce the mature dimer of approximately 500 kDa found in plasma. *S. aureus* binding sites are indicated by an asterisk. **B.** Schematic respresentation of the *S. aureus* fibronectin-binding proteins FnBPA and FnBPB. Their distinct binding domains for fibronectin and fibrinogen/elastin are indicated. Percentage amino acid identities between the binding domains of FnBPA and FnBPB are shown.

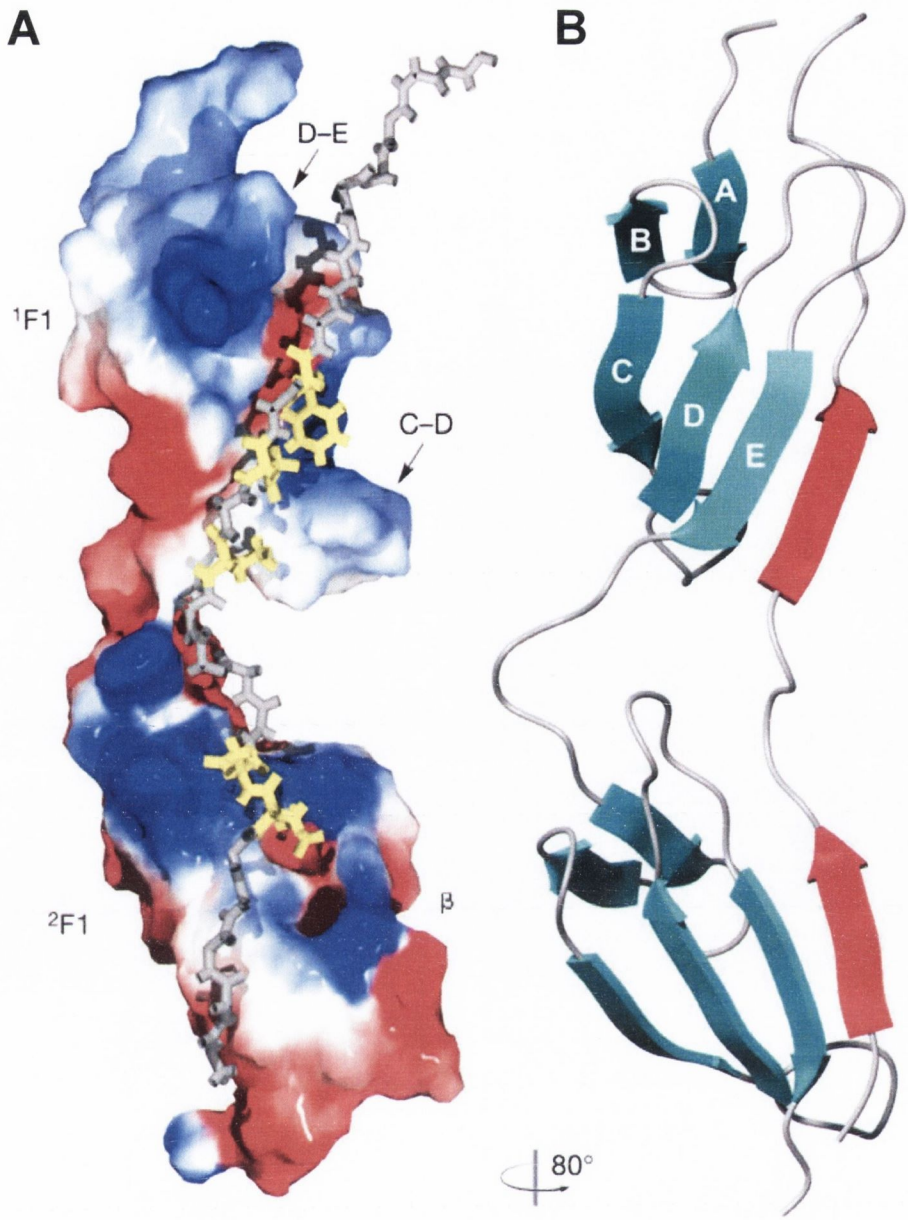


Figure 1.5 Structure of the *Strep. dysgalactiae* B3 peptide in complex with ^{1}F1^{2}F1 type I fibronectin modules

A. Surface potential of ^{1}F1^{2}F1 fibronectin modules with bound B3 peptide (grey). Negatively and positively charged regions of the ^{1}F1^{2}F1 surface are shown in red and blue, respectively. Side chains of hydrophobic and acidic B3 residues are shown in yellow

B. Ribbon diagram of the lowest-energy β-zipper structure showing strands of the F1 modules (cyan) and the fourth strand formed by B3 (red). The difference in orientation between the two views is indicated.

Adapted from Schwarz-Linek *et al.*, 2003.

The *fnbA* gene was present significantly more often in invasive *S. aureus* isolates compared to carriage strains (Peacock *et al.*, 2002). There is also evidence that individuals who have suffered invasive infection contain higher serum antibody levels than healthy individuals (Dryla *et al.*, 2005), indicating that FnBPA is expressed *in vivo* during infection. FnBPs have been demonstrated to be important virulence factors in different infection models. FnBP-expression by *S .aureus* was not responsible for the development of experimental septic arthritis, but contributed to systemic inflammation, weight loss and mortality (Palmqvist *et al.*, 2005). FnBPA expressed by *Lactococcus lactis* was sufficient for heart valve colonization, invasion of surrounding endothelium and dissemination to the spleen of infected animals in experimental endocarditis (Que *et al.*, 2001, 2005). *S. aureus* is a major cause of infections associated with indwelling catheters and implanted devices (Lowy, 1998). It is thought that adhesion to fibronectin that becomes deposited on these devices is an important step in establishing such infections (Vaudaux *et al.*, 1993).

1.3.2.4 Fibrinogen-binding surface proteins

S. aureus expresses at least five proteins on its cell surface that promote interactions with fibrinogen. A 340 kDa plasma glycoprotein, fibrinogen is involved in blood clot formation through its polymerization to form fibrin and in mediating platelet adhesion and aggregation at sites of vascular damage. Implanted prostheses become rapidly covered in plasma fibrinogen, providing an adhesive surface allowing for *S. aureus* colonization (Herrmann *et al.*, 1988; Cheung and Fischetti, 1990). Fibrinogen is composed of 6 polypeptides, two α-chain peptides, two β-chains and two γ-chains (Doolittle, 1984). These are arranged symmetrically, with the C-termini of the α,β and γ chains at each end of the mature molecule (D domains) and the N-termini are found linked by disulphide bonds in the central (E) domain (Figure 1.6).

It has long been known that *S. aureus* cells form clumps when suspended in plasma (Much, 1908). It is now known that this reaction is mediated by fibrinogen (Hawiger *et al.*, 1983). Clumping in fibrinogen solution initially was thought to be mediated by cell-bound coagulase (Boden and Flock, 1989), but this has been shown not to be the case (McDevitt *et al.*, 1992).

1.3.2.4.1 ClfA

The first fibrinogen-binding surface protein of *S. aureus* to be identified was clumping factor A (ClfA; McDevitt *et al.*, 1994; Figure 1.3). ClfA expression confers the ability of bacterial cells to adhere to immobilized fibrinogen and to form cell-aggregates in soluble fibrinogen (clumping) (McDevitt *et al.*, 1994). ClfA contains all the characteristics of gram-positive bacterial surface-anchored proteins. It has a 40 residue signal sequence for secretion, a wall-spanning region, a hydrophobic membrane-spanning region and an LPDTG motif for cell-wall linkage (McDevitt *et al.*, 1994). The 520 residue fibrinogen binding domain (A domain) is located at the mature N-terminus of ClfA and is exposed on the cell surface (McDevitt *et al.*, 1995). C-terminal to the A domain is a region consisting of serine-aspartate (SD) dipeptide repeats (McDevitt *et al.*, 1994). The length of this repeat region is variable between different strains (McDevitt and Foster, 1995). The function of the SD repeats is to project the A domain away from the cell surface allowing interaction with fibrinogen (Hartford *et al.*, 1997). Surface proteins with SD repeats (Clf-Sdr family) are found in other staphylococci, with four others (ClfB, SdrC, SdrD, SdrE) present in *S. aureus*.

ClfA specifically recognizes the extreme C-terminus of the fibrinogen γ-chain (McDevitt *et al.*, 1997). This region of the γ-chain recognizes GPIIb/IIIa on platelets and is required for platelet aggregation (Farrell *et al.*, 1992; Hettasch *et al.*, 1992). Fibrinogen-binding by ClfA is regulated by Ca^{2+} (O'Connell *et al.*, 1998). An IC_{50} of 2.5 mM Ca^{2+} was determined for *S. aureus* cells binding fibrinogen. Similar concentrations of Ca^{2+} (1.5 mM) occur in blood (Mousa *et al.*, 2000; May and Heptinstall, 2004) and suggests that *in vivo* ClfA remains partially unoccupied by soluble fibrinogen. This would allow bacteria to adhere to immobilized fibrinogen, such as in blood clots, through the unoccupied receptors.

The A domain of ClfA is composed of three subdomains, N1, N2 and N3, based on the similar domain organization of the A domain of ClfB (Perkins *et al.*, 2001). The minimum fibrinogen-binding region of ClfA is domain N2N3 (McDevitt *et al.*, 1995). The crystal structure of ClfA N2N3 revealed that each subdomain has a similar structure (Figure 1.7) comprising a novel type of fold (DEv-IgG fold) similar to the immunoglobulin fold (Deivanayagam *et al.*, 2002; Figure 1.7). The DEv-IgG fold consists of nine anti-parallel β-sheets linked by flexible loops. A hydrophobic trench is formed between the two folded domains. It was predicted using docking software to be the binding site for the γ-chain of fibrinogen. Residues predicted to be important in ligand binding were verified by site-directed

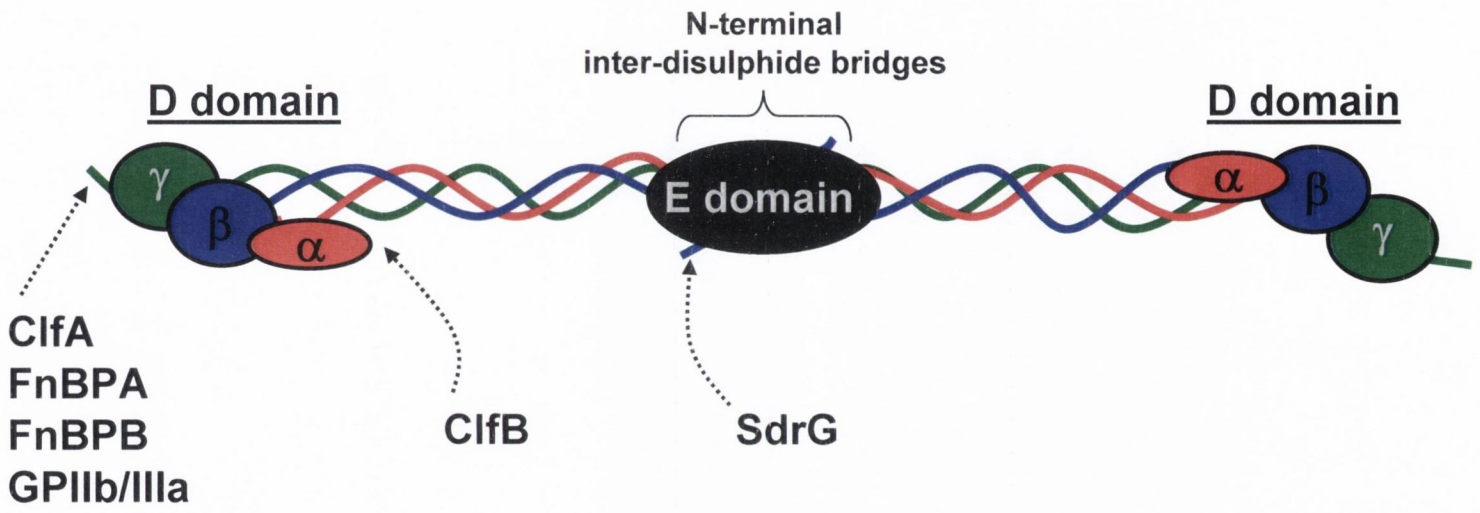


Figure 1.6 Structure of human fibrinogen.

Binding sites for *S. aureus* surface proteins and the *S. epidermidis* SdrG protein are indicated. The binding site in fibrinogen for ClfA, FnBPA and FnBPB is the same as the GPIIb/IIIa platelet integrin binding site required for platelet aggregation.

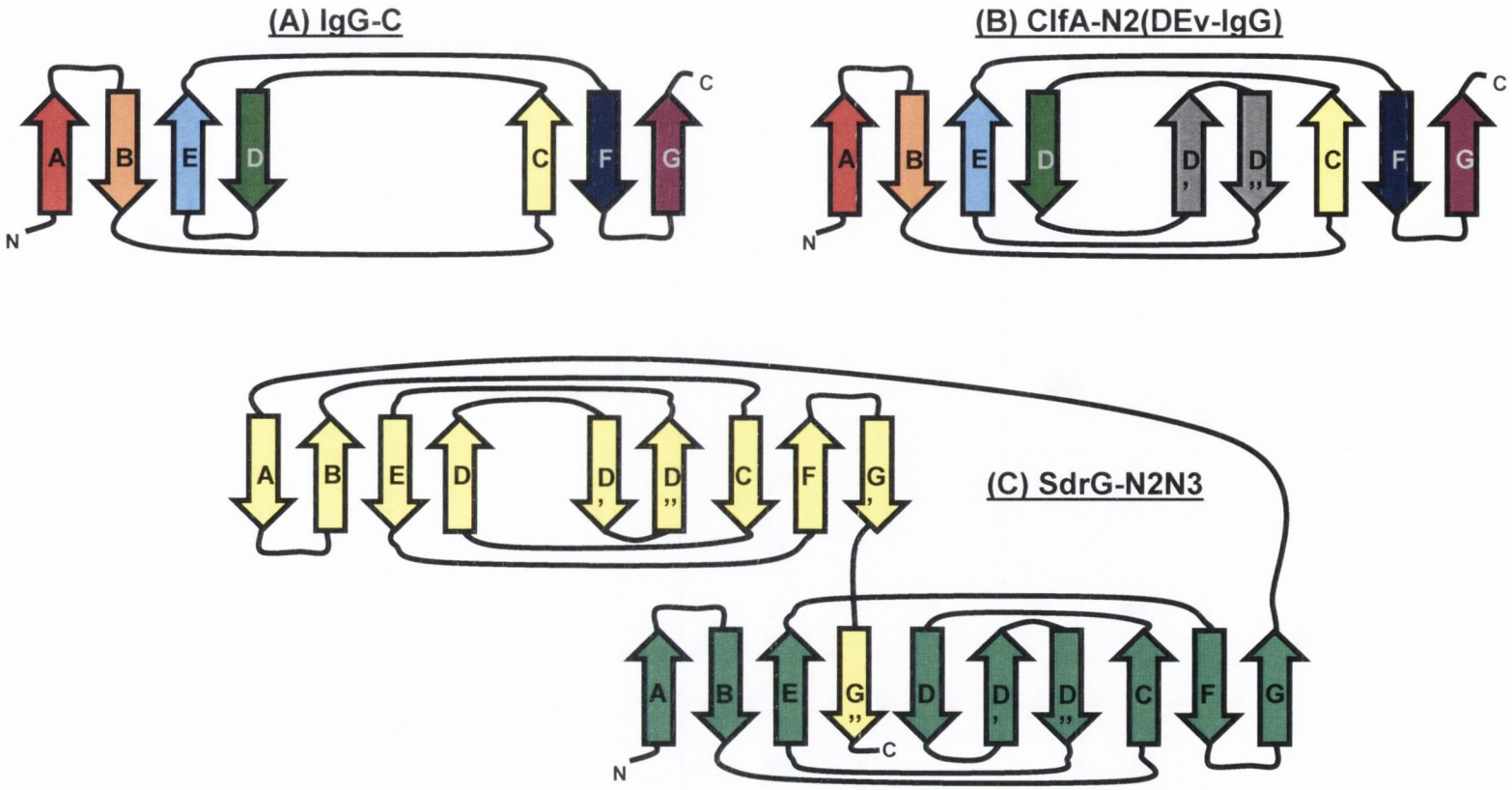


Figure 1.7 Topology of the DEv-IgG fold

Individual MSCRAMM domains show a novel variation of the immunoglobulin fold, designated the DEv-IgG fold. (**A**) The IgG-C fold. (**B**) The DEv-IgG fold of the N2 domain of ClfA region A contains additional β-strands, D' and D'' (coloured grey). (**C**) The N2 and N3 domains of SdrG A domain each comprise a DEv-IgG fold similar to that observed for ClfA. Upon ligand binding, the latching peptide (G'') of the N3 domain (yellow) latches into the space created between strands E and D in the N2 domain (green). The β-strand complementation stabilizes the SdrG-fibrinogen interaction. Nomenclature and colouration of the SdrG β-sheets and subdomains matches that of the SdrG peptide complex in Figure 1.8

Modified from Deivanayagam *et al.*, 2002 and Ponnuraj *et al.*, 2003.

mutagenesis (Deivanayagam *et al.*, 2002), suggesting that the γ-chain of fibrinogen binds in the hydrophobic trench lining domains N2 and N3 (Figure 3.1).

ClfA has been shown to be an important virulence factor in a number of studies. *S. aureus clfA* mutants have been shown to be less infective in endocarditis models than the parental strain (Moreillon *et al.*, 1995). Heterologous expression of ClfA in *Streptococcus gordonii* and *Lactococcus lactis* drastically increased infectivity (Stutzmann-Meier *et al.*, 2001; Que *et al.*, 2001, 2005). Administration of human immunoglobulin containing elevated levels of anti-ClfA antibodies was shown to increase sterilization of valvular vegetations and clearance of bacteremia in rabbit endocarditis infections (Vernachio *et al.*, 2003). ClfA has also been shown to be a virulence factor in murine models of septic arthritis (Josefsson *et al.*, 2001; Palmqvist *et al.*, 2005). Active immunization with recombinant ClfA or passive immunization with polyclonal anti-ClfA antibodies protected mice from arthritis and sepsis-induced death (Josefsson *et al.*, 2001). ClfA is expressed by nearly all strains of *S. aureus* (Peacock *et al.*, 2002; Patti, 2004) and is expressed *in vivo* (Colque-Navarro *et al.*, 2001; Dryla *et al.*, 2005), although a correlation between infection and level of anti-ClfA antibodies has not been proven. It is the target of several novel prophylactic and therapeutic strategies to combat *S. aureus* infections (see Section 1.4).

1.3.2.4.2 ClfB

Clumping factor B shares similar domain organization with ClfA (Figure 1.3). The A domain of ClfB possesses fibrinogen-binding activity but only shares 26 % sequence identity with the A domain of ClfA (Ni Eidhin *et al.*, 1998). In common with ClfA, the binding of ClfB to fibrinogen is inhibited by millimolar concentrations of Ca^{2+}. Whereas ClfA is expressed throughout the growth cycle, ClfB is only expressed in early exponential phase, and its contribution to fibrinogen-binding is masked by ClfA (Ni Eidhin *et al.*, 1998). ClfB recognizes a different part of the fibrinogen molecule (α-chain) than ClfA (γ-chain) and, unlike ClfA, it can also mediate binding to cytokeratin-10, recognizing GS-loops in the tail region of the keratin molecule (O'Brien *et al.*, 2002b; Walsh *et al.*, 2004). Cytokeratin-10 is expressed on the surface of desquamated nasal epithelial cells (O'Brien *et al.*, 2002b) and ClfB-mediated binding to this ligand may play a role in nasal colonization by *S. aureus*. The titres of anti-ClfB antibodies have been observed to be higher in non-carriers than in individuals who have *S. aureus* present in the nares (carriers) (Dryla *et al.*, 2005) suggesting

that anti-ClfB antibodies may help protect against nasal colonization. Recent studies using a murine model of nasal colonization demonstrated that immunization (both intranasally and systemically) with recombinant ClfB was protective (Schaffer *et al.*, manuscript submitted). In addition, systemic administration of a monoclonal antibody directed against ClfB that inhibited *S. aureus* binding to mouse cytokeratin-10 protected against colonization in naïve mice. ClfB may prove to be a suitable candidate antigen for vaccination against nasal colonization by *S. aureus* as the *clfB* gene was present in all strains examined (Peacock *et al.*, 2002). Cytokeratin-10, and not fibrinogen, may be the most physiologically relevant ligand for ClfB.

1.3.2.4.3 SdrG

The *Staphylococcus epidermidis* surface protein SdrG is a member of the Sdr-Clf protein family characterized by a repeat region (R) made up of SD repeats (McCrea *et al.*, 2000; Hartford *et al.*, 2001; Figure 1.3). The A domain of SdrG mediates attachment to the N-terminus of the fibrinogen Bβ-chain (Figure 1.6) (Davis *et al.*, 2001). Although it shares only 24 % and 23 % homology with the A domains of ClfA and ClfB, respectively, the crystal structure of the SdrG N23 A domain truncate, which is functional (Davis *et al.*, 2001) bears striking resemblance to that of the ClfA A domain N23 structure (Figure 1.8; Deivanayagam *et al.*, 2002) and the ClfB A domain N23 structure (Figure 1.8; M. Höök, unpublished data). Each sub-domain consists of the DEv-IgG fold (Figure 1.7) with a cleft of approximately 3 nm separating the two folded domains (Ponnuraj *et al.*, 2003). The crystal structure of the SdrG N23 protein was determined in complex with a synthetic peptide representing the fibrinogen β-chain N-terminus (β6-20), which was bound in the trench between domains N2 and N3 (Figure 1.8; Ponnuraj *et al.*, 2003). Comparison of this structure to that of the apo-SdrG structure (no ligand bound) allowed a dynamic model for ligand binding ('dock, lock and latch' model) to be proposed. Initially, the fibrinogen peptide 'docks' in the trench that separates the N2 and N3 folded domains, and is stabilized by protein-protein interactions between residues in the trench and the ligand. This binding event triggers a structural rearrangement at the C-terminus of the N3 domain. β-strand G'' (the latching peptide; Figure 1.8) in the apo-form of the structure extends into the solvent region. Upon ligand 'docking', the G'' strand undergoes a directional change and crosses over the binding trench. The binding trench becomes covered by part of the G' β-sheet and the linker separating β-sheets

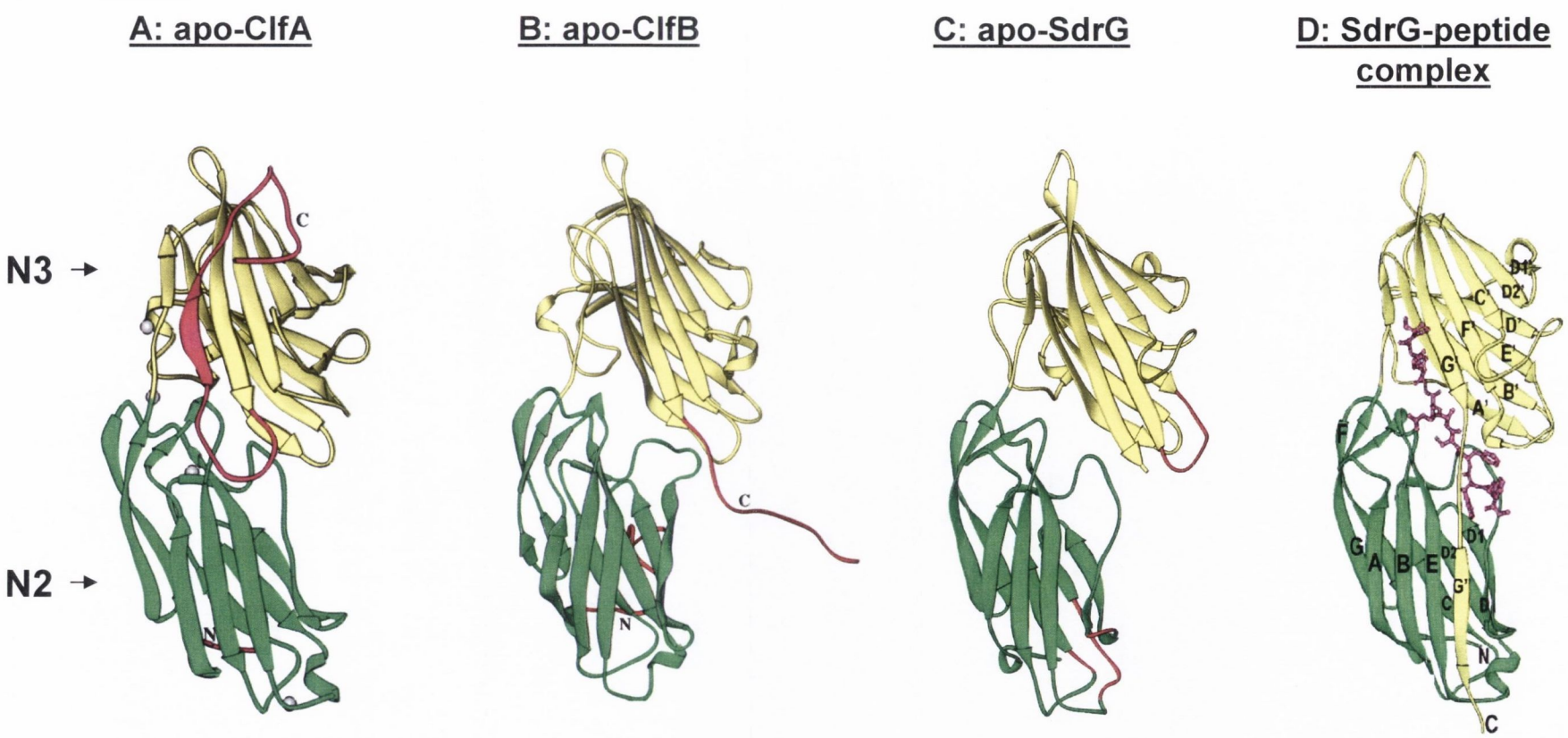


Figure 1.8 Apo structures of domains N23 of ClfA, ClfB and SdrG and SdrG-peptide complex

Ribbon representations of rClfA$_{(221-559)}$, rClfB$_{(282-542)}$ and rSdrG$_{(276-597)}$. Regions of poor resolution are shown in red. Domains N2 (green) and N3 are indicated. **A.** The C-terminus of rClfA$_{(221-559)}$ (latching peptide shown in red) loops back and folds into the N3 domain, partially blocking the proposed ligand-binding cleft. **B.** In rClfB$_{(282-542)}$ this peptide is located in the latching cleft of another rClfB molecule. **C.** In apo-SdrG the peptide (G'') is free in solution but interacts with N2 in the SdrG-peptide complex. **D:** Fibrinogen β-chain peptide analogue in complex with SdrG is shown in ball and stick form. Strand designations and colours correspond to the DEv-IgG fold representation in Figure 1.7. SdrG-peptide complex is taken from Ponnuraj *et al.*, 2003.

G' and G'', and thus 'locks' the peptide in place. Hydrogen bonding between the bound fibrinogen peptide and β-strand G'/linker regions of the adhesin occurs, securing the ligand in the trench. The final step is β-strand complementation, where the C-terminal β-strand G'' of the N3 domain 'latches' on to the neighbouring N2 domain, where it inserts between strands E and D (the latching cleft) creating a new β-sheet in the N2 domain (Figures 1.7 and 1.8). This serves to stabilize the overall structure (Ponnuraj *et al.*, 2003). A conserved TYTFTDYVD-like motif forms the back of the latching cleft in rSdrG, and is found in a similar location in the structures of rClfA and rClfB. This motif is likely to be involved in binding of the latching peptide to the latching cleft in domain N2 (Ponnuraj *et al.*, 2003).

It is proposed that surface proteins bearing DEv-IgG folded domains that bind to fibrinogen (ClfA, ClfB, SdrG, Can, and possibly FnBPA and FnBPB) all do so by this 'dock, lock, latch' mechanism.

1.3.2.4.4 FnBPA/FnBPB

In addition to their role in fibronectin binding (section 1.3.2.3), both FnBPA and FnBPB can promote bacterial adhesion to the C-terminus of the fibrinogen γ chain (Wann *et al.*, 2000), the same site as that recognized by ClfA (McDevitt *et al.*, 1997). Binding is mediated by the N-terminal A domains of FnBPA or FnBPB (Figure 1.3). These domains are predicted to form independently folded DEv-IgG subdomains similar to that seen for ClfA and SdrG (Deivanayagam *et al.*, 2002; Ponnuraj *et al.*, 2003), and may bind fibrinogen by the 'dock, lock, latch' mechanism. It is likely that *S. aureus* FnBPs can function in a similar manner to ClfA in infection due to their fibrinogen-binding activity.

Unlike ClfA, the A domains of FnBPA and FnBPB can also mediate bacterial binding to elastin, a major component of the extracellular matrix (Roche *et al.*, 2004). Therefore FnBPs may be important factors in the colonization of elastin-rich tissue such as lung and heart valves, potentially leading to the development of pneumonia and native-valve endocarditis, respectively.

1.3.2.5 Collagen-binding protein

Collagen is the main component of the extracellular matrix of connective tissue. Some strains of *S. aureus* express a collagen-binding adhesin, Cna, that is necessary and sufficient for adhesion of *S. aureus* to collagen substrates and collagenous tissues (Patti *et al.*, 1992).

Cna shares similar domain organization to other *S. aureus* surface proteins (Figure 1.3). Collagen-binding activity is located in the approximately 500 amino acid A domain, and the minimum ligand-binding truncate ($Cna_{151-315}$) has been crystallized (Patti *et al.*, 1995; Symerski *et al.*, 1997). $Cna_{151-315}$ forms a DEv-IgG folded domain containing a surface trench in one of its β-sheets that can accommodate the collagen triple helix (Symerski *et al.*, 1997; Deivanayagam *et al.*, 2002). Cna may be an important virulence determinant of infections involving collagen-rich bone tissue such as arthritis and osteomyelitis. It is a virulence factor in animal models of septic arthritis, endocarditis and osteomyelitis (Patti *et al.*, 1994; Hienz *et al.*, 1996; Elasri *et al.*, 2002), and vaccination against Cna is protective in septic arthritis (Nilsson *et al.*, 1998). However, the *cna* gene is only present in approximately 30 to 50 % of *S. aureus* isolates (Smeltzer *et al.*, 1997; Peacock *et al.*, 2002; Arciola *et al.*, 2005) so it does not represent a useful target for vaccination or immunotherapy.

1.3.2.6 Iron-regulated surface proteins

A subset of *S. aureus* genes encoding 4 surface proteins are expressed only under iron-limitation, likely to resemble growth conditions in serum and within the host during infection. The genes encoding 3 of the iron-surface-determinant (Isd) proteins (IsdA, IsdB and IsdC) are found in a locus encoding an iron-transport system (Mazmanian *et al.*, 2003). The gene encoding IsdH is found outside the main *isd* cluster (Dryla *et al.*, 2003). Expression is controlled by the ferric-uptake-response (Fur) transcriptional regulator, which prevents transcription of *isdA*, *isdB*, *isdC* and *isdH* in the presence of iron (Horsburg *et al.*, 2001; Mazmanian *et al.*, 2003; Dryla *et al.*, 2003). IsdA, IsdB and IsdH each contain the sortase A LPXTG sorting signal, while IsdC contains an NPQTN motif for sortase B-catalysed cell wall sorting (Mazmanian *et al.*, 2002; Dryla *et al.*, 2003). A number of iron-containing ligands have been identified for these proteins. IsdA has been shown to bind transferrin, haemoglobin, and hemin (Taylor and Heinrichs, 2002; Mazmanian *et al.*, 2003; Clarke *et al.*, 2004). IsdB contains hemin-binding and hemoglobin-binding activities (Mazmanian *et al.*, 2003), and IsdH binds haptoglobin and haptoglobin-haemoglobin complexes (Dryla *et al.*, 2003). It is proposed that the expression of Isd proteins in response to limiting levels of free iron results in binding of heme-containing ligands to Isd proteins. Heme molecules are liberated and transferred across the cell wall and membrane into the cytoplasm (Mazmanian *et al.*, 2003; Skaar and Schneewind, 2004). IsdC appears to be buried in the cell wall and is not displayed on the cell surface (Mazmanian *et al.*, 2002). It is proposed to function in the

passage of heme iron across the cell wall (Skaar and Schneewind, 2004). Several other iron-uptake mechanisms have been identified in *S. aureus*, including another heme-iron uptake system (*hts* locus) and numerous siderophore-dependent systems (Skaar *et al.*, 2004). This indicates that the Isd iron-uptake system is redundant in function, as evidenced by the ability of *isd* mutants to continue to utilize haem-iron (Mazmanian *et al.*, 2003).

Other non-iron based ligands have been identified for some of the Isd surface proteins. IsdA is a physiologically relevant adhesin for fibrinogen and fibronectin when *S. aureus* is grown in iron-depleted conditions (Clarke *et al.*, 2004). IsdH has been shown to specifically bind complement component C3 (N. Yanasigisawa, P. Speziale and T.J. Foster, unpublished data), which may interfere with complement deposition on the bacterial surface. The binding domains in IsdA and IsdH are known as NEAT domains (near transporter), which are found in variable numbers in bacterial genes that are in the vicinity of putative iron-siderophore transporters in Gram-positive bacteria (Andrade *et al.*, 2002). IsdA and IsdC contain a single NEAT domain, while IsdB and IsdH each contain two NEAT domains (Andrade *et al.*, 2002). The NEAT domains of IsdA and IsdH share low sequence identity (20 %; Clarke *et al.*, 2004), but are predicted to form structures composed mostly of β-sheets (Andrade *et al.*, 2002). The single NEAT domain in IsdA was shown to be responsible for fibrinogen binding (Clarke *et al.*, 2004) and both of the NEAT domains in IsdH could bind to haptoglobin (Dryla *et al.*, 2003).

1.3.2.7 Other surface proteins

S. aureus contains three additional genes (in addition to *clfA* and *clfB*) that encode surface proteins with Ser-Asp (SD) repeats (Josefsson *et al.*, 1998; Figure 5.8). The *sdrC*, *sdrD*, and *sdrE* genes are closely linked in a tandem-array in the *S. aureus* chromosome, although some strains do not contain all three genes (Josefsson *et al.*, 1998). No biological activities have been attributed to SdrC or SdrD. SdrE was shown to promote platelet aggregation by *S. aureus* (O'Brien *et al.*, 2002a; section 1.5.3). A surface protein with significant homology to SdrE that could bind bone sialoprotein was isolated from *S. aureus* strain O24 (Tung *et al.*, 2000).

Analysis of finished *S. aureus* genome sequences predicted 10 novel surface proteins bearing LPXTG motifs (Mazmanian *et al.*, 2001; Roche *et al.*, 2003). These were designated Sas proteins (*S. aureus* surface). Like other *S. aureus* surface proteins, many of these proteins contain repeat domains. SasE, SasI and SasJ, which were present in all the genome sequences

analysed, have been characterized and were renamed IsdA, IsdH and IsdB respectively (section 1.3.2.6). SasG is homologous to the Pls (plasmin-sensitive) surface protein, which is encoded within the type I SCC*mec* element of some MRSA strains. Pls impairs bacterial adhesion to immobilized ligands (Savolainen *et al.*, 2001). In addition SasG and Pls can promote *S. aureus* adherence to nasal epithelial cells (Roche *et al.*, 2003), which may be important in nasal colonization. SasA (also known as SraP) is encoded in a chromosomal locus that resembles that encoding the *Strep. gordonii* platelet adhesin GspB (section 1.5.2.2). SraP was recently shown to act as an *S. aureus* platelet-binding protein (Siboo *et al.*, 2005). No functions have been attributed to other Sas proteins (SasC, SasD, SasF, SasH, SasK). Antibodies against many Sas proteins have been detected in convalescent sera from patients with documented *S. aureus* infections (Roche *et al.*, 2003) indicating that expression of these proteins occurs during infection.

1.3.3 Capsule

More than 90 % of *S. aureus* clinical isolates produce capsular polysaccharide (CP) on their cell surface, of which there are 11 distinct serotypes. Heavily encapsulated strains express serotype 1 or 2 macrocapsules and form mucoid colonies on solid media. They are seldom encountered among clinical isolates. Approximately 25 % of human isolates express serotype 5 microcapsules and 50 % express type 8 microcapsule, both of which are polymers of hexosaminuronic acids (O'Riordan and Lee, 2004). The role of CP in immune evasion (section 1.3.5.4) is discussed below. A bicomponent vaccine comprising the major CP serotypes has been developed for use in individuals at high risk of *S. aureus* infection (section 1.4.1).

1.3.4 Extracellular proteins

More than half of the actual or potential virulence determinants in *S. aureus* are secreted extracellular proteins (Kuroda *et al.*, 2001). Some of these are described in Table 1.1. Most *S. aureus* strains secrete several superantigen toxins (Fraser *et al.*, 2000; McCormick *et al.*, 2001). Superantigens have the ability to bind the exterior surface of the MHC class II protein on the surface of antigen-presenting cells and to link it to T cell receptors on the surface of a T helper cell (Proft and Fraser, 2003). Binding occurs without the requirement for the MHCII molecule to present an antigenic peptide to a suitable T cell receptor. Up to 30%

Table 1.1 Extracellular proteins of *S. aureus*

Secreted protein	Effect	Reference
alpha-toxin	Pore forming toxin. Lyses mammalian erythrocytes.	Montoya and Gouaux, 2003
beta-toxin	Sphingomyelinase. Lysis of cells with high content of sphingomyelin in their plasma membranes	Doery, 1963 Walev *et al.*, 1996
gamma-toxin	2-component cytolytic toxin. Lyses mammalian erythrocytes	Kaneko and Kamio, 2004
Panton-Valentine leukocidan	2-component cytolytic toxin highly specific for leukocytes.	Kaneko and Kamio, 2004
CHIPS	Inhibits neutrophil chemotaxis	de Haas *et al.*, 2004 Postma *et al.*, 2004
SCIN	Inhibits complement fixation on bacterium surface	Rooijakkers *et al.*, 2005
Efb	Secreted fibrinogen-binding protein. Inhibitor of complement fixation. Blocks platelet aggregation	Lee *et al.*, 2004 Shannon and Flock, 2004.
Staphylokinase	Plasminogen activator. Binds to and inactivates human defensins.	Sakharov *et al.*, 1996 Jin *et al.*, 2004
Aureolysin	Metalloprotease. Tissue damage Inactivation of antimicrobial peptides	Sieprawska-Lupa *et al.*, 2004
Enterotoxins	Superantigen, food poisoning	Bohach *et al.*, 1990
TSST-1	Superantigen, toxic shock	McCormick *et al.*, 2001
Coagulase	Activates prothrombin, causes clotting of fibrinogen	Boden and Flock, 1992
V8 protease	Serine protease. Modification of *S. aureus* surface proteins	Shaw *et al.*, 2004 McGavin *et al.*, 1997
Staphopain A and B	Cysteine proteases. Cause vascular leakage leading to septic shock	Shaw *et al.*, 2004 Imamura *et al.*, 2005

of T cells can become activated, leading to proliferation and massive release of cytokines which mediates toxic shock, causing tissue damage and multiorgan dysfunction (Marrack and Kappler, 1990; Bone, 1994). Low-level expression of superantigen toxins can cause immune suppression by the local depletion of T-cells. Enterotoxins also cause staphylococcal food poisoning if ingested (Bohach *et al.*, 1990).

S. aureus produces a number of pore-forming cytolytic toxins that destroy host cells such as erythrocytes, leukocytes and platelets (Bhakdi *et al.*, 1988; Montoya and Gouaux, 2003; Kaneko and Kamio, 2004). A number of secreted enzymes and proteases are produced that destroy tissue and may facilitate the spread of infection to adjoining tissue (Lowy, 1998). Proteases may also play a role in detachment of *S. aureus* from surfaces by processing of cell surface proteins (McGavin *et al.*, 1997).

1.3.5 Immune evasion by *S. aureus*

1.3.5.1 Host defences against infection

When *S. aureus* breaches the outer physical barriers of the body, comprising the skin and mucous surfaces, it encounters the host's immune system comprising both innate and induced responses. *S. aureus* infection of the skin stimulates a strong inflammatory response involving the migration of neutrophils and macrophages to the site of infection. These cells will attempt to engulf and dispose of the invading organisms with the help of available antibodies that are present in the host's serum, and complement. The complement cascade is described in detail in Chapter 5. The main purpose of complement fixation on *S. aureus* is to promote phagocytosis by professional phagocytes (neutrophils and macrophages). Formylated peptides released by growing bacteria and chemoattractant molecules released during complement activation (C3a and C5a) attract phagocytes to the site of infection. Phagoctyes express specific receptors for complement fragments and formylated peptides that enhance the efficiency of phagocytosis. Neutrophils also carry specific receptors that can recognize the Fc region of IgG and complement proteins bound to the bacterial surface that facilitate efficient uptake and killing.

At the initial stage of infection, uptake of *S. aureus* by macrophages stimulates the acquired immune response. B cells in the lymph nodes are stimulated to differentiate and secrete antibodies that neutralise toxins and recognise bacterial targets, promoting more

efficient phagocytosis of bacterial cells. It is clear that this system does not work properly in the case of *S. aureus*. Antibodies to *S. aureus* antigens are present in all humans and there is evidence that titres rise following infection (Roche *et al.*, 2003; Dryla *et al.*, 2005). However, these antibodies and immunological memory seem to be insufficient to prevent subsequent infections.

S. aureus has evolved numerous strategies to thwart the host immune response to facilitate its survival and pathogenesis in superficial and invasive disease conditions. Selected examples of such mechanisms are discussed below.

1.3.5.2 Inhibition of neutrophil chemotaxis

The chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a 14.1-kD secreted protein encoded by bacteriophages that occur in about 60% of clinical isolates (Veldkamp *et al.*, 2000; de Haas *et al.*, 2004). Two separate active sites on CHIPS are involved in binding to the receptors for complement fragment C5a (C5R) and the formylated peptide receptors (FPR) on neutrophils and monocytes, which block the cognate agonist from binding and results in potent, specific inhibition of chemotaxis (Haas *et al.*, 2004; Postma *et al.*, 2004; 2005). It is hypothesized that CHIPS plays a major role early in infection by inhibiting leukocyte migration to the site of infection, and it is an important virulence factor (de Haas *et al.*, 2004).

1.3.5.3 Toxins that kill leukocytes

The expression of cytolytic leukotoxins by *S. aureus* contributes to development of abscesses by killing neutrophils that are attempting to engulf and kill bacteria. Leukotoxins are likely to be the major cause of pyogenicity which is a hallmark of many *S. aureus* infections. There are four types of bicomponent leucotoxin, the γ-haemolysin (Hlg), the Panton-Valentine leucocidin (PVL), leucocidin E/D and leucocidin M/F-PV-like. Bicomponent leukotoxins comprise two subunits that are secreted separately and assemble on the leukocyte membrane, beginning with binding of monomers, followed by oligomerisation to form a single heptameric pore, culminating in the formation of clusters of pores (Kaneko and Kamio, 2004). The γ-toxin lyses both erythrocytes and leucocytes whereas PVL is toxic only for leucocytes (Menestrina *et al.*, 2003). PVL expression is strongly associated with severe skin infections and necrotizing pneumonia (see section 1.2.2.2).

1.3.5.4 Resistance to opsonophagocytosis

S. aureus expresses surface–associated anti-opsonic proteins and a polysaccharide capsule that both can interfere with the deposition of antibodies and complement formation by classical and alternative pathways, or with their access by neutrophil complement receptors and Fc receptors. Thus efficient phagocytosis by neutrophils that requires recognition of bound complement and antibody is compromised.

Binding of the Fc-region of IgG to staphylococcal protein A results in the coating of bacteria with IgG molecules in the incorrect orientation to be recognized by neutrophil Fc-receptors. This could explain why *S. aureus spa* mutants are phagocytosed more efficiently *in vitro* (Gemmell *et al.*, 1991), and exhibit decreased virulence in several animal infection models (Patel *et al.*, 1987; Palmqvist *et al.*, 2002).

ClfA is another cell-surface associated protein that displays anti-phagocytic properties (Palmqvist *et al.*, 2004). Bacteria expressing a non-fibrinogen binding ClfA mutant were phagocytosed more efficiently *in vitro* than bacteria expressing the wild-type protein (J. Higgins and T.J. Foster, unpublished results). This suggests that the coating of bacteria expressing ClfA with fibrinogen inhibits deposition of or access to opsonins. This may be important in the pathogenesis of conditions such as sepsis and septic arthritis. Other fibrinogen-binding surface proteins (ClfB, FnBPA and FnBPB) may exhibit anti-phagocytic properties in a similar manner.

The majority of *S. aureus* clinical isolates express one of three different serotypes of capsular polysaccharide on their cell surface (CP5, CP8, CP336; O'Riordan and Lee, 2004; Roghmann *et al.*, 2005). Capsular polysaccharide expression reduces phagocytosis in the presence of normal serum opsonins *in vitro* and is associated with increased virulence in animal infection models (Thakker *et al.*, 1998; Luong and Lee, 2002). Capsular polysaccharide is likely to prevent access of phagocyte complement receptors to complement components assembled beneath the capsule layer, thus impairing phagocytosis (Cunnion *et al.*, 2003).

S. aureus also secretes a number of proteins that interfere with complement fixation on the bacterial surface and thus impair killing by human neutrophils. Extracellular fibrinogen binding protein (Efb) binds the α-chain of the central complement component C3 inhibiting its deposition on the cell surface and complement-mediated opsonophagocytosis (Lee *et al.*, 2004). A recently described staphylococcal complement inhibitor (SCIN) protein was shown to bind to activator-bound C3 convertase complexes, inhibiting further C3b deposition by all

complement pathways resulting in a substantial reduction in *S. aureus* killing by neutrophils (Rooijakkers *et al.*, 2005).

1.3.5.5 Resistance to killing by antimicrobial peptides

In addition to its ability to inhibit opsonophagocytosis, *S. aureus* has developed mechanisms to inhibit bacterial killing by defensins and other antimicrobial peptides once it becomes phagocytosed. These antimicrobial peptides form an important part of the innate immune response. Antimicrobial peptides include small anionic peptides (e.g. dermicidin), small cationic peptides (e.g. cathelicidin LL-37, platelet microbicidal proteins), anionic and cationic peptides that form disulphide bonds (e.g. α-defensins and β-defensins) and some small peptides derived from larger proteins (e.g. lactoferricin from lactoferrin) (Brogden, 2005). Antimicrobial activity is generally due to disruption of the integrity of lipid bilayers, but in some cases more specific inhibitory modes of action may occur (Brogden, 2005). The *S. aureus dlt* operon (*dltABCD*) is associated with the addition of D-alanine moieties to cell surface structures such as wall teichoic acids and lipoteichoic acids (Peschel *et al.*, 1999). The MprF protein of *S. aureus* is associated with the modification of membrane phosphatidylglycerol with L-lysine residues (Peschel *et al.*, 2001). In both instances, these modifications serve to reduce the net negative charge of the cell surface and repel a broad range of cationic antimicrobial peptides away from the cytoplasmic membrane.

S. aureus also secretes proteins which neutralize cationic peptides. The extracellular metalloprotease aureolysin cleaves and inactivates the human defensin peptide cathelicidin LL-37 and contributes significantly to resistance to the peptide *in vitro* (Sieprawska-Lupa *et al.*, 2004). Staphylokinase, a small protein secreted by *S. aureus*, has potent defensin peptide binding activity. It induces the release of defensins from leukocyte granules and effectively neutralises them. One molecule of staphylokinase can bind up to 6 defensin peptides (Jin *et al.*, 2004). Interestingly, the genes encoding staphylokinase, CHIPS, SCIN and the superantigen enterotoxin A are found on a pathogenicity island (SaPI5) carried by lysogenic bacteriophages (Rooijakkers *et al.*, 2005). The activities of each protein appear to be specific to the human immune response.

Platelets, in addition to their role in blood coagulation (section 1.5.1), contain a number of small, microbicidal cationic peptides termed platelet-microbicidal-proteins (PMP) that are released upon stimulation by platelet agonists such as thrombin (Yeamen *et al.*, 1997). These peptides target the cytoplasmic membrane, resulting in permeabilization and uptake into

the cytoplasm, and are proposed to inhibit synthesis of macromolecules such as DNA and proteins leading to cell death (Xiong *et al.*, 2002). *S. aureus* endocarditis isolates are frequently more resistant to PMPs than isolates from soft-tissue infections (Yeamen *et al.*, 1992b; Bayer *et al.*, 1998). PMP resistant *S. aureus* strains have been demonstrated to be more virulent than PMP sensitive strains in a number of experimental endocarditis studies (Dhawan *et al.*, 1997; 1998; Mercier *et al.*, 2000). PMPs are likely to play an antimicrobial defense role in preventing or limiting bacterial proliferation in endovascular infections. Resistance of *S. aureus* to PMPs is likely to play a role in the success of this pathogen in causing endovascular infections (see section 1.5).

1.4 Treatment of staphylococcal infections

S. aureus is a major pathogen associated with infections of the bloodstream, skin and soft tissue. Penicillin remains the drug of choice to treat infections if the causative isolate is sensitive to it (Lowy, 1998). The acquisition of multiple antimicrobial resistance genes (such as SCC*mec* elements and associated resistance determinants, see section 1.2.2.1) has resulted in the emergence of *S. aureus* strains that exhibit resistance to most classes of antimicrobials. MRSA strains have become endemic in many hospitals and are emerging as significant pathogens in the community. Vancomycin was, until recently, the only drug to which *S. aureus* remained uniformly susceptible. Clinical infections by *S. aureus* isolates harbouring the enterococcal *vanA* gene mediating high-level vancomycin resistance have recently been reported (Cosgrove *et al.*, 2004). New approaches to treat or prevent *S. aureus* infections are urgently required. The identification of new targets for novel-antistaphylococcal therapies is an active area of research (Garcia-Lara *et al.*, 2005).

Very few novel classes of antimicrobial agents have become available for treating infections caused by multiply-resistant bacterial strains. Linezolid is a synthetic oxazolidinone that binds to bacterial ribosome and inhibits translation initiation. The streptogramins quinupristin/dalfopristin (Synercid) act synergistically to inhibit protein synthesis by targeting bacterial ribosomes (Hancock, 2005). Both linezolid and Synercid display antibacterial action against MRSA isolates, however isolated instances of *S .aureus* resistance to these agents have

been documented (Hancock, 2005). Past experience suggests that it is only a matter of time before resistance to these agents becomes widespread.

An individual who has suffered from an *S. aureus* infection is usually not protected from a subsequent infection. This is because the host is prevented from mounting a strong antibody response and immunological memory is compromised by the immunosuppressive properties of the organism. However, there is mounting evidence that it is possible to generate a robust antibody response to highly purified surface components of *S. aureus*. Strategies based on active or passive immunization against some *S. aureus* surface components are discussed below.

1.4.1 Capsular polysaccharide vaccine – StaphVax

The expression of capsular polysaccharide by *S. aureus* inhibits phagocytosis in the presence of normal serum opsonins (section 1.3.3.4). However, high levels of specific anti-capsular polysaccharide antibodies promote opsonophagocytosis and protect against infection (Lee *et al.*, 1997; Thakker *et al.*, 1998; O'Riordan and Lee, 2004). Capsular polysaccharides are poorly immunogenic, and the levels of anti-capsular polysaccharide antibodies in normal human serum are too low to be protective. Increased immunogenicity is observed upon conjugation of capsular polysaccharide to a protein carrier molecule, and generates a booster response upon repeated immunisation (Fattom *et al.*, 1993). Capsular polysaccharide (CP) serotypes 5 and 8 are the predominant serotypes found among human isolates, representing 85 % of clinical strains (O'Riordan and Lee, 2004). A bivalent vaccine comprising CP5 and CP8 conjugated to recombinant *Pseudomonas aeruginosa* exotoxin A (StaphVax) was developed by Nabi, Inc (Florida, U.S.A) intended for immunization of individuals at high risk of *S. aureus* infection. It has undergone phase I, II and III clinical trials (Fattom *et al.*, 1993; Welch *et al.*, 1996; Shinefield *et al.*, 2002). Immunization of haemodialysis patients with StaphVax was successful in reducing the incidence of infection in these vulnerable patients over an 8 month period (Fattom *et al.*, 2004). Clinical trials are underway determining the protective effect of purified antibodies to CP5 and CP8 (AltaStaph; O'Riordan and Lee, 2004).

1.4.2 Immunotherapies targeting ClfA

ClfA is an important virulence factor in several animal infection models and is expressed by 100 % of invasive *S. aureus* strains (Peacock *et al.*, 2002). ClfA therefore

represents a good vaccine candidate. Studies in animal infection models have shown that both active and passive immunisation against ClfA gives protection (Josefsson *et al.*, 2001; Brouillette *et al.*, 2002) indicating that protection is antibody-mediated. High serum levels of anti-ClfA antibodies may protect against infection by both inhibiting ClfA-mediated bacterial adhesion to fibrinogen immobilized on surfaces and by promoting efficient opsonophagocytosis of bacteria by neutrophils (Patti, 2004).

Inhibitex Inc. (Georgia, USA) have developed a hyperimmunoglobulin, SA-IGIV (Veronate), derived from plasma donors with high titres of antibodies against ClfA and the fibrinogen-binding *S. epidermidis* SdrG protein (see section 1.3.2.4.3) for the prevention of staphylococcal infections in very low weight neonates (Vernachio *et al.*, 2003). Veronate recognised the *S. aureus* cell surface, specifically inhibited bacterial adherence to immobilised fibrinogen, and acted as an efficient opsonin in an *in vitro* phagocytosis assay. When used in conjunction with vancomycin, it was therapeutically effective in a rabbit model of catheter-induced infective endocarditis caused by an MRSA strain (Vernachio *et al.*, 2003). Promising results were obtained in a phase II trial for the prevention of staphylococcal infections in low birth weight infants (Bloom *et al.*, 2005) and a phase III trial is currently underway.

A humanised monoclonal antibody against the fibrinogen-binding A domain of ClfA (Aurexis) has been developed for use as a novel therapeutic by Inhibitex (Patti, 2004). Aurexis inhibited bacterial adhesion to immobilized fibrinogen, enhanced opsonophagocytosis, and protected against intravenous challenge in a murine sepsis model (Hall *et al.*, 2003). In combination with vancomycin, it reduced bacterial densities in infected vegetations, kidneys and spleens in a therapeutic infective endocarditis model (Patti, 2004). Aurexis is currently undergoing phase II clinical trials aimed at use in the therapy of *S. aureus* bacteraemia, in conjunction with conventional antimicrobial therapies (Patti, 2004).

1.5 Bacteria, platelets and cardiovascular disease

Occasionally, opportunistic bacterial pathogens gain entry to the human circulatory system resulting in a bacteremic infection. Bacteremia can, in some instances, result in the development of serious cardiovascular complications, such as life-threatening infective endocarditis (IE), disseminated intravascular coagulation (DIC), thrombocytopenia,

atherosclerosis and myocardial infarction. The interaction between bacterial pathogens and human blood platelets is postulated to play an important role in the development of IE, and may be important in the pathogenesis of other disease states.

IE is characterized by the formation of vegetative growths on heart valves containing a collection of bacteria, platelets, fibrin and inflammatory cells which may shield bacteria against the host immune response and antibiotics (Mylonakis and Calderwood, 2001). Complications of IE include congestive heart failure as a result of infection-induced vavular damage, neurologic complications such as stroke, and systemic embolism resulting in infection of organs such as the kidneys and spleen. Patients presenting with IE often have symptoms such as fever, weight loss, malaise and night sweats. IE is lethal if not aggressively treated with antibiotics, in combination with surgery where necessary. Despite this, a significant mortality rate is seen in studies of patients with IE; in particular IE due to *S. aureus* is associated with high mortality rates (25-47 %; Mylonakis and Calderwood, 2001). Despite improvements in healthcare over the past 20 years, the incidence of IE has not decreased. This is the result of a progressive change in risk factors and the emergence of antibiotic-resistant bacterial strains. Chronic rheumatic heart disease was the main risk factor in the pre-antibiotic era. Nowadays, new at-risk groups include intravenous drug users, patients with prosthetic valves, haemodialysis patients, elderly people with valve sclerosis and those exposed to nosocomial disease (Moreillon and Que, 2004). While many bacterial pathogens can cause IE, staphylococci, streptococci and enterococci together account for > 80 % of all instances of this disease (Moreillon and Que, 2004). Staphylococci have now overtaken streptococci as the most prevalent cause of IE (Mylonakis and Calderwood, 2001). Successful treatment of IE depends on effective antibiotic therapy. Endocarditis caused by methicillin-sensitive *S. aureus* (MSSA) is usually treated with nafcillin or oxacillin, while vancomycin is the treatment of choice when the causative bacterium is an MRSA strain (Mylonakis and Calderwood, 2001). The emergence of *S. aureus* strains that have some degree of vancomycin resistance (VISA/VRSA strains; Kuroda *et al.*, 2001; Cosgrove *et al.*, 2004) may result in cases of IE that are untreatable with currently available antibiotics. A full understanding of the pathogenesis of IE is crucial for the developments of novel non-antibiotic based therapeutics to combat this disease.

The ability of bacteria to bind to and activate platelets may contribute to IE development in a number of ways. Bacteria in the bloodstream could bind to activated platelets in a sterile developing thrombus on damaged valve surfaces, facilitating colonization.

Bacteria within this thrombus may then capture and activate circulating platelets from the bloodstream, leading to enhancement of the vegetation. Alternatively, bacteria and platelets may co-localize at the site of valvular damage, resulting in activation leading to thrombus development. *S. aureus* is an important cause of IE in patients with no known valvular damage. In this scenario, the formation of bacteria-platelet microaggregates in the bloodstream and their subsequent deposition on the valve surface may be important in the initiation of IE. A role for platelets in the progression of IE is suggested by studies using the rabbit model of *S. aureus* endocarditis. Rabbits treated with aspirin, a cyclo-oxygenase inhibitor that prevents platelet aggregation, had significantly lower bacterial titres and smaller vegetations than in control untreated rabbits (Nicolau *et al.*, 1993; Kupferwasser *et al.*, 1999). Strains of *Streptococcus sanguis* that could cause platelet aggregation *in vitro* gave rise to endocarditis with larger vegetations, a more severe clinical course and greater mortality than a strain of *S. sanguis* that could not aggregate platelets (Herzberg *et al.*, 1992).

1.5.1 Biology and function of platelets

Platelets are the most numerous cells in the bloodstream, with normal platelet counts ranging from 1.5×10^8 to 4.5×10^8 per ml (Marcus, 1999). The primary role of platelets is in hemostasis, although a number of other functions, including inflammation and anti-microbial defense, are attributed to them (Ni and Freedman, 2003). Damage to the vascular endothelium triggers the response of platelets, which eventually results in the formation of a hemostatic plug (thrombus) containing aggregated platelets and fibrin to arrest bleeding. Although platelets are anucleate and can perform only limited translation of stable megakaryocyte mRNA, they are a highly complex cell-type, and contain a number of surface receptors that allow response to environmental factors (Clemetson, 2002). Deficiencies in either the platelet GPIb/V/IX complex (Bernard-Soulier Syndrome) or the platelet GPIIb/IIIa integrin (Glanzmann thrombasthenia) lead to bleeding disorders as a result of impaired platelet function (Clemetson, 2002). Granules within platelets contain a number of important mediators, such as ADP, serotonin, von Willebrand factor (vWF), fibrinogen and calcium that help regulate thrombus formation (Marcus, 1999).

A number of different receptor types are found on the platelet membrane. Among these are several members of the integrin family (αIIbβ3, αVβ3, α2β1, α5β1, α6β1), the leucine-rich repeat GPIb/V/IX complex, members of the seven transmembrane receptor family

(e.g. thrombin receptors PAR-1 and PAR-4 and ADP receptors $P2Y_1$ and $P2Y_{12}$) and members of the immunoglobulin superfamily (e.g. GPVI and FcγRIIa) (Clemetson, 2002). The principle adhesive surface for platelets is the extracellular matrix (ECM) which becomes exposed in injured blood vessels (Shattil and Newman, 2004). Receptors recognizing exposed subendothelial components and extracellular matrix (ECM) proteins mediate platelet adhesion to sites of tissue damage and platelet spreading to cover the exposed tissue (Gibbins, 2004). Spreading is accompanied by secretion of several prothrombotic factors such as ADP and serotonin which serve to activate approaching platelets. Platelet activation results in inside-out signaling that upregulates integrin affinity. Binding of fibrinogen to the activated αIIbβ3 integrin (also known as the glycoprotein (GP) IIb/IIIa complex) results in the cross-linking of adjacent platelets into aggregates leading to thrombus assembly (Gibbins, 2004).

Thrombus formation at sites of vascular damage is a complex process, with many platelet receptors acting synergistically to regulate clot formation. Arguably the most important receptors involved in regulating thrombus formation are the GPIIb/IIIa integrin and the GPIb/V/IX complex. Thrombus formation is initiated by platelet adhesion to exposed subendothelium. The nature of the adhesive ligand involved is determined by the prevailing rheological conditions. Under low shear conditions, such as those found in larger arteries and veins, platelet adhesion is mediated through collagens (platelet receptors α2β1 and GPVI), fibronectin (through α5β1) and laminin (through α6β1) (Clemetson, 2002; Jackson *et al.*, 2003). However, under high shear conditions found in small arteries and arterioles, platelet adhesion is critically dependent on binding of GPIb/V/IX to subendothelial bound von Willebrand factor (Jackson *et al.*, 2003).

Following firm platelet adhesion, subsequent platelet-platelet cohesion (platelet aggregation) occurs on the layer of adherent platelets to form a hemostatic plug. Central to this process is GPIIb/IIIa. Under conditions of high shear, von Willebrand factor is the major ligand promoting platelet aggregate formation by cross-linking activated platelets through GPIIb/IIIa, with fibrinogen and/or fibrin playing a stabilizing role. At low shear rates, binding of the bivalent glycoprotein fibrinogen to GPIIb/IIIa is the dominant factor mediating thrombus formation by bridging GPIIb/IIIa receptors on adjacent platelets (Jackson *et al.*, 2003). Secretion of soluble agonists (ADP, thromboxane (TX) A_2) from platelet granules and thrombin generation on the platelet surface further the rate of platelet activation and aggregation (Ni and Freedman, 2003). ADP is thought to be involved in the progressive

recruitment of platelets within developing aggregates, while thrombin may be important in both promoting initial thrombus growth and in stabilizing formed thrombi through fibrin formation (Jackson *et al.*, 2003). A schematic representation of platelet aggregate formation on a von Willebrand factor substratum is shown in Figure 1.9.

Platelet aggregation is critically dependent on GPIIb/IIIa. This receptor, like all integrins, is a heterodimeric type I transmembrane receptor consisting of an α and a β subunit. Each subunit contains a relatively large extracellular domain, a single-pass transmembrane domain, and a short (20 – 60 amino acid) cytoplasmic tail. GPIIb/IIIa exists in both resting (low-affinity) and activated (high-affinity) conformations, the resting state predominanting in unstimulated platelets and the activated state in stimulated platelets. Platelet activation by an agonist generates intracellular signals that are transmitted to the β3 cytoplasmic tail (inside-out signaling). This results in conformational changes in the extracellular domain of GPIIb/IIIa converting it to its activated form (the crystal structure of which is known), increasing its affinity for adhesive ligands such as fibrinogen and von Willebrand factor (Xiao *et al.*, 2004). Binding of such ligands to the extracellular face of GPIIb/IIIa promotes integrin clustering and stimulation of protein tyrosine kinase activity (outside-in signaling). This drives further integrin activation by transmitting signals to the cytoplasmic tails of GPIIb/IIIa stimulating conformational change. In this way, GPIIb/IIIa ligand binding becomes progressively irreversible and results in irreversible platelet aggregation (Ni and Freedman, 2003; Shattil and Newman, 2004; Xiao *et al.*, 2004). This bidirectional type of signaling is depicted in Figure. 1.10.

1.5.2 Bacterial interactions with platelets

A number of bacterial species have been shown to interact with platelets *in vitro*, including members of the genera *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Helicobacter*, *Borrelia*, *Chlamydia* and *Listeria* to name but a few. Staphylococci and streptococci, which are major causes of IE, are probably the most intensively studied bacteria with regard to their ability to interact with platelets. *In vitro*, some bacteria mediate platelet aggregation. This is thought to be the result of a multistep process, with initial bacterial adhesion to resting platelets triggering intracellular signaling resulting in platelet activation. Conformation modulation of GPIIb/IIIa increases its affinity for fibrinogen, and fibrinogen binding to activated GPIIb/IIIa receptors resulting in cross-linking of adjacent platelets into platelet

aggregates (depicted schematically in Figure 1.11). Bacteria-mediated platelet aggregation *in vitro* is preceded by a lag phase, the length of which is variable depending on (i) the bacterium-platelet ratio, (ii) the bacterial species being tested and (iii) the surface components expressed by particular strains of a single species. A thorough understanding of the bacterial factors mediating platelet binding and aggregation along with the platelet receptors involved in recognizing bacteria is required if novel therapeutics are to be developed to inhibit this process. The major findings in this area of research will be reviewed here, with special attention paid to the interaction of staphylococci and streptococci with human platelets.

1.5.2.1 *S. aureus*-platelet interactions

Platelet binding by bacteria is a prerequisite for subsequent platelet activation and aggregation stimulated by pathogenic organisms (Clawson, 1973). The ability of *S. aureus* cells to bind directly to the platelet surface in the absence of plasma co-factors has been demonstrated in a number of studies (Yeamen *et al.*, 1992a; Herrmann *et al.*, 1993; Sullam *et al.*, 1996; Nguyen *et al.*, 2000; Siboo *et al.*, 2001, 2005). Herrmann and co-workers noted that binding of *S. aureus* to platelets was extensively promoted in the presence of plasma, which would be more representative of *in vivo* conditions. Adhesion was sensitive to both an anti-fibrinogen antibody and an anti-GPIIb/IIIa monoclonal antibody (Herrmann *et al.*, 1993), suggesting that fibrinogen acted as a bridging molecule between the *S. aureus* receptors (such as ClfA, ClfB, FnBPA or FnBPB) and GPIIb/IIIa on the platelet. It was recently reported that soluble fibrin is a major mediator of *S. aureus* adhesion to activated platelets in solution (Niemann *et al.*, 2004), likely as a result of a similar bridging mechanism.

It has long been known that *S. aureus* can cause platelet aggregation in a manner dependent on surface-expressed proteins (Hawiger *et al.*, 1972; 1979; Kessler *et al.*, 1991). ClfA has been shown to be an important factor in promoting platelet binding and aggregation *in vitro* (Siboo *et al.*, 2001; O' Brien *et al.*, 2002a). It is also a virulence factor in experimental endocarditis studies (Moreillon *et al.*, 1995; Sullam *et al.*, 1996; Stutzmann-Meier *et al.*, 2001; Que *et al.*, 2001, 2005). ClfA is the major surface component responsible for platelet aggregation by *S. aureus* Newman cells grown to stationary phase (O'Brien *et al.*, 2002a), and exerts a potent pro-aggregatory effect when expressed on the surface of the non-aggregating surrogate host *Lactococcus lactis* (O'Brien *et al.*, 2002a). It was found that the molecular interaction between *S. aureus* and platelets under physiological hydrodynamic shear

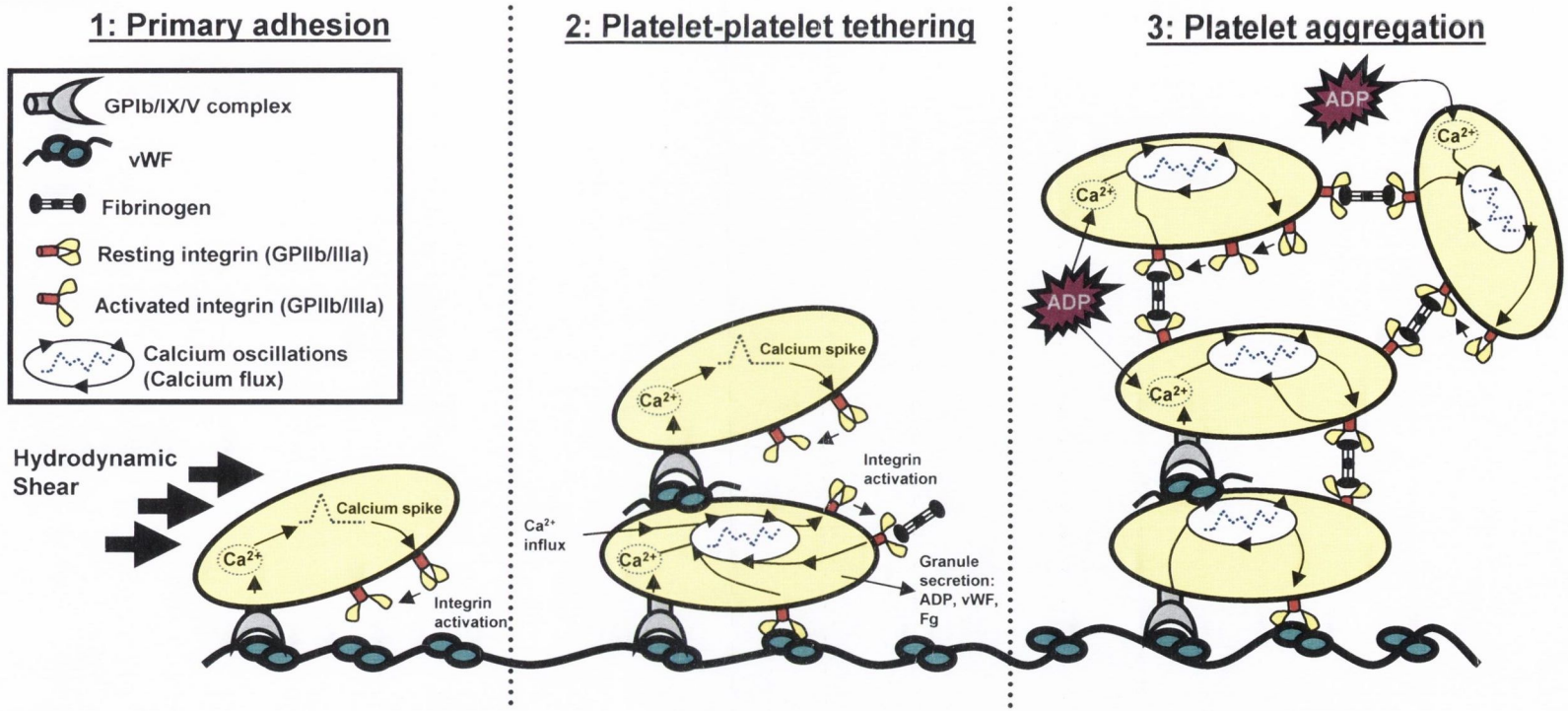


Figure 1.9 Platelet thrombus formation on a von Willebrand factor surface

1: Primary adhesion. The GPIb complex mediates platelet binding to immobilized von Willebrand factor (vWF) exposed in injured blood vessels under high shear forces in flowing blood. This binding is accompanied by a transient calcium mobilization event leading to low-level integrin activation.

2: Platelet-platelet tethering. Integrin activation in adherent platelets leads to stabilizing adhesion by activated GPIIb/IIIa binding immobilized vWF. Sustained calcium flux occurs, leading to integrin activation and binding of adhesive molecules (vWF and fibrinogen (Fg)) to the platelet surface, forming a highly efficient surface for the subsequent platelet adhesion (tethering).

3: Platelet aggregation. Platelets bound to the primary adherent platelets become activated. Secreted granule components such as ADP trigger platelet activation in the local platelet population. Binding of fibrinogen (and vWF) to activated GPIIb/IIIa receptors on platelets crosslinks adjacent platelets into aggregates. This results in the formation of a thrombus at the site of injury to arrest bleeding.

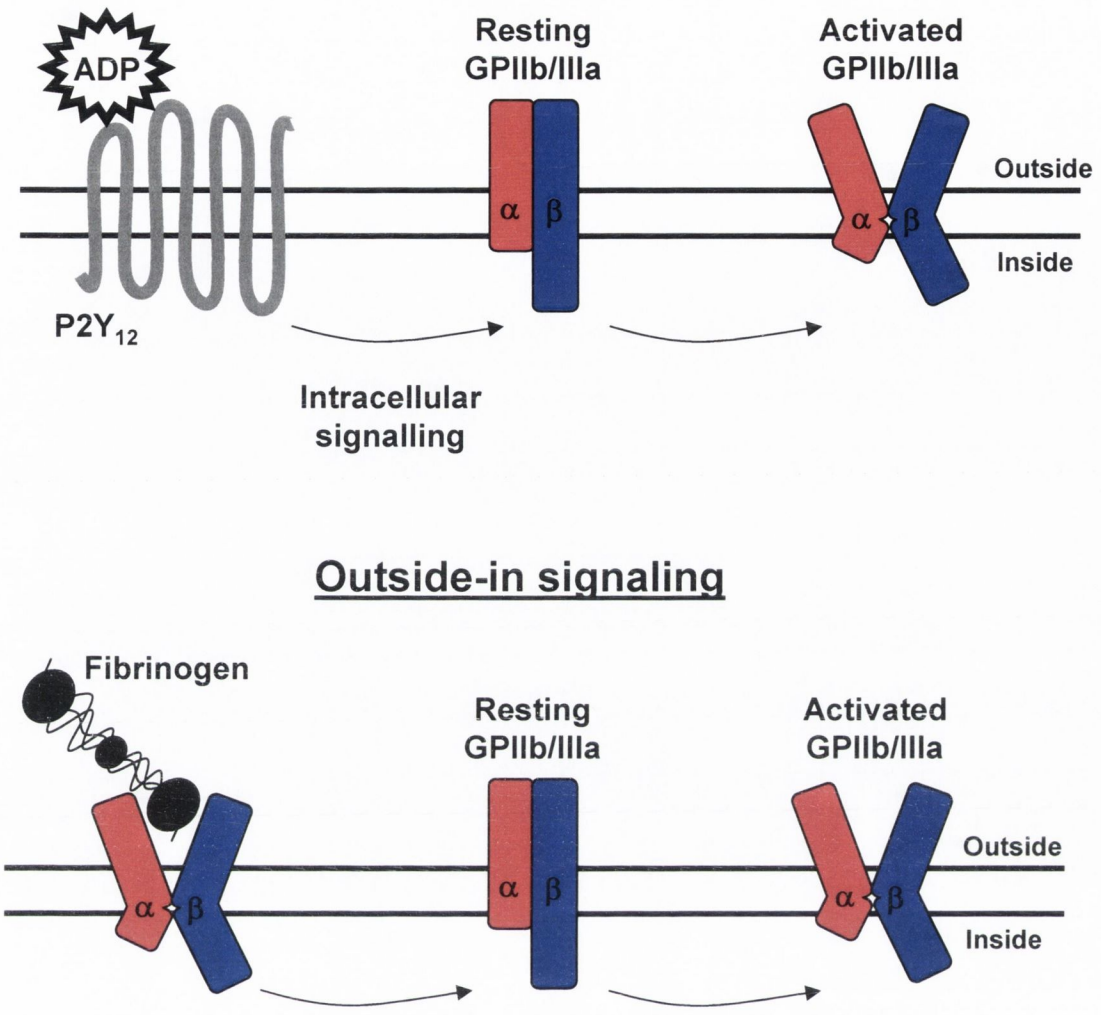


Figure 1.10 Bidirectional signalling events leading to integrin GPIIb/IIIa activation.

Inside-out signalling. The binding of a platelet agonist (ADP) to its receptor ($P2Y_{12}$) on the platelet surface generates intracellular signals resulting in platelet activation. These signals are relayed to the cytoplasmic tails of GPIIb/IIIa resulting in a conformational change increasing its affinity for fibrinogen.

Outside-in signalling. Fibrinogen-binding to activated GPIIb/IIIa generates signals driving further integrin activation. GPIIb/IIIa ligand binding becomes progressively more irreversible and results in irreversible platelet aggregation

Adapted from Shattil and Newman, 2004.

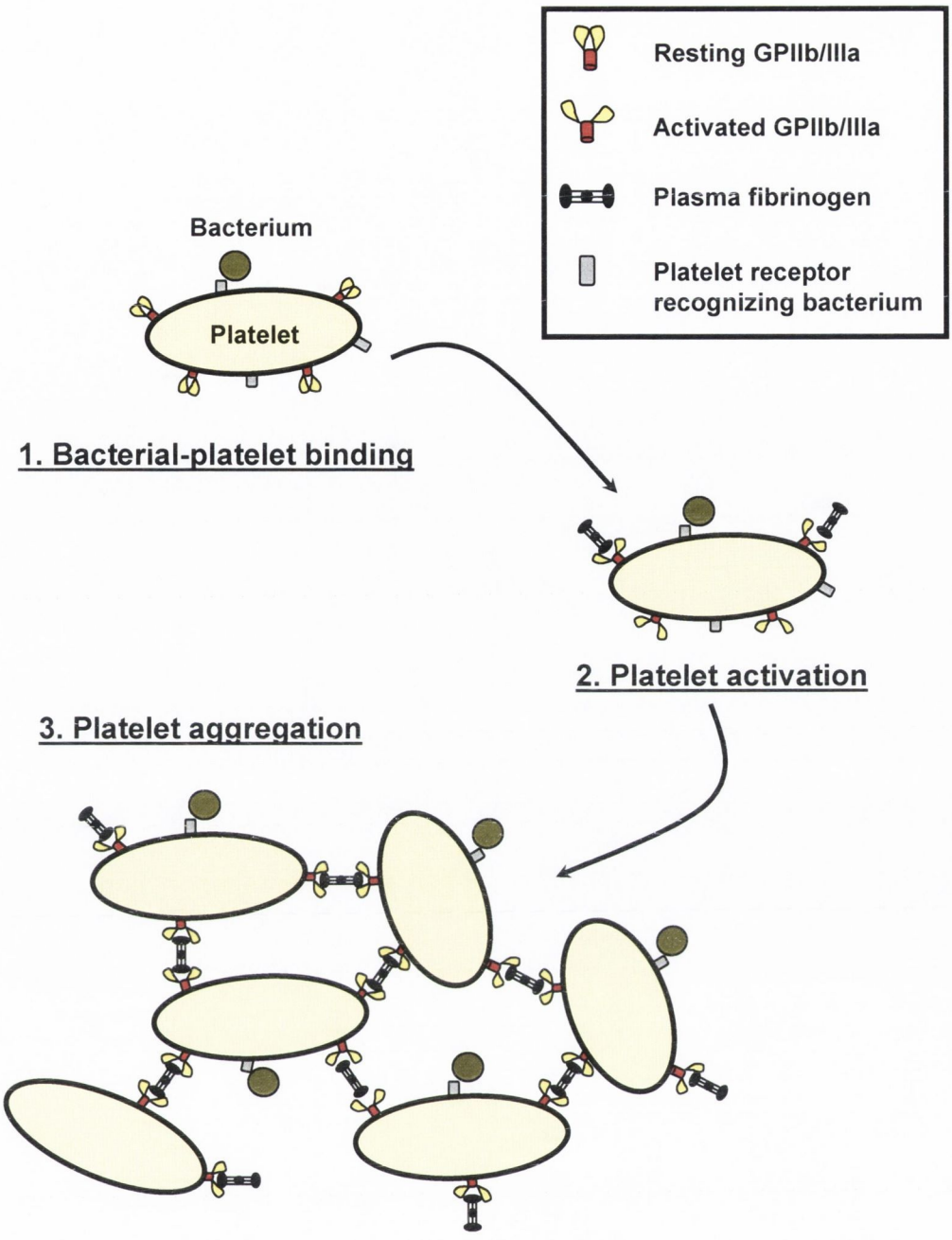


Figure 1.11 Platelet activation and aggregation mediated by bacteria.

Bacterial-platelet binding is mediated by bacterial surface components recognizing specific platelet receptors. These interactions trigger intracellular signals causing platelet activation. GPIIb/IIIa undergoes a conformational change increasing its affinity for soluble fibrinogen. Fibrinogen-dependent cross-linking of activated GPIIb/IIIa receptors on adjacent platelets results in the formation of platelet aggregates.

forces leading to thrombus formation required ClfA and platelet GPIIb/IIIa (Pawar *et al.*, 2004). Another study examining real-time thrombus formation under shear conditions demonstrated thrombus formation upon exposure of whole blood to immobilized *S. aureus* Newman cells, likely mediated by ClfA (Sjöbring *et al.*, 2002). In agreement with this, it was demonstrated that immobilized recombinant ClfA protein supported thrombus formation to the same degree as whole bacteria (Sjöbring *et al.*, 2002). Thrombus formation was inhibited either by GPIIb/IIIa antagonists or by a function-blocking antibody to the platelet Fc receptor FcγRIIa (Sjöbring *et al.*, 2002). ClfA has been shown to interact directly with a platelet membrane protein of 118 kDa which did not appear to be GPIIb/IIIa (Siboo *et al.*, 2001). The identity of this protein (designated p118) is not known and the role that this interaction plays in ClfA-mediated platelet aggregation is unclear. It is widely believed that the ability of ClfA to bind plasma fibrinogen is important in stimulating platelet aggregation. However the molecular mechanisms by which ClfA interacts with platelets leading to their activation and aggregation remain ill-defined and unclear.

O'Brien and co-workers demonstrated roles for ClfB and SdrE in promoting platelet aggregation when expressed in *S. aureus* or *L. lactis* (O'Brien *et al.*, 2002a). It was recently demonstrated that *S. carnosus* cells expressing the fibronectin-binding protein FnBPA could stimulate rapid aggregation of platelets suspended in plasma (Heilmann *et al.*, 2004). *S. aureus* appears to express a number of surface components that interact with platelets leading to their activation and aggregation. Such redundancy in function suggests that this may play an important role in pathogenesis.

A direct binding mechanism involving staphylococcal protein A (SpA) was recently characterized. SpA was shown to bind to the platelet C1q complement receptor gC1qR/p33 (Nguyen *et al.*, 2000). gC1qR/p33 expression on the platelet surface can only be detected following platelet activation by agonists such as epinephrine or ADP (Peerschke and Ghebrehiwet, 2001; Peerschke *et al.*, 2003). It is therefore unlikely that this interaction plays a direct role in platelet activation caused by *S. aureus*. The expression of SpA by *S. aureus* Newman appeared to play a role in platelet aggregation in the absence of ClfA, however SpA expression by *L. lactis* was not sufficient to cause aggregation (O'Brien *et al.*, 2002a). SpA-mediated binding of *S. aureus* to gC1qR/p33 on activated platelets may play a role in the colonization of developing sterile thrombi *in vivo*. The ability of SpA to bind to von Willebrand factor (the major ligand for the platelet GPIb complex) appears to play a role in the

interaction between *S. aureus* and platelets leading to their aggregation under high shear conditions, through the formation of a von Willebrand factor bridge linking SpA with GPIb (Hartlieb *et al.*, 2000; Pawar *et al.*, 2004).

The genomes of the sequenced *S. aureus* strains contain a homolog of the *Streptococcus gordonii* platelet binding-proteins GspB and Hsa (see section 1.5.2.2) known as SasA (Roche *et al.*, 2003; Figure 1.12). It was recently demonstrated that expression of this protein (also called SraP (Serine-rich adhesin for platelets)) by *S. aureus* contributed to direct platelet binding and virulence in experimental endocarditis (Siboo *et al.*, 2005). The gene encoding SraP is present in all isolates examined (Roche *et al.*, 2003) and was expressed by the majority of clinical isolates examined (Siboo *et al.*, 2005). It was not determined if SraP-mediated binding of *S. aureus* to platelets plays a role in platelet aggregation (Siboo *et al.*, 2005).

The platelet receptors involved in *S. aureus*-mediated platelet activation have not been satisfactorily identified. Some conflicting reports have been published on the role of GPIIb/IIIa in the response of platelets to *S. aureus*. Bayer and co-workers found that the aggregation of rabbit platelets by *S. aureus* was insensitive to GPIIb/IIIa antagonists (Bayer *et al.*, 1995). In contrast, numerous other studies have shown that aggregation of platelets by *S. aureus* is crucially dependent on GPIIb/IIIa (O'Brien *et al.*, 2002a; Sjöbring *et al.*, 2002; Pawar *et al.*, 2004; Liu *et al.*, 2005). The failure of GPIIb/IIIa antagonists to inhibit *S. aureus*-induced rabbit platelet aggregation (Bayer *et al.*, 1995) may result from the fact that these agents are highly species-specific, with most having very little activity against platelets from rabbits or rodents (Cox *et al.*, 1992). The role of GPIIb/IIIa in platelet aggregation by *S. aureus* is not entirely clear. It is possible that platelet activation occurs independently of GPIIb/IIIa. Activation would result in the expression of the high-affinity form of GPIIb/IIIa, resulting in fibrinogen-binding and GPIIb/IIIa-dependent aggregation. Alternatively, GPIIb/IIIa could be involved in recognizing *S. aureus*-bound fibrinogen, and such an interaction could result in activation and aggregation (which would also be GPIIb/IIIa-dependent). Some recent evidence suggests that the latter may be true (Liu *et al.*, 2005). A snake venom-derived GPIIb/IIIa antagonist, crotavirin, as well as two clinically available GPIIb/IIIa inhibitors (abciximab and tirofiban), inhibited platelet aggregation by several clinical *S. aureus* isolates. Platelets treated with these agents also failed to undergo shape change characteristic of activation in response to *S. aureus*, or lacked synthesis of the

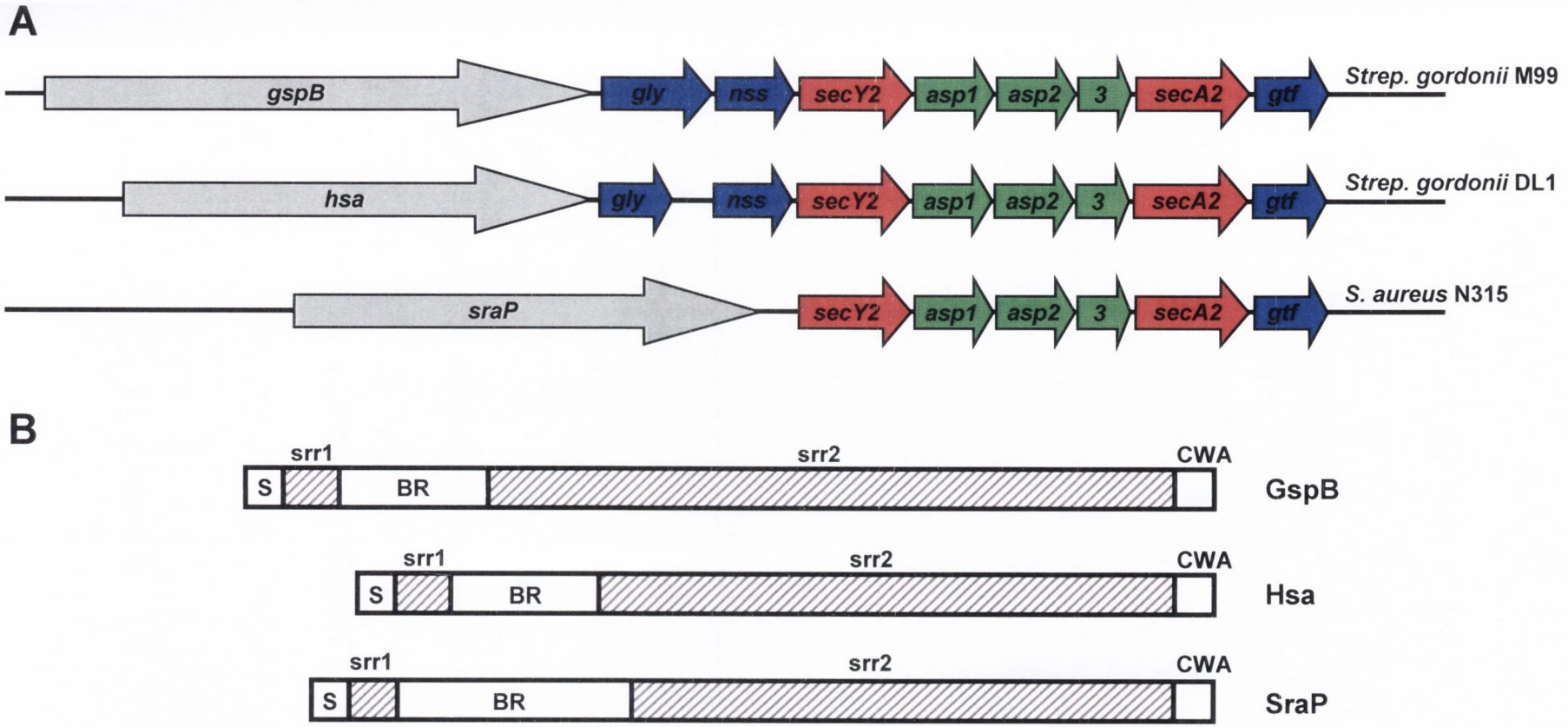


Figure 1.12 GspB, Hsa and SraP platelet binding proteins from *Strep. gordonii* and *S. aureus*

A. Organisation of the genes involved in production of serine-rich glycoprotein adhesins from *Strep. gordonii* and *S. aureus*. Genes are colour coded according to their function. Grey: structural genes. Blue: genes involved in glycosylation. Red: SecA and SecY homologs. Green: export associated genes.

B. Domain organisation. A unusually long signal sequence (S) of approximately 90 amino acids is present at the N-terminus. This may be required for export through the accessory SecA2-SecY2 system. Two serine-rich-repeat repeat domains (srr) are separated by a basic non-repeat domain (BR). Cell-wall anchoring domains (CWA) at the C-terminus are required for anchoring of the secreted proteins to the cell wall.

Adapted from Bensing *et al.*, 2004, Takamatsu *et al.*, 2004.

activation markers PDGF/ TXB_2 (Liu *et al.*, 2005). The likely role of GPIIb/IIIa in *S. aureus*-induced activation is mediating a fibrinogen bridge between *S. aureus* and the platelet (Liu *et al.*, 2005) as previously suggested (Herrmann *et al.*, 1993).

S. aureus α-toxin is secreted as a momomeric protein that, upon binding to target membranes, oligomerises into a mushroom-shaped heptameric pore resulting in cell lysis (Montoya and Gouaux, 2003). Rabbit erythrocytes are highly sensitive to α-toxin, whereas human erythrocytes are relatively resistant to α-toxin attack. Human platelets, however, appear to be much more sensitive to lysis by α-toxin than human erythrocytes (Siegel and Cohen, 1964; Bernheimer and Schwarz, 1965). Rapid aggregation of human platelets was observed at sub-hemolytic concentrations of toxin. Aggregation was preceded by platelet shape change, granule and ATP secretion, and assembly of the prothrombinase complex on platelet membranes (Bhakdi *et al.*, 1988; Arvand *et al.*, 1990). Pore formation by mature α-toxin likely results in of Ca^{2+} influx across the platelet membrane causing activation, and prothrombinase formation on the platelet surface will generate thrombin, a potent procoagulant molecule.

At least one *S. aureus* factor, a secreted 15.8 kDa fibrinogen-binding protein named Efb (extracellular fibrinogen binding protein), has been shown to interact with platelets in a manner that prevents platelet aggregation (Shannon and Flock, 2004). The fibrinogen binding domain of Efb shares homology with the fibrinogen binding C-terminal repeats of staphylococcal coagulase (Boden and Flock, 1994). Efb binds to ADP-stimulated platelets by recognizing the α-chain of platelet-bound fibrinogen (Palma *et al.*, 2001). It also appears that Efb binds directly to activated platelets in a fibrinogen-independent manner. However the nature of the platelet receptor involved is not known (Shannon and Flock, 2004). The interaction(s) of Efb with activated platelets resulted in inhibition of platelet aggregation (Palma *et al.*, 2001; Shannon and Flock, 2004). In a rat wound-infection model, an *efb* mutant strain was less pathogenic than the Efb producing parental strain, suggesting Efb contributes to a delay in wound healing, possibly through its inhibition of platelet function (Palma *et al.*, 1996). A murine acute thrombosis model, where intravenous administration of collagen and epinephrine results in rapid death due to massive systemic coagulation, was used to assess the anti-thrombogenic potential of Efb (Shannon *et al.*, 2005). Efb administration completely

protected mice from collagen/epinephrine challenge. Mice treated with Efb also displayed significantly longer bleeding times that control mice (Shannon *et al.*, 2005), suggesting the potential development of Efb as a novel anti-thrombotic agent.

The fact that *S. aureus* has numerous mechanisms for binding to and activating platelets suggests that the interaction with platelets is an important aspect of *S. aureus* pathogenesis in the establishment of endocardial infections. Direct binding to platelets in sterile vegetations may be mediated by interactions involving ClfA (Sullam *et al.*, 1996; Siboo *et al.*, 2001), protein A (Nguyen *et al.*, 2000) and SraP (Siboo *et al.*, 2005). Binding may also be mediated by soluble plasma components, such as fibrinogen/fibrin, fibronectin or vWF bridging ClfA/ClfB/ FnBPA/FnBPB/protein A to the platelet surface (Herrmann *et al.*, 1993; Heilmann *et al.*, 2004; Niemann *et al.*, 2004; Pawar *et al.*, 2004; Liu *et al.*, 2005). The interaction of some *S. aureus* factors (ClfA, ClfB, SdrE, FnBPA) and platelets results in platelet activation leading to aggregate formation, although the precise mechanisms by which individual surface components interact with platelets leading to activation and aggregation has not been satisfactorily elucidated in any study thus far. One reason for this is the fact that multiple *S. aureus* factors are involved in this process, resulting in difficulties interpreting results using isogenic mutants. This could be circumvented by the use of a heterologous expression system such as *L. lactis*. The platelet receptors involved in mediating platelet activation by *S. aureus* have not been fully characterized, but strong evidence exists that GPIIb/IIIa plays an important role (O'Brien *et al.*, 2002a; Sjobring *et al.*, 2002; Pawar *et al.*, 2004; Liu *et al.*, 2005). Other receptors implicated in aggregation by *S. aureus* include GPIb recognizing protein A-bound vWF at high shear (Pawar *et al.*, 2004) and the platelet FcγRIIa receptor (Sjobring *et al.*, 2002). It is clear that further work is required to elucidate the pathways by which factors such as ClfA and FnBPA interact with platelets leading to aggregation and thrombus formation.

1.5.2.2 *Streptococcus*-platelet interactions

In contrast to *S. aureus*, much research has focused on the ability of *Streptococcus* species to interact with platelets. Streptococci, especially members of the viridans group of streptococci, are the second leading cause of IE (Moreillon and Que, 2004). The viridans streptococci are a large, heterogenous group that are commonly found as commensals in the human oral cavity. Streptococcal bacteremias leading to IE can occur as a result of

periodontal disease and poor dental hygiene practices. Species of streptococci associated with IE are the viridans group (*Streptococcus sanguis*, *Streptococcus oralis*, *Streptococcus gordonii*, *Streptococcus mutans*, *Streptococcus salivarius* and the *Streptococcus milleri* group), *Streptococcus pyogenes* (group A streptococcus) and *Streptococcus agalactiae* (group B streptococcus).

Strep. gordonii is an important cause of IE (Douglas *et al.*, 1993). *Strep. gordonii* strain M99 expresses a large, serine-rich glycoprotein (GspB) on its cell surface that mediates direct bacterial binding to human platelets (Bensing and Sullam, 2002). The chromosomal locus encoding GspB contains a dedicated transport system required for GspB export (SecA2 and Sec Y2; Bensing and Sullam, 2002; Figure 1.12) and a number of other genes involved in export (*asp1-5*; Takamatsu *et al.*, 2004a, 2005a) and glycosylation (*gly*, *nss*, *gtfA*, *gtfB*; Takamatsu *et al.*, 2004a, 2004b) of GspB. It is heavily glycosylated, containing approximately 10 % (wt/wt) carbohydrate, mostly made up of glucose and *N*-acetylglucosamine, with trace amounts of galactose and *N*-acetylgalactosamine (Bensing *et al.*, 2004a). A GspB homolog (Hsa) was identified in *Strep. gordonii* strain DL1 (Takahashi *et al.*, 2002). Hsa was predicted to be a large cell-wall anchored protein with a similar domain organization to GspB (see Figure 1.12), and was shown to bind sialic acid-containing molecules (Takahashi *et al.*, 2002). Hsa expression by *Strep. gordonii* DL1 was linked to the ability of this strain to aggregate platelets. Hsa-mediated platelet binding was eliminated by treatment of platelets with neuraminidase, suggesting that Hsa recognizes sialic acid-containing receptors on the platelet surface (Takahashi *et al.*, 2004). The platelet receptor recognizing both GspB and Hsa was recently identified as GPIb (Bensing *et al.*, 2004b). *Strep. gordonii* strains expressing either GspB or Hsa were found to bind sialic-acid moieties of platelet GPIbα (Bensing *et al.*, 2004b). The binding domains of GspB and Hsa were recently localized to the basic regions located between the two serine-rich-repeat domains (Takamatsu *et al.*, 2005b; Figure 1.12).

Strep. sanguis is a predominant cause of IE due to the viridans streptococci (Douglas *et al.*, 1993). Certain strains of *Strep. sanguis* are capable interacting directly with platelets, while other strains do not bind (Kerrigan *et al.*, 2002). Direct platelet binding by *Strep. sanguis* strains 133-79 and SK36 was dependent on the interaction between a serine-rich surface glycoprotein SrpA expressed by *Strep. sanguis* and the GPIb complex on platelets (Kerrigan *et al.*, 2002; Plummer *et al.*, 2005). Platelets adhered to bacteria expressing SrpA

under both static and shear conditions (Plummer *et al.*, 2005). SrpA shows similar structural organization to the GPIb-binding proteins GspB and Hsa of *Strep. gordonii*, and mediates bacterial adhesion to purified glycocalacin (the extracellular GPIbα domain; Kerrigan *et al.*, 2002; Plummer *et al.*, 2005). Adhesion could be inhibited by the addition of exogenous sialic acid (Plummer *et al.*, 2005), indicating that SrpA interacts with sialic-acid moieties on GPIb, similar to GspB and Hsa of *Strep. gordonii*.

The expression of GspB/Hsa/SrpA proteins by *Streptococcus* species may be an important determinant in establishing endovascular infection. Homologs of GspB and genes associated with its export and glycosylation are found in genomes of other streptococci (*Strep. pneumoniae* and *Strep. agalactiae*) and all sequenced strains of *S. aureus* (Takamatsu *et al.*, 2005). These large surface-expressed glycoproteins may be conserved determinants in the interaction of Gram-positive species with platelets.

Much *in vitro* analysis on bacteria-mediated platelet aggregation has focused on *Strep. sanguis*. Strains of *Strep. sanguis* capable of adhering to platelets displayed maximal aggregation responses in a range of human donors, while adhesion-defective strains caused prolonged or no aggregation of human platelets (Herzberg *et al.*, 1983). Studies on platelets from donors with Glanzmann thrombasthenia demonstrated that GPIIb/IIIa is important in the platelet response to *Strep. sanguis* (Soberay *et al.*, 1987; Ford *et al.*, 1993; Kerrigan *et al.*, 2002). When tested in a rabbit endocarditis model, a strain of *Strep. sanguis* capable of aggregating platelets caused endocarditis with larger vegetations, a more severe clinical course and greater mortality than a non-aggregating *Strep. sanguis* strain (Herzberg *et al.*, 1992).

Plasma co-factors were shown to be required for platelet aggregation by strains of *Strep. sanguis* and *Strep. salivarius* (Sullam *et al.*, 1988). Crucial roles for bacterium specific IgG and the platelet Fc receptor FcγRIIa were identified (Sullam *et al.*, 1988). This suggested that streptococcal-bound IgG interacting with FcγRIIa was critical for platelet aggregation caused by these strains. It has been demonstrated in numerous other studies that many streptococcal species utilize specific antibodies against the bacterium present in plasma and platelet FcγRIIa in their stimulation of resting platelets into aggregates, including *Strep. sanguis* (Ford *et al.*, 1997; Kerrigan *et al.*, 2002), *Strep. pyogenes* (Sjöbring *et al.*, 2002), *Strep. mutans* (Chia *et al.*, 2004), *Strep. pneumoniae* (Zimmerman and Spiegelberg, 1975), and *Strep. agalactiae* (Pietrocola *et al.*, 2004). It was recently demonstrated that signaling

mediated through FcγRIIa occurred during *Strep. sanguis*-induced platelet activation (Pampolina and McNicol, 2005).

Platelet aggregation studies performed using a panel of *Strep. sanguis* isolates demonstrated that 3 distinct platelet-interactive phenotypes existed (Kerrigan *et al.*, 2002). *Strep. sanguis* strains capable of binding platelet GPIb (SrpA positive) induced aggregation in a FcγRIIa-dependent manner with lag times of approximately 5 – 7 minutes (Herzberg *et al.*, 1983; Kerrigan *et al.*, 2002). Some *Strep. sanguis* strains that were defective in platelet binding (SrpA negative) induced platelet aggregation with relatively long lag times (approximately 16 minutes; Kerrigan *et al.*, 2002). This involved multiple plasma components such as IgG and complement proteins binding to the bacterial cell in order to interact with the platelet to induce aggregation, which was FcγRIIa-dependent (Ford *et al*, 1996, 1997). Isogenic *srpA* knockout mutants displayed a significant increase in the lag time before platelet aggregation occurred (Plummer *et al.*, 2005). This demonstrates that SrpA-mediated platelet binding by *Strep. sanguis* is required for efficient platelet aggregation. It appears that multiple stimuli are required for *Strep. sanguis*-induced platelet activation. It seems that SrpA-mediated binding of bacteria to GPIb and recognition of streptococcal-bound IgG by platelet FcγRIIa together generate signals required for rapid activation, due to the high affinity of SrpA for GPIb. In the absence of direct binding of *Strep. sanguis* to platelets, assembly of complement and IgG on the bacterial cell is required for interactions with FcγRIIa and a complement receptor on platelets (Ford *et al.*, 1996, 1997). The long lag time associated with this mechanism is likely the result of the time taken for complement assembly on the bacterial cell surface (Ford *et al.*, 1996). The platelet-aggregation-associated protein (PAAP) of *Strep. sanguis* is also involved in inducing platelet aggregation, but does not contribute to the initial binding of *Strep. sanguis* to platelets (Herzberg *et al.*, 2005). PAAP is a rhamnose-rich glycoprotein of 115 kDa which contains a collagen-like platelet-interactive domain comprising residues Pro-Gly-Glu-Gln-Gly-Pro-Lys (Erickson and Herzberg, 1993). Gong and colleagues reported that PAAP interacts with platelet membrane proteins of 175 kDa and 230 kDa to mediate platelet aggregation (Gong *et al.*, 1995). Some *Strep. sanguis* isolates did not adhere to or aggregate platelets at all (Ford *et al.*, 1996; Kerrigan *et al.*, 2002). It is unclear at the moment why some *Strep. sanguis* strains consistently fail to aggregate platelets.

The expression of fibrinogen-binding surface proteins on the surface of certain *Streptococcus* species is an important factor in triggering platelet aggregation. *S. pyogenes* (group A streptococcus) has been reported to stimulate rapid aggregation of platelets in plasma (Kurpiewski *et al.*, 1983). The expression of cell-surface fibrinogen-binding protein M proteins by *Strep. pyogenes* is required to induce platelet thrombus formation under physiological shear conditions (Sjobring *et al.*, 2002). This was dependent on the fibrinogen-binding activity of M protein and the presence of IgG recognizing M proteins in plasma. FcγRIIa and GPIIb/IIIa were shown to be crucial to the development of mature thrombi. A model was proposed based on these findings whereby *Strep. pyogenes*-bound fibrinogen aided bacterial adhesion to resting platelets through GPIIb/IIIa, and streptococcal-bound IgG interacting with FcγRIIa induced activation of platelets resulting in thrombus formation (Sjöbring *et al.*, 2002).

Strep. agalactiae (group B streptococcus) is associated with endocarditis in immunocompromised adults. The ability of *S. agalactiae* to stimulate platelet aggregation is dependent on the expression of a fibrinogen-binding surface protein, FbsA (Pietrocola *et al.*, 2004). Isogenic *fbsA* mutants failed to stimulate rapid aggregation associated with FbsA expression. It was also shown that expression of FbsA in *L. lactis* conferred the ability to stimulate rapid aggregation. Aggregation was dependent on GPIIb/IIIa, FcγRIIa, and antibody to FbsA (Pietrocola *et al.*, 2004), indicating a similar mechanism as that proposed for *Strep. pyogenes* (Sjobring *et al.*, 2002).

A recurrent theme of the streptococcal-platelet interaction is the crucial importance of the platelet FcγRIIa receptor and streptococcal-specific IgG in triggering platelet activation and aggregation. However, differences are also seen in the binding of different streptococcus species to platelets, and even differences are observed between strains of a single species (e.g. *Strep. sanguis*), which apparently reflects differences in their profile of surface expressed proteins (Plummer *et al.*, 2005). The recent work demonstrating the identities of the GPIb-binding proteins of *Strep. sanguis* and *Strep. gordonii* may be useful in development of novel therapeutics to prevent binding of these organisms to platelets in the treatment of endocarditis. The genome sequence of *S. sanguis* is currently under construction which, when available, will allow identification of other potential surface components that mediate platelet binding and activation by streptococci.

1.5.2.3 Interactions of other bacterial species with platelets

Helicobacter pylori infection is associated with the development of peptic ulcers, gastric carcinoma and atrophic gastritis. Cardiovascular conditions such as artherosclerotic vascular disease, myocardial infarction and thrombocytopenia are thought to be associated with *H. pylori* infection. Remission of thrombocytopenia in patients has been observed upon eradication of *H. pylori* infection (Handin, 2003). Some strains of *H. pylori* have been shown to stimulate platelet aggregation (Byrne *et al.*, 2003). Aggregation was a genuine response, as it was blocked by GPIIb/IIIa antagonists and aspirin. *H. pylori* interaction with platelets involved binding to platelet GPIb, but unlike strains of *S. sanguis* this involved bacterial-bound vWF bridging to the receptor. IgG specific for *H. pylori* was also required for activation and aggregation through binding FcγRIIa (Byrne *et al.*, 2003).

Borrelia burgdorferi, a tick-borne spirochete, is the etiological agent of Lyme disease, a chronic multisystemic infection affecting humans in specific geographical locations, especially in North America and northern parts of Europe such as Scandinavia.
The related *Borrelia hermsii* is the causative agent of relapsing fever, which is also transmitted to humans via ticks. Relapsing fever is characterized by waves of fever that coincide with high concentrations of spirochetes in the bloodstream (spirochetemia). High density of *B. hermsii* in the bloodstream is also associated with decreased platelet counts (thrombocytopenia). Both *B. burgdorferi* and *B. hermsii* have been shown to interact with platelets via GPIIb/IIIa (Coburn *et al.*, 1993, 1994; Alugupalli *et al.*, 2001). Platelet binding by *B. burgdorferi* may be mediated through its outer membrane protein p66 (Defoe and Coburn, 2001). *B. burgdorferi* only bound GPIIb/IIIa on activated platelets, and did not bind to resting platelets (Coburn *et al.*, 1993). In contrast, *B. hermsii* binds resting platelets independently of GPIIb/IIIa, stimulates platelet activation, and subsequently binds at high levels to activated GPIIb/IIIa (Alugupalli *et al.*, 2001). Mice infected with *B. hermsii* suffered from severe thrombocytopenia, attributed to the formation of spirochete-platelet complexes followed by rapid clearance (Alugupalli *et al.*, 2003). Platelet binding by species of *Borrelia* may participate in establishing infection in diverse tissues affected by Lyme disease or help localize spirochetes at sites where ticks are feeding by binding activated platelets, and thus facilitate spreading of infection to other hosts.

1.6 Rationale for this study

S. aureus is the leading cause of infective endocarditis (Fowler *et al.*, 2005). This condition is associated with high mortality rates, even with aggressive antibiotic therapy (Mylonakis and Calderwood, 2001; Moreillon and Que, 2004). Mortality rates will no doubt increase with the emergence of multiply-resistant *S. aureus* strains that are refractory to treatment with clinically available antibiotics. It is widely believed that the ability of endovascular pathogens such as *S. aureus* to interact with platelets is a crucial virulence determinant in the pathogenesis of infective endocarditis (Sullam *et al.*, 1996). Platelet binding and activation by *S. aureus* resulting in the formation of platelet aggregates is likely to play a fundamental role in thrombus formation on the endocardial surface.

The fibrinogen-binding surface protein ClfA of *S. aureus* is the major mediator of platelet activation and aggregation by bacteria grown to stationary phase (O'Brien *et al.*, 2002a). Recent studies in this laboratory demonstrated that the fibronectin-binding proteins FnBPA and FnBPB are the predominant pro-aggregatory factors expressed by exponentially grown bacteria (J.R. Fitzgerald, personal communication). Both ClfA and FnBPA act as virulence factors in experimental endocarditis models (Que *et al.*, 2001, 2005). The goal of this study was to determine the molecular mechanisms by which bacteria expressing ClfA and FnBPA interact with platelets to trigger their activation and aggregation. This may provide new insights on the pathogenesis of *S. aureus* infective endocarditis and identify novel interactions that may serve as attractive targets for new therapeutic approaches.

1.6.1 Aims and objectives

- Functional expression of ClfA and FnBPA on the surface of the surrogate host *Lactococcus lactis*, which does not naturally cause platelet aggregation.
- Comparison of the ability of *L. lactis* expressing ClfA and a non-fibrinogen-binding ClfA mutant (ClfA PY) to cause platelet aggregation. This will establish if the fibrinogen-binding activity of ClfA is important in triggering rapid platelet activation.
- Expression of truncated FnBPA proteins on the surface of *L. lactis* – this will allow identification of the FnBPA domains responsible in triggering platelet aggregation.
- Measurement of cytosolic calcium flux in platelets exposed to bacteria expressing ClfA or FnBPA. This will demonstrate if the aggregation response is the result of genuine platelet activation.

• Inhibition studies using function-blocking antibodies to platelet receptors. This will allow identification of platelet receptors involved in recognizing bacteria expressing ClfA or FnBPA.

• Identification of plasma cofactors involved in platelet binding and aggregation promoted by ClfA-expressing or FnBPA-expressing bacterial cells.

Chapter 2

Materials and Methods

2.1 Bacterial strains and growth conditions

Escherichia coli, *Staphylococcus aureus* and *Lactococcus lactis* strains are listed in Table 2.1. *E. coli* was routinely grown on L-agar or in L-broth at 37°C. *S. aureus* was grown on Trypticase Soy agar (TSA, Oxoid) or on Brain-Heart Infusion (BHI, Oxoid) agar or broth at 37°C. *E. coli* and *S. aureus* broth cultures were grown in an orbital shaker at 200 rpm at 37°C. *L. lactis* was grown on M17 agar or in M17 broth supplemented with 0.5 % (w/v) glucose (GM17). *L. lactis* broth cultures were grown statically at 30°C. For platelet aggregation studies, *S. aureus* and *L. lactis* were grown in 20 ml and 5 ml culture volumes respectively. *S. aureus* cells were harvested in either exponential phase (OD_{600} of 0.5 - 0.6) or stationary phase (16 h). *L. lactis* cells were harvested after 16 h. Stocks of bacterial strains were made by supplementing broth cultures with 20 % (v/v) glycerol and snap-freezing in liquid nitrogen.

The following antibiotics were incorporated into the media where appropriate: ampicillin (Amp), 100 μg/ml; chloramphenicol (Cm), 5 or 10 μg/ml; erythromycin (Em), 5 μg/ml; kanamycin (Kan), 100 μg/ml. Antibiotics were purchased from Sigma Chemical Co.

2.2 Plasmids

All plasmids and derivatives are listed in Table 2.2.

2.3 DNA manipulation

DNA manipulation techniques were performed using standard methods (Sambrook *et al.*, 1989). Enzymes for DNA manipulation were purchased from New England Biolabs, Stratagene and Promega and were used according to the manufacturers' instructions. *E. coli* transformants were screened for the presence of recombinant plasmids using the rapid colony screening procedure developed by Le Gouill and Dery (1991). Recombinant plasmids, isolated from *E. coli*, were initially established in *S. aureus* by electroporation into the restriction-deficient RN4220 strain as previously described (Augustin and Gotz, 1990). All confirmatory DNA sequencing was performed by Lark Technologies.

2.3.1 Polymerase chain reaction (PCR)

PCR reactions were performed in 100 µl final volumes with 100 pM forward and reverse primers (Sigma Genosys), 10 ng plasmid DNA or 20 ng genomic DNA as template, 2.5 mM dNTPs and 5 U *Pfu* polymerase (Promega) in a standard *Pfu* reaction buffer (Promega). Primer sequences are given in Table 2.3. Reactions typically consisted of 30 cycles with 1 min denaturation (at 95°C), 1 min annealing (temperature dependent on the primers used), and an extension step (at 72°C). 2 minutes were allowed per 1 kb of DNA to be amplified in the extension cycle. PCR products were purified using the High Pure PCR Product purification kit (Roche) according to the manufacturers' protocol.

2.3.2 Isolation of plasmid and genomic DNA

Plasmid DNA was purified using Promega Wizard SV Plus Minipreps DNA purification system as recommended by the supplier, except that *L. lactis* cells were treated with mutanolysin (Sigma; 500 U) and lysozyme (Sigma; 200 µg) for 30 min at 37°C to digest the cell-wall peptidoglycan prior to alkaline lysis of the cells.

Preparation of genomic DNA was performed using the Genomic DNA purification kit (Edge Biosystems) as per the suppliers protocol, except that *S. aureus* cells were treated with 200 µg lysostaphin (AMBI, New York) for 20 min at 37°C to digest the cell-wall peptidoglycan.

2.4 Phage transduction

Chromosomal mutations and plasmids, both marked with antibiotic-resistance cassettes, were transferred between *S. aureus* strains by transduction using phage 85. Recombinant plasmids were initially established in the restriction deficient *S. aureus* RN4220 strain by electroporation. An overnight culture of the strain containing the marker to be transduced was diluted 1:100 into phage broth (20 g/L Nutrient broth No. 2 (Oxoid)) supplemented with 10 mM $CaCl_2$ and grown for 4 h at 37°C with shaking. 200 µl of a phage 85 stock was used to infect 300 µl of this culture. Phage 85 was diluted prior to infection to the highest dilution that provided confluent lysis, and was incubated with the cells at room temperature for 30 min. The culture was mixed with 10 ml molten phage top agar (20 g/L

Table 2.1 Bacterial strains

Strain	Relevant Properties	Source/ Reference
E. coli		
XL-1 Blue	Propagation of plasmids	Stratagene
Topp3	Protease deficient strain. Used for recombinant protein expression	Stratagene
L. lactis		
MG1363	Plasmid-free derivative of strain NCDO 712	Gasson, 1983
NZ9800	MG1363 derivative containing the nisin-sucrose conjugative transposon *Tn5276*, Δ*nisA*	Kuipers *et al.*,1993
S. aureus		
8325-4	NCTC 8325 cured of prophages	Novick, 1967
RN4220	Restrition-deficient derivative of 8325-4	Kreiswirth *et al.*, 1983
Newman	Clumping factor positive strain	Duthie and Lorenz, 1952
Newman *clfA*	Newman derivative deficient in clumping factor A. *clfA*2::Tn*917*, Em^R	McDevitt *et al.*, 1994
Newman *spa*	Newman derivative deficient in protein A. *spa*::Ka^R	O'Brien *et al.*, 2002
Newman *clfA clfB*	Newman derivative deficient in clumping factor A and B. *clfA*2::Tn*917* Em^R, *clfB*::Tc^R	Ni Eidhin *et al.*, 1998
Newman *clfA clfB spa*	Newman derivative deficient in clumping factors A and B and protein A. *clfA*2::Tn*917* Em^R, *clfB*::Tc^R, *spa*::Ka^R	This work

Table 2.1 Bacterial strains, continued

Strain	Relevant Properties	Source/ Reference
S. aureus		
Newman *clfA clfB spa sdrC sdrD sdrE*	Newman derivative deficient in ClfA, ClfB, protein A, SdrC, SdrD, and SdrE. Silent frameshift mutation in *clfA* (*clfA5*), *clfB*::Em^R, *spa*::Ka^R, *sdrCDE*::Tc^R	J.R. Fitzgerald
P1	Rabbit virulent strain. Strong adhesion to extracellular matrix proteins.	Sheretz *et al.*, 1993
P1 *fnbA fnbB*	P1 derivative deficient in FnBPA and FnBPB *fnbA*::Tc^R, *fnbB*::Em^R	Roche *et al.*, 2004
P1 *fnbA fnbB spa*	P1 derivative deficient in FnBPA, FnBPB and protein A. *fnbA*::Tc^R, *fnbB*::Em^R, *spa*::Ka^R	M. Knobel

Table 2.2 Plasmids

Plasmid	Features	Marker(s)	Source/ Reference
pCUI	Shuttle vector derived from pC194 and pUC19, maintains high copy number in *E. coli* and *S. aureus*	Amp^R in *E. coli* Cm^R in *S. aureus*	Augustin *et al.*, 1992
pCF77	pCUI derivative containing an entire copy of the *clfA* gene	Amp^R in *E. coli* Cm^R in *S. aureus*	Hartford *et al.*, 1997
pCF77 PY	pCF77 derivative containing an entire copy of *clfA* with mutations encoding the changes $P_{336}S$ and $Y_{338}A$	Amp^R in *E. coli* Cm^R in *S. aureus*	J. Higgins
pFnBA4	pSK265 derived multicopy plasmid containing entire *fnbA* gene from *S. aureus* 8325-4. Expression of FnBPA in *S. aureus*	Cm^R	Greene *et al.*, 1995
pFnBB4	pSK265 derived multicopy plasmid containing entire *fnbB* gene from *S. aureus* 8325-4. Expression of FnBPB in *S. aureus*	Cm^R	Greene *et al.*, 1995
pQE30	*E. coli* vector for the expression of hexa-histidine tagged recombinant proteins	Amp^R	Quigen
pCF40	pQE30 derivative encoding the full length A domain of ClfA (N123)	Amp^R	O'Connell *et al.*, 1998
pQE30*clfA* PY	pQE30 derivative encoding the full length A domain of ClfA PY (N123)	Amp^R	This work
pNZ8037	Plasmid for controlled expression of heterologous proteins in *L. lactis*. *Nco*I site translationally fused to *nisA* nisin-inducible promoter	Cm^R	De Ruyter *et al.*, 1996

Table 2.2 Plasmids, continued

Plasmid	Features	Marker(s)	Source/ Reference
pNZ8037*clfA*	pNZ8037 containing the full-length *clfA* gene, cloned from *S. aureus* Newman in-frame with the ATG start codon within the *Nco*I site	Cm^R	R. Downer
pNZ8037*clfA* PY	pNZ8037 containing the full-length *clfA* PY gene, cloned from pCF77 PY in-frame with the ATG start codon within the *Nco*I site	Cm^R	This work
pNZ8037*clfB*	pNZ8037 containing the full-length *clfB* gene, cloned from *S. aureus* Newman in-frame with the ATG start codon within the *Nco*I site	Cm^R	This work
pNZ8037*fnbA*	pNZ8037 containing the full-length *fnbA* gene, cloned from *S. aureus* 8325-4 in-frame with the ATG start codon within the *Nco*I site	Cm^R	This work
pNZ8037*fnbA*-A	pNZ8037 containing a truncated *fnbA* gene encoding an FnBPA protein lacking the BCD domain	Cm^R	This work
pNZ8037*fnbA*-BCD	pNZ8037 containing a truncated *fnbA* gene encoding an FnBPA protein lacking the A domain	Cm^R	This work
pNZ8037*fnbB*	pNZ8037 containing the full-length *fnbB* gene, cloned from *S. aureus* 8325-4 in-frame with the ATG start codon within the *Nco*I site	Cm^R	This work
pNZ8037*sdrC*	pNZ8037 containing the full-length *sdrC* gene, cloned from *S. aureus* Newman in-frame with the ATG start codon within the *Nco*I site	Cm^R	This work

Table 2.2 Plasmids, continued

Plasmid	Features	Marker(s)	Source/ Reference
pNZ8037*sdrD*	pNZ8037 containing the full-length *sdrD* gene, cloned from *S. aureus* Newman in-frame with the ATG start codon within the *Nco*I site	Cm^R	This work
pNZ8037*sdrE*	pNZ8037 containing the full-length *sdrE* gene, cloned from *S. aureus* Newman in-frame with the ATG start codon within the *Nco*I site	Cm^R	This work
pKS80	Plasmid for high-level expression of heterologous proteins in *L. lactis*	Em^R	Hartford *et al.*, 2001
pKS80*sdrC*	pKS80 containing the entire *sdrC* gene, cloned from *S. aureus* Newman, in-frame with the ATG start codon within the *Bcl*I site	Em^R	O'Brien *et al.*, 2002
pKS80*sdrD*	pKS80 containing the entire *sdrD* gene, cloned from *S. aureus* Newman, in-frame with the ATG start codon within the *Bcl*I site	Em^R	O'Brien *et al.*, 2002
pKS80*sdrE*	pKS80 containing the entire *sdrE* gene, cloned from *S. aureus* Newman, in-frame with the ATG start codon within the *Bcl*I site	Em^R	O'Brien *et al.*, 2002
pBluescript KS+	High copy number plasmid for *E. coli*. Used for general cloning	Amp^R	Stratagene
pBluescript::*fnbA* P1	pBluescript plasmid containing a 2.2 kb *Bam*HI-*Hind*III PCR-generated fragment of the *fnbA* gene from *S. aureus* P1	Amp^R	This work
pBluescript::*fnbB* P1	pBluescript plasmid containing a 2.0 kb *Bam*HI-*Hind*III PCR-generated fragment of the *fnbB* gene from *S. aureus* P1	Amp^R	This work

Table 2.3 Primers

Primer	Sequence (5' – 3') [a, b]	5' restriction site
pClfA F1	CATG*CCATGG*AGATGAAGAAAAAAGAAAAACCAC	*Nco*I
pClfA R1	TGC*TCTAGA*CTCAGATACATATGGATTCTTC	*Xba*I
pClfA F2	CGC*GGATCC*AGTGAAAATAGTGTTACGCAATCT	*Bam*HI
pClfA R2	CGC*AAGCTT*CTCTGGATTGGTTCAATTTCAC	*Hind*III
pClfB F1	CTTG*CCATGG*AAAAAAGAATTGATTATTTGTCG	*Nco*I
pClfB R1	TGC*TCTAGA*TTCTTCCGGTAAAATGACTG	*Xba*I
pFnBPA F1	CGTG*CCATGG*AAAACAATCTTAGGTACGGC	*Nco*I
pFnBPA R1	TGC*CTCGAG*TTAAAATTCTAACTTTATCTCTC	*Xho*I
pFnBPA F2	CCG*AAGCTT*GAAGAGGAATATGATTCATCAAC	*Hind*III
pFnBPA R2	CGG*AAGCTT*TGCAGCTTCTTTGTCTTGTCC	*Hind*III
pFnBPA F3	CGG*AAGCTT*ATCGTGCCACCAACGCCAC	*Hind*III
pFnBPA R3	CCG*AAGCTT*ATTTTTCTCATTTCCGTTCGC	*Hind*III
pFnBPA F4	CCG*GGATCC*CCATCTTAGGTACGGCATTAG	*Bam*HI
pFnBPA R4	CCG*GGATCC*TGTCTTCCTCGAATGACTGG	*Bam*HI
pFnBPB F1	CGTG*CCATGG*AAAGCAATCTTAGATACGGC	*Nco*I
pFnBPB R1	TGC*TCTAGA*CGCCTTCATAGTGTCATTG	*Xba*I
pFnBPB F2	CCG*GGATCC*AAGAAAACACAAATTGGGAGC	*Bam*HI
pFnBPB R2	CGG*GGATCC*CCACCTTGTTCATATTTCGG	*Bam*HI
pSdrC F1	CTTG*CCATGG*ATAATAAAAAGACAGCAAC	*Nco*I
pSdrC R1	CCG*TCTAGA*TTATTTATTTTGTTTTTTACGACG	*Xba*I
pSdrD F1	CTTG*CCATGG*TAAACAGAGAAAATAAAACG	*Nco*I
pSdrD R1	CCG*TCTAGA*TTATTTATTTTGTTTTTTACGACGAC	*Xba*I
pSdrE F1	CTTG*CCATGG*TTAAGAAGGATAATAAAAAG	*Nco*I
pSdrE R1	CCG*TCTAGA*TTATTTGTTTTGTTTTTTGCGAGG	*Xba*I
pSdrE F2	CAATGGT**G**CA**C**GGTGATTC	None
pSdrE R2	GATCTTGATAATCAACAGTGAC	None

[a] **Restriction sites are in italics** [b] **Nucleotides in primers for site-directed mutagenesis are in bold**

Nutrient broth No. 2 (Oxoid), 3.5 g/L Agar No. 1 (Oxoid)) supplemented with 10 mM $CaCl_2$ at 50°C and spread over two phage base plates (20 g/L Nutrient broth No. 2 (Oxoid), 7 g/L Agar No. 1 (Oxoid)) supplemented with 10 mM $CaCl_2$. Plates were incubated overnight at 37°C. Phage plate stocks containing the marker to be transduced were prepared by transferring the top agar to an Oakridge tube and centrifuged for 10 min at 4°C at 15,000 rpm. The supernatant was removed and filtered through a 0.45 μm filter. Phage plate stocks were stored at 4°C.

A 20 ml culture of the recipient strain was grown for 16 h at 37°C. Cells were harvested and resuspended in 1 ml TSB. 500 μl cells were mixed with 500 μl phage plate stock (with marker for transduction) in 1 ml L-broth supplemented with 10 mM $CaCl_2$. A control tube was included that contained 500 μl cells mixed with 1.5 ml L-broth supplemented with 10 mM $CaCl_2$ but no phage plate stock. The mixtures were incubated statically at 37°C for 25 min and then with shaking at 37°C. 1 ml of 0.02 M ice-cold Na_2citrate was added and the mixtures centrifuged. Cells were resuspended in 1 ml 0.02 M ice-cold Na_2citrate and incubated on ice for 2 h. The transduction mixture was plated on TSA containing 0.05 % (w/v) Na_2citrate and the appropriate antibiotic. Colonies obtained were restreaked twice on TSA containing 0.05 % (w/v) Na_2citrate and antibiotic.

2.5 Cloning of *S. aureus* genes encoding surface proteins into *L. lactis*

Genes encoding surface proteins were amplified by PCR using *Pfu* polymerase, using specific primer pairs (Table 2.3) designed to amplify the entire open reading frame of the gene of interest from the appropriate DNA template. Forward primers incorporated an *Nco*I restriction site which facilitated the translational fusion of the ATG start codon to the nisin-inducible *nisA* promoter in the pNZ8037 expression vector (Figure 2.1).

2.5.1 ClfA

The entire *clfA* gene was amplified using the primer pair pClfA F1/pClfA R1 and *S. aureus* Newman genomic DNA as the template. *Nco*I/*Xba*I cleaved DNA was ligated with pNZ8037 DNA cleaved with the same enzymes to generate the plasmid pNZ8037*clfA*.

2.5.2 ClfA PY

The entire *clfA* PY gene was amplified using the primer pair pClfA F1/pClfA R1 and pCF77 PY plasmid DNA as the template. *Nco*I/*Xba*I cleaved DNA was ligated with pNZ8037 DNA cleaved with the same enzymes to generate the plasmid pNZ8037*clfA* PY.

2.5.3 ClfB

The *clfB* gene was amplified using the primer pair pClfB F1/pClfB R1 and *S. aureus* Newman genomic DNA as the template. *Nco*I/*Xba*I cleaved DNA was ligated with pNZ8037 DNA cleaved with the same enzymes to generate the plasmid pNZ8037*clfB*.

2.5.4 FnBPA

The *fnbA* gene was amplified using the primer pair pFnBPA F2/pFnBPA R2 and *S. aureus* 8325-4 genomic DNA as the template. *Nco*I/*Xho*I cleaved DNA was ligated with pNZ8037 DNA cleaved with the same enzymes to generate the plasmid pNZ8037*fnbA*.

2.5.5 FnBPB

The *fnbB* gene was amplified using the primer pair pFnBPB F1/pFnBPB R1 and *S. aureus* 8325-4 genomic DNA as the template. *Nco*I/*Xba*I cleaved DNA was ligated with pNZ8037 DNA cleaved with the same enzymes to generate the plasmid pNZ8037*fnbB*.

2.5.6 FnBPA truncates

Genes encoding truncated derivatives of FnBPA were generated using an inverse PCR strategy with pNZ8037*fnbA* DNA as the template. The PCR product obtained with primer pair pFnBPA F3/pFnBPA R3 was purified, digested with *Hind*III, and ligated to yield the plasmid pNZ8037*fnbA*-A. The PCR product obtained with primer pair pFnBPA F2/pFnBPA R2 was manipulated in the exact same manner to generate the plasmid pNZ8037*fnbA* BCD domain.

2.5.7 SdrC

The *sdrC* gene was amplified using the primer pair pSdrC F1/pSdrC R1 and *S. aureus* Newman genomic DNA as the template. *Nco*I/*Xba*I cleaved DNA was ligated with pNZ8037 DNA cleaved with the same enzymes to generate the plasmid pNZ8037*sdrC*.

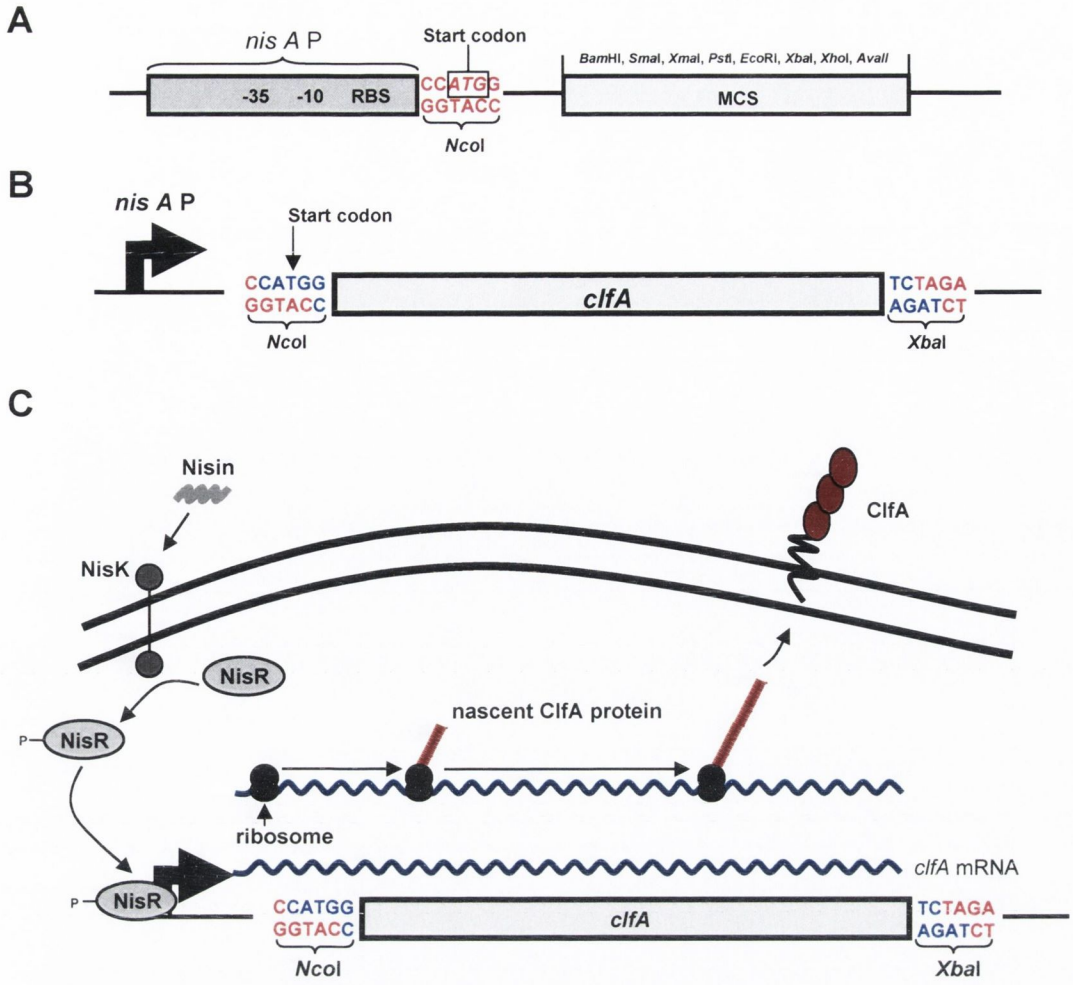


Figure 2.1. Nisin-inducible expression vector pNZ8037

A. pNZ8037 contains the nisin-inducible *nisA* promoter (*nisA* P). Promoter sequences and the ribosome-binding site are found upstream of the ATG start codon within the *Nco*I restriction site. This site facilitates translational in-frame fusions of target genes to the promoter sequences. A multiple-cloning-site (MCS) containing eight unique restriction sites facilitates directional cloning of target genes to the promoter

B. Fusion of target genes to *nisA* P in pNZ8037. The gene of interest (*clfA* is shown as an example here) is amplifled by PCR from an appropriate template. The forward primer incorporates an *Nco*I site in-frame with the *clfA* ATG start codon. The reverse primer, complementary to approximately 200 bp downstream of the *clfA* stop codon to include transcritptional termination sequences, incorporates a unique MCS site (*Xba*I) . Both the amplified gene and pNZ8037 vector are cleaved with *Nco*I and *Xba*I and ligated together. This results in the insertion of the *clfA* gene between the *Nco*I and *Xba*I sites in pNZ8037 and fuses the ATG start codon of *clfA* to the *nis* A promoter. Nucleotides shown in red are derived from the pNZ8037 vector and those shown in blue are derived from the PCR-amplified *clfA* gene. The insertion of the *Nco*I site in *clfA* (and other surface protein genes) results in mutation of the second amino acid in the translated protein.

C. Expression of target genes from *nisA* P in pNZ8037. Extracellular nisin binds to the sensor-histidine kinase NisK. This causes phosphorlylation of the NisR response regulator, which binds to promoter sequences and activates transcription. The translational fusion of *clfA* to *nisA* P results in efficient translation of *clfA* mRNA sequences. ClfA is sorted to the cell wall and is displayed in a functional manner on the cell surface.

2.5.8 SdrD

The *sdrD* gene was amplified using the primer pair pSdrD F1/pSdrD R1 and *S. aureus* Newman genomic DNA as the template. *Nco*I/*Xba*I cleaved DNA was ligated with pNZ8037 DNA cleaved with the same enzymes to generate the plasmid pNZ8037*sdrD*.

2.5.9 SdrE

The *sdrE* gene was amplified by PCR in two separate fragments and ligated together to yield the full *sdrE* gene as depicted in Figure 2.2. This was necessary due to an internal *Nco*I site within the *sdrE* gene 1284 nucleotides downstream of the ATG start codon. Mutation of this site facilitated fusion of the entire gene to the *Nco*I site within the nisin-inducible promoter *nisA* P in pNZ8037. The amplified gene had two silent mutations in the internal *Nco*I site. PCR reactions with the primers pSdrE FI and pSdrE R2 yielded a PCR product of 1276 nucleotides (*sdrE* 5'). PCR reactions with primers pSdrE F2 (mutagenic primer) and pSdrE R1 yielded a PCR product of 2225 nucleotides (*sdrE* 3') with the *Nco*I site mutated. The *sdrE* 5' DNA fragment was digested with *Nco*I and the *sdrE* 3' fragment digested with *Xba*I. Cleaved fragments were ligated in a blunt-end ligation and the ligated DNA was pelleted by ethanol precipitation and resuspended in 10 µl nuclease-free H_2O. 1 µl (50 ng) of ligated DNA was used as the template in a second PCR reaction with primers flanking the *sdrE* open reading frame (pSdrE F1 and pSdrE R1) to produce the full-length *sdrE* gene lacking the internal *Nco*I site. This PCR product was cleaved with *Nco*I/*Xba*I and ligated with pNZ8037 DNA cleaved with the same enzymes to generate the plasmid pNZ8037*sdrE*.

2.5.10 Preperation and transformation of electrocompetant *L. lactis* NZ9800

L. lactis NZ9800 cells were made competent for DNA transformation (Wells *et al.*, 1993) by growth in 100 ml GM17 broth containing 2.5 % (w/v) glycine. Cells were harvested by centrifugation (7000 rpm for 10 min) and washed twice in 10 ml ice-cold 0.5 % sucrose/ 10 % (v/v) glycerol. Cells were finally resuspended in 1 ml of the same solution and kept chilled on ice. Electrocompetant cells (45 µl) were mixed with pNZ8037 DNA ligated to the gene of interest in a microfuge tube. DNA ligations were concentrated by ethanol precipitation and the DNA pellets resuspended in 5 µl nuclease-free H_2O before mixing with competent cells. Cell/ DNA mixtures were transferred to pre-chilled 1 mm gap electroporation cuvettes (Flowgen) and electroporated using a Gene Pulsar apparatus (BioRad) with the following parameters: 2.0 kV, 200 Ω and 25 µF. Cells were immediately mixed with 950 µl recovery

broth (GM17, 0.5 M sucrose, 20 mM $MgCl_2$, 2 mM $CaCl_2$), transferred to a microfuge tube, incubated on ice for 10 min and finally incubated at 30°C for 2 h. The cells were harvested by centrifugation, resuspended in 100 µl recovery broth and plated on GM17 agar incorporating 5 µg/ml Cm. Plates were incubated for 48 h at 30°C. Transformant colonies were inoculated into 3 ml GM17 containing 10 µg/ml Cm and 1.6 ng/ml nisin and grown for 16 h. Clones expressing the gene of interest were identified by whole-cell dot immunoblotting (Section 2.6) or by ligand adherence assays (Section 2.8).

2.5.11 Induction of *L. lactis* strains carrying pNZ8037 derivatives with nisin

L. lactis NZ9800 cells carrying pNZ8037 derivatives were grown in 2 ml GM17 medium, incorporating 10 µg/ml Cm, for 16 h at 30°C. Cultures were diluted $^1/_{100}$ into 5 ml fresh GM17 medium with 10 µg/ml Cm and grown to OD_{600} of 0.5. Nisin (Sigma) was prepared at a stock concentration of 500 µg/ml in PBS and diluted into exponential phase cultures to the desired final concentration, typically in the range from 0.025 to 3.2 ng/ml. Induced cultures were grown at 30°C for 16 h. Cells were harvested by centrifugation at 3000 rpm for 10 min. Cells were washed twice in PBS and resuspended in 1 ml PBS. The optical density of cell suspensions (OD_{600}) was determined in a spectrometer.

2.6 Whole-cell dot immunoblotting

S. aureus or *L. lactis* cells were washed twice in PBS and adjusted to an OD_{600} of 1.0 in PBS. Cells were serially diluted with PBS in 96-well flat bottomed plate (Sarstedt) to an OD_{600} of 0.002. Five µl of each cell suspension to be analyzed was applied to a nitrocellulose membrane (Protran, Schleicher & Schull) and allowed to dry. Membranes were blocked for 2 h in TS buffer (10 mM Tris-HCl, 0.9 % NaCl, pH 7.4) containing 10 % (w/v) skimmed milk powder (Marvel). Rabbit polyclonal antibodies specific for *S. aureus* surface proteins were diluted to a suitable concentration in TS buffer/ 10 % milk and incubated with the membrane for 1.5 h with shaking. Membranes were washed three times in TS buffer to remove unbound antibody. Membranes were then incubated with goat-anti-rabbit antibodies conjugated to horseradish peroxidase (Dako) diluted 1:2000 in TS buffer/ 10 % milk for 1.5 h. Membranes were then washed three times in TS buffer to remove unbound antibody. The membrane was developed in the dark using the chemiluminescent substrate LumiGlo (New England BioLabs)

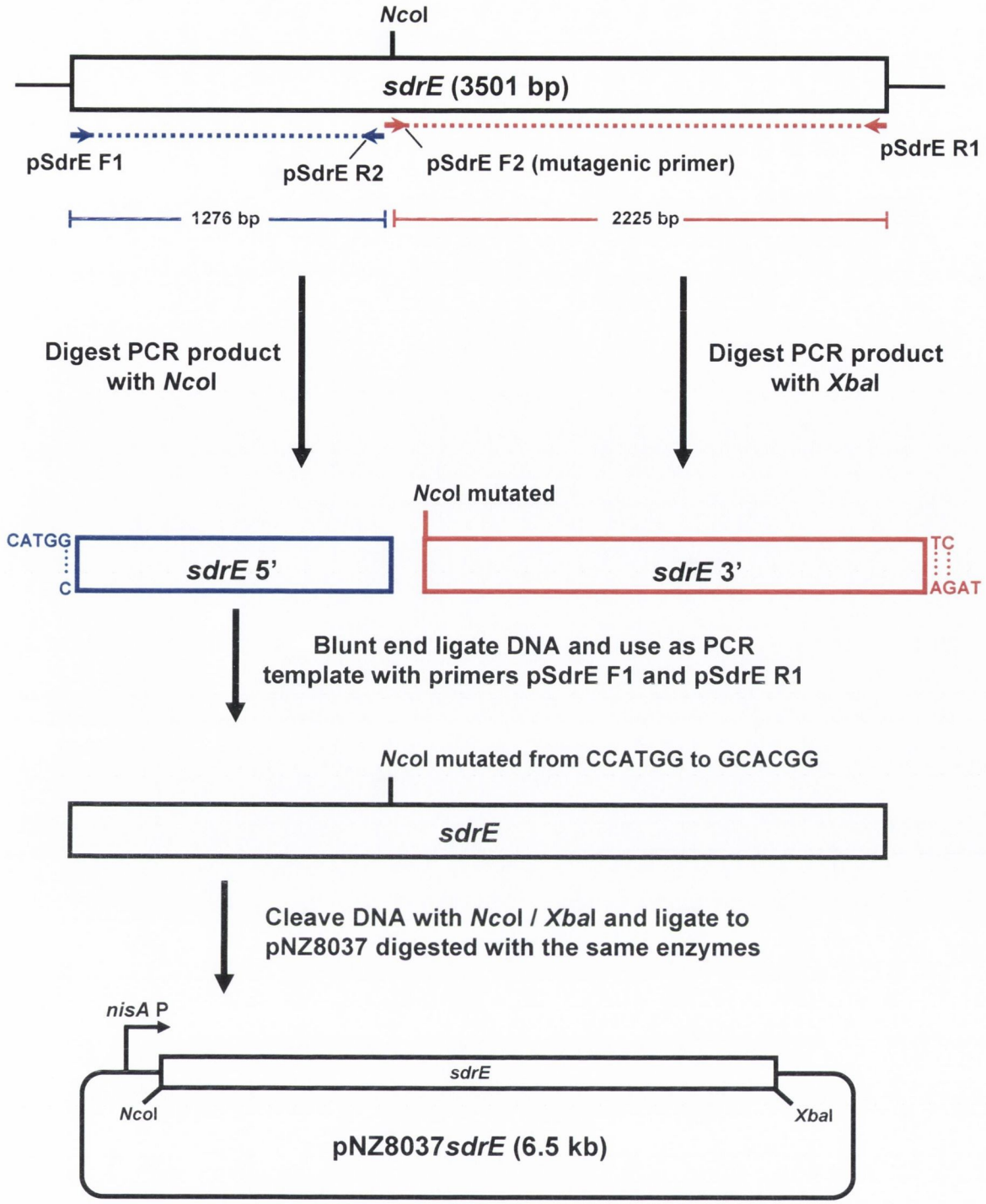


Figure 2.2. Cloning of the *sdrE* gene into pNZ8037

The *sdrE* gene of *S. aureus* Newman contains an *Nco*I site at position 1284 downstream of the ATG start codon. The *sdrE* gene was amplified in 2 separate fragments. PCR reactions with primers pSdrE F1 and pSdrE R2 generated a fragment of 1284 bp (*sdrE* 5') with an *Nco*I site in frame with the *sdrE* ATG start codon. PCR reactions with pSdrE F2 and pSdrE R1 generated a fragment of 2225 bp (*sdrE* 3'). The F2 primer incorporated nucleotide mismatches to remove the natural *Nco*I site. *sdrE* 5' and *sdrE* 3' DNA fragments were cleaved with *Nco*I or *Xba*I respectively. Cleaved DNA fragments were blunt end ligated together, which served as the template for a final PCR reaction with primers pSdrE F1 and pSdrE R1. The final PCR product encompassed the entire *sdrE* containing a 5' *Nco*I site and a 3' *Xba*I site. The *sdrE* gene also lacked the internal *Nco*I site (mutated from CCATGG to GCACGG). The *sdrE* gene was cleaved with *Nco*I and *Xba*, and was than ligated to pNZ8037 DNA cleaved with the same enzymes to generate plasmid pNZ8037*sdrE*.

as recommended by the manufacturer, exposed to X-Omat autoradiographic film (Kodak) and visualized using a Kodak X-OMAT 1000 Processor developing machine.

2.7 Antibodies to *S. aureus* surface proteins

Polyclonal antibodies to *S. aureus* surface proteins were generated by immunizing rabbits with purified, recombinant proteins. Antibodies against ClfA were generated by immunization with the A domain protein of ClfA and were kindly provided by Judy Higgins (Trinity College Dublin). Antibodies against the A domains of FnBPA and FnBPB were kindly provided by Fiona Keane (Trinity College Dublin). Antibodies recognizing the A domains of SdrC, SdrD and SdrE were provided by Louise O'Brien (Trinity College Dublin). For Western blotting and dot-immunoblotting experiments, antibodies were generally diluted between $^{1}/_{1000}$ and $^{1}/_{5000}$.

2.8 Adherence assays to immobilized fibrinogen and fibronectin

Bacterial adherence to immobilized fibrinogen and fibronectin was measured using a previously described assay (Wolz *et al.*, 1996) with a modified protocol. In brief, human fibrinogen (Calbiochem) or human fibronectin (Calbiochem) were diluted to a specific concentration in PBS and 100 µl per well used to coat a 96-well flat bottomed plate (Sarstedt) for 16 h at 4°C. Control wells were included that only contained PBS. Wells were washed three times with 200 µl PBS and blocked with 100 µl filter-sterilized 2% (w/v) bovine serum albumin (BSA, Sigma) for 2 h at 37°C. The wells were washed three times with PBS and 100 µl washed bacterial cells in PBS (OD_{600nm} of 1.0) were added. The plates were incubated at 37°C for 2 h. Non-adherent cells were removed by washing the wells three times with PBS and adherent cells were fixed using 100 µl 25 % (v/v) formaldehyde per well for 15 min. The wells were washed three times in PBS and the adherent cells stained with 100 µl 0.5 % (w/v) crystal violet per well for 1 min. The wells were extensively washed in PBS to remove excess stain. Cell-bound crystal violet was solubilized using 100 µl 5 % (v/v) acetic acid per well and the plates gently shaken. Plates were then read after 5 min in an ELISA plate reader at 570 nm.

2.9 Cell clumping in soluble fibrinogen

The clumping of bacterial cells in a solution of fibrinogen was measured by turbidometry in a PAP-4 aggregometer (BioData). Washed bacterial cells were adjusted to OD_{600} of 2.0 in PBS and 200 µl of cell suspension was incubated in siliconized flat-bottom glass cuvettes (BioData) at 37°C with stirring (900 rpm). This stirred suspension was used to establish the baseline light transmission in the aggregometer. Human fibrinogen (1 mg) was added to the bacterial suspension and clumping was measured by increases in optical transmission. For routine screening of clumping activity, assays were performed in 96-well flat bottomed dishes (Sarstedt). Human fibrinogen was dissolved in PBS to a concentration of 2 mg/ml. 20 µl washed bacterial cells (approximately 1.5 x 10^8 cells, OD_{600nm} of 6.0) were added to the wells and the plates were shaken briskly for 5 min. Cell clumping was determined by visual inspection of the wells.

2.10 Isolation of *S. aureus* and *L. lactis* cell wall proteins

Cell wall-associated proteins of *S. aureus* and *L. lactis* were isolated as described by Hartford *et al.*, 2001. In brief, cells from cultures of *S. aureus* or *L. lactis* were washed twice in PBS and adjusted to an OD_{600nm} of approximately 40 in 250 µl PBS (approximately 10^{10}cells). The cells were pelleted by centrifugation and resuspended in 250 µl of 20 mM Tris (pH 8.0), 10 mM $MgCl_2$ containing 30 % raffinose (Sigma) in a microfuge tube. Complete ® Mini Protease Inhibitor Cocktail (Roche, 40 µl of a 10 x stock) and 5 mM EDTA were added, along with 200 µg lysostaphin (for *S. aureus*) or 250 U mutanolysin/100 µg lysozyme (for *L. lactis*) and the reaction was incubated at 37°C for 15 min with gentle mixing by inversion every 5 min. During this step, the cell wall-associated proteins are released due to the breakdown of the cell wall peptidoglycan by the murolytic enzymes and the formation of protoplasts occurs. The cell-wall proteins were then harvested by pelleting of the protoplasts by centrifugation (8000 rpm for 10 min) and removal of the supernatant containing the solubilized cell wall-associated proteins. Cell wall-associated protein samples were stored at -20°C.

2.11 Protein electrophoresis and immunodetection

2.11.1 SDS-PAGE

Protein samples for electrophoresis by SDS-PAGE were adjusted to specific final concentrations in PBS and diluted 2-fold in final sample buffer (10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol, 3 % (w/v) SDS, 0.01 % bromophenol blue in 62.5 mM Tris-HCl, pH 6.8). 20 µl volumes were separated by SDS-PAGE (Laemlli, 1970) using 4.5 % stacking and 10 % separating acrylamide gels, except in the case of cell wall-associated proteins, which were separated through 7.5 % separating acrylamide gels. An aliquot of prestained protein molecular weight marker (New England Biolabs) containing markers of the sizes: 175 kDa, 83 kDa, 62 kDa, 47.5 kDa, 32 kDa, 25.5 kDa, 16 kDa and 6.5 kDa, was run on each gel. Protein samples were electroporesed at 130 V. Fibrinogen samples were adjusted to 250 µg/ sample before electrophoresis. Human serum samples were diluted 1:20 before electrophoresis. Cell wall-associated protein samples were run as neat (undiluted) samples. Purified recombinant proteins were adjusted to 150 µg/ sample prior to electrophoresis. After separation, proteins were either visualized using Coomassie blue stain or electroblotted onto PVDF membranes (Roche) for 1 h at 100 V using a wet transfer cell (BioRad) for detection.

2.11.2 Western immunoblotting

PVDF membranes containing electroblotted proteins were incubated in TS Tween buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05 % (v/v) Tween 20 (Sigma)) containing 10 % (w/v) skimmed milk for 16 h at 4°C to block any non-specific interactions. Primary antibodies were diluted to the required working concentration in TS Tween/ 5 % (w/v) skimmed milk and incubated with the membrane for 1.5 h at room temperature with shaking. Polyclonal anti-ClfA A domain antibodies were used at a dilution of 1:5000. Polyclonal antibodies against FnBPA and FnBPB A domains were used at a dilution of 1:1000. Unbound antibody was removed by washing the membrane three times in TS buffer containing 0.05 % Tween 20. The appropriate secondary antibody was diluted in TS Tween/ 5 % (w/v) skimmed milk and incubated with the membrane for 1.5 h with shaking. Excess secondary antibody was removed by washing three times in TS Tween buffer. The membrane was developed in the dark using the chemiluminescent substrate LumiGlo (New England BioLabs) as

recommended by the manufacturer and exposed to X-Omat autoradiographic film (Kodak). Exposed films were visualized using a Kodak X-OMAT 1000 Processor developing machine.

2.11.3 Ligand-affinity blotting

Fibronectin-binding proteins in *S. aureus* cell wall extracts were detected by probing membranes with biotinylated fibronectin. Human fibronectin (1 mg in 2 ml PBS) was incubated with 2 mg NHS-biotin for 15 min at room temperature. The reaction was stopped by addition of 10 mM NH_4Cl. Excess biotin was removed by dialysis against PBS for 16 h at 4°C. Membranes were blocked with 10 % (w/v) skimmed milk in TS Tween buffer for 16 h at 4°C. Biotinylated fibronectin was diluted to 30 µg/ml in TS Tween/ 5 % (w/v) skimmed milk and was incubated with the membranes for 2 h. Excess fibronectin was removed by washing with TS Tween and bound fibronectin detected using peroxidase-conjugated streptavidin (Roche; diluted $^1/_{10000}$ in 5 % slimmed milk / TS Tween) and developed by chemiluminescence.

2.12 Purification of His-tagged recombinant proteins by immobilized metal chelate affinity chromatography

Recombinant domains of *S. aureus* surface associated proteins were expressed with an N-terminal hexa-histidine (His_6) affinity tag proteins to allow high degree purification by nickel affinity chromatography. DNA encoding the region of interest was amplified by PCR and cloned into the pQE30 expression vector. pQE30 contains an IPTG inducible promoter to allow controlled expression of the recombinant protein and also contains sequences located 5 ' to the MCS that encodes the 6 x His residues. A pQE30 construct encoding the full-length A domain of ClfA (N123; residues 40-559; plasmid pCF40) has been previously described (O'Connell *et al.*, 1998). A pQE30 construct was constructed to produce the corresponding A domain protein of ClfA PY (pQE30*clfA* PY). Plasmid pCF77 PY was used as the template in a PCR reaction with primers pClfA F2 / R2. The product was cleaved with *Hind*III and *Bam*HI, ligated with pQE30 DNA cleaved with the same enzymes and cloned into *E. coli* XL1-Blue. All pQE30 constructs were transformed into the protease-deficient *E. coli* Topp 3 strain for large-scale purification. Cultures were grown to OD_{600} of 0.6 and then induced with 1 mM IPTG for a further 4 h at 37°C. Cells were harvested by centrifugation and resuspended

in $^{1}/_{200}$ original culture volume of PBS before lysis in a French pressure cell. The lysate was centrifuged at 19000 *g* for 20 min to remove cellular debris and then filtered through a 0.45 μm Sartorius filter. A HiTrap™ Chelating HP column (5 ml; Amersham Pharmacia) was equilibrated in binding buffer (5 mM imadazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and then charged with 150 mM Ni^{2+}. The filtered cleared cell lysate was applied to the column at a flow rate of 0.5 ml/min. The column was then washed with binding buffer at a flow rate of 1.5 ml/min until the $A_{280\ nm}$ of the eluate was < 0.001. Bound protein was eluted from the column with a continuous linear gradient of imadazole (5- 100 mM; total volume of 100 ml) in 0.5 M NaCl and 20 mM Tris-HCl (pH 7.9). Elution was carried out at a flow rate of 1.5 ml/min and eluted protein was collected in 3 ml fraction. Elution was monitored by measuring the $A_{280\ nm}$ of the eluate. Peak fractions were analysed by SDS-PAGE as described in section 2. 11. Ten μl of each fraction was separated through 10 % acrylamide gels and visualized by Coomassie blue staining. Fractions containing the purified recombinant protein were dialysed against PBS for 16 h at 4°C. Protein concentrations were determined using the BCA assay kit (Pierce) in accordance with the manufacturers' instructions.

2.13 Purification of human fibrinogen

Commercial sources of human fibrinogen are contaminated by other plasma proteins to some extent. Analysis of commercial fibrinogen sources by SDS-PAGE and Coomassie staining reveal a number of low-intensity contaminating bands in addition to the three major bands observed for fibrinogen (the α, β and γ chains). For platelet aggregation assays in which the roles of individual plasma factors were analysed, removal of as many contaminating proteins as possible from fibrinogen preparations was required, with particular attention paid to the depletion of IgG and fibronectin

2.13.1 Removal of contaminating IgG by protein A-sepharose

A column was poured containing 1 ml packed protein A-sepharose (Sigma) and maintained in a solution of 20 % (v/v) ethanol at 4°C. Human fibrinogen (Calbiochem) was dissolved in PBS at a concentration of 10 mg/ml and filtered through a 0.45 μm filter. Prior to addition of fibrinogen to the column, the column was washed with 10 ml PBS at room temperature. After washing, 2 ml of fibrinogen solution was added to the column, and the

flow-through collected in 15 ml tubes. A further 5 ml of PBS was added to wash out any non-specifically bound fibrinogen. IgG that was bound to the column was eluted using 5 ml of 100mM glycine, pH 2.5 and collected in a 15 ml tube. The column was then washed with PBS (10 ml) and finally with 10 ml 20 % (v/v) ethanol before storage in 20 % (v/v) ethanol at 4°C. IgG depleted fibrinogen was concentrated to 10 mg/ml using a centrifugal filtration device (Amicon) as recommened by the manufacturer. Depletion of IgG was verified by Western immunoblotting using a goat-anti-human IgG specific for the Fc portion of human IgG (Dako, Glostrup, Denmark).

2.13.2 Removal of contaminating fibronectin by anion-exchange chromatography

IgG-depleted human fibrinogen was further purified by anion-exchange chromatography to remove fibronectin and other contaminating proteins. A Hi Trap^{TM} Q sepharose column (5 ml; Amersham Pharmacia) was equilibrated in binding buffer (250 mM Tris, pH 7.8). 20 mg of IgG depleted human fibrinogen dissolved in 10 ml PBS, prepared as described in section 2.13.1, was loaded onto the column at a flow rate of 0.5 ml/min. The column was then washed with binding buffer at a flow rate of 1.5 ml/min until the $A_{280\ nm}$ of the eluate was < 0.001. Bound proteins were eluted (flow rate of 1.5 ml/min) from the column with a gradient (0 – 35 % in 100 ml) of elution buffer (25 mM Tris, pH 7.8, 1 M NaCl). Purified fibrinogen normally eluted from the column between 20 % and 30 % elution buffer. A second gradient was used (35 – 100 % in 50 ml) of elution buffer to remove any remaining proteins bound to the column. Eluted proteins were collected in 3 ml volumes and analysed by reduced SDS-PAGE and Coomassie staining. Fractions containing purified fibrinogen were pooled and dialysed against PBS for 16 h at 4°C. Protein concentrations were determined using the BCA assay kit (Pierce), concentrated to 10 mg/ml using a centrifugal filtration device (Amicon) and stored in aliquots at -20°C. Absence of contaminating fibronectin was verified by Western immunoblotting using a rabbit-anti-fibronectin antibody (Sigma).

2.14 Platelet aggregation

2.14.1 Platelet preparation

Blood was drawn from healthy human volunteers that had abstained from non-steroidal anti-inflammatory drugs during the previous 10 days using a 19-gauge butterfly needle. For

the preparation of platelet-rich plasma (PRP), 54 ml of blood was drawn into 6 ml of 3.8 % (w/v) Na citrate. The blood was aliquoted into 5 ml volumes in 15 ml tubes and centrifuged for 10 min at 150 x *g*. The PRP contained in the upper layer of each tube was carefully removed using a pasteur pipette and the samples pooled. The remaining blood was centrifuged at 720 x *g* for 10 min to yield the platelet-poor-plasma (PPP) which was used as the 100 % light transmission reference in aggregation assays using PRP as the platelet source.

For the preparation of washed gel-filtered platelets (GFP), 51 ml of blood was drawn into 9 ml ACD buffer (25 mM citric acid, 75 mM Na citrate, 135 mM D-glucose). The blood was aliquoted into 5 ml volumes in 15 ml plastic tubes and centrifuged for 10 min at 150 x *g*. The PRP contained in the upper layer of each tube was carefully removed using a pasteur pipette and the samples pooled. The pH of the PRP was adjusted to 6.5 with ACD and prostaglandin E1 (Sigma) was added at a final concentration of 1 μM. The PRP was centrifuged at 630 x *g* for 10 min to pellet the platelets. The supernatant (PPP) was removed using a pasteur pipette and the platelet pellet was carefully 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 washed platelet suspension was filtered by gel-filtration on a Sepharose 2B column (Sigma) containing 10 ml packed sepharose that had been equilibrated in JNL buffer. Flow-through fractions containing the GFPs were collected in 15 ml tubes.

2.14.2 Aggregation of platelet-rich-plasma (PRP)

PRP was prepared as described above. Activation of platelet aggregation by bacterial strains was measured by light transmission at 37°C using a PAP-4 aggregometer (BioData). A sample of PPP was used as the 100 % light transmission reference. Reactions were performed in siliconized flat-bottom glass cuvettes (BioData) at 37°C with stirring (900 rpm). Bacterial cells were washed and adjusted to OD_{600nm} of 1.6 in PBS (approximately 2 x 10^9 cells/ml). 50 μl of bacterial suspension (1 x 10^8 cells) was added to 450 μl PRP and the reaction was allowed to proceed. This resulted in a bacterium-platelet ratio of approximately 1:1. If no aggregation had occurred after 25 min the reaction was considered negative. Antibodies against platelet receptors were incubated with PRP suspensions for 20 min at 37°C prior to the addition of bacterial cells. The anti-GPIIb/IIIa monoclonal Fab fragment antibody abciximab was provided by Eli Lily. The anti-FcγRIIa monoclonal antibody IV-3 was kindly provided

by R. Klimkowski (Medarex). The anti-GPIb monoclonal antibody AN51 was purchased from Dako.

2.14.3 Aggregation of gel-filtered platelets (GFP)

GFPs were prepared as described in section 2.14.1 and used at a final concentration of 2 x 10^8 platelets/ ml. Ca^{2+} ($CaCl_2$) was added at a concentration of 1 mM. Reactions were carried out in 250 µl volumes using 225 µl GFP (containing 5 x 10^7 platelets) and 25 µl bacterial suspension (OD_{600} of 1.6) in siliconized flat-bottom glass cuvettes (BioData) at 37°C with stirring (900 rpm). Aggregation was assayed by light transmission with a sample of JNL buffer used as the 100 % light transmission reference. GFPs were supplemented with various combinations of plasma proteins prior to the addition of bacterial cells. Purified fibrinogen (prepared as described in section 2.13) was added at a final concentration 1 mg/ml. Pooled human IgG (Gammagard IVIG, Baxter) and pooled IgG samples specifically depleted of IgG recognizing bacterial antigens (see section 2.15) were used at a final concentration of 2 mg/ml. Human serum samples were diluted to a final concentration of 20 %. Human serum specifically depleted of complement components C1q, C9, and Factor B were purchased from Calbiochem.

2.15 Depletion of anti-staphylococcal antibodies from pooled human IgG samples

Pooled human IgG was depleted of antibodies recognising *S. aureus* surface proteins by either incubation with *L. lactis* cells expressing the protein of interest at high levels or by passage over the antigen that had been immobilized on a Ni^{2+}-sepharose affinity column.

2.15.1 Depletion of specific IgG with bacterial cells

A 10 ml culture of *L. lactis* NZ9800 cells expressing high levels (induced with 1.6 ng/ml nisin) of the surface protein of interest was grown for 16 h. Cells were harvested by centrifugation (3000 rpm for 10 min), washed twice in PBS and finally resuspended in 1 ml PBS in a microfuge tube. The cells were pelleted by centrifugation and resuspended in 1 ml of pooled human IgG (Gammagard IVIG, Baxter) containing 90 mg antibody. The mixture was

incubated with rotation in a blood tube rotator (Stuart Scientific) for 2 h at 4°C. The cells were pelleted by centrifugation and the IgG contained in the supernatant was removed, filtered through a 0.45 μm filter and stored at 4°C. Depletion of the target IgG was verified by ELISA as described in section 2.15.3.

2.15.2 Depletion of specific IgG by column chromatography

Recombinant staphylococcal proteins (1 mg in 5 ml PBS) containing an N-terminal His_6 affinity tag were applied to a 5 ml HiTrap™ Chelating HP column previously charged with 150 mM Ni^{2+} and equilibrated in binding buffer (5 mM imadazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The column was then washed with 20 ml binding buffer. 1 ml of pooled human IgG (100 mg protein) was repeatedly passaged over the column in a continuous flow system (0.1 ml/min) for 24 h at 4°C. Unbound IgG was collected by washing through the system with 20 ml PBS. IgG bound to the column was eluted using 10 ml of 2.5 % (w/v) glycine, pH 2.5. The unbound IgG, depleted of specific IgG for the protein of interest, was then concentrated by centrifugation using an Amicon centrifugal filter device (Millipore) to its original concentration (100 mg in 1 ml PBS) and stored at 4°C. Depletion of the target IgG was verified by ELISA as described in section 2.15.4.

2.15.3 Depletion of specific IgG recognizing the FnBPA BCD domain/fibronectin complex

The hexa-histidine recombinant protein encompassing the fibronectin-binding domains BCD of FnBPA (rBCD) was kindly supplied by Morris Knobel. The immune response against the fibronectin-binding domains of FnBPA is predominantly directed at the complex formed between the BCD domains and the 29 kDa N-terminal fibronectin domain (Casolini *et al.*, 1998). Two hundred μg rBCD was incubated with 400 μg of the purified N-terminal 29 kDa fibronectin domain (kindly provided by Pietro Speziale) with rotation for 30 minutes to form the BCD/fibronectin complex. This complex was covalently linked to a NHS-activated sepharose column (Amersham Pharmacia) following the manufacturers' protocol. 1 ml of pooled human IgG (100 mg protein) was repeatedly passaged over the column in a continuous flow system (0.1 ml/min) for 24 h at 4°C. The rest of the procedure was identical to that described in section 2.15.2.

2.15.4 Determination of specific antibody levels in IgG samples by ELISA

IgG samples were tested for reactivity with recombinant staphylococcal proteins by ELISA. Microtitre wells (Nunc Maxisorb) were incubated with 100 µl of 50 mM sodium carbonate, pH 9.5, containing 0.04 – 10 µg/ml recombinant protein for 16 h at 4°C. The wells were subsequently blocked with 100 µl BSA (2 % w/v) for 1 h at 37°C. The wells were then washed three times with PBST (0.1 % Tween 20 in PBS) and incubated with 5 µg human IgG in 100 µl 2 % (w/v) BSA in PBS for 1.5 h at room temperature. Control wells coated with recombinant staphylococcal proteins were incubated with 2 % (w/v) BSA in PBS without added antibody. Unbound antibody was removed by washing the wells five times with PBST. Bound antibody was detected by incubation (1 h at 37°C) with a goat-anti-human IgG conjugated to peroxidase (Dako, Glostrup, Denmark). After washing, binding was quantified using the substrate *p*-phenylenediamine dihydrochloride (Sigma) and measuring the absorbance at 450 nm in an ELISA plate reader. Absorbance values for control wells were subtracted from values obtained for the test wells.

ELISA assays detecting antibodies recognizing FnBPA were performed similarly. Both the recombinant FnBPA A domain protein (kindly provided by Fiona Keane) and BCD domain/fibronectin complex were immobilized in microtitre wells for 16 h at 4°C. Each well contained 500 ng of the staphylococcal antigen diluted in 100 µl 50 mM sodium carbonate, pH 9.5. After blocking of the wells with 2 % w/v BSA, IgG samples were serially diluted (0.08 – 100 µg/well) across a range of protein-coated wells and incubated for 1.5 h at room temperature. Bound antibody was detected as described above.

2.16 Depletion of IgG from human serum

IgG was depleted from human serum by passage over a column containing 1 ml packed protein A-sepharose (Sigma). The column was first washed with 10 ml PBS before the addition of a sample of human serum (1 ml). The flow-through was collected along with any remaining non-specifically bound proteins that were washed off the column with PBS into a total final volume of 10 mls. The sample was concentrated by centrifugation using an Amicon centrifugal filter device (Millipore) to its original volume (1 ml) to yield the IgG-depleted serum. IgG that was bound to the column was removed by washing with 10 ml of 2.5 % (w/v) glycine, pH 2.5. IgG-depleted and normal serum samples were analysed for the presence of IgG by Coomassie stained polyacrylamide gels (10 % separating gels) and Western

immunoblotting using a goat-anti-human IgG specific for the Fc portion of human IgG (Dako). Serum samples were diluted 1:20 before separation by SDS-PAGE.

2.17 Depletion of complement from human serum or PRP samples

2.17.1 Heat inactivation of complement

Human serum samples were thawed at room temperature. Two hundred µl samples were separated into microfuge tubes and heated in a water bath at 56°C for at least 10 min. Samples were centrifuged at 14000 rpm for 1 min to remove any precipitated material. Heated serum was left to cool at room temperature before addition to GFPs (20 % final concentration) in platelet aggregation assays (section 2.14.3).

2.17.2 Adsorption of complement from serum by zymosan treatment

Complement was removed from serum samples by treatment with the yeast polysaccharide zymosan (Sigma). Human serum samples were thawed at room temperature. Five hundred µl of serum was mixed with the complement-activating polysaccharide zymosan (100 mg; Sigma) in a microfuge tube. This mixture was transferred to a siliconized glass tube (BioData) and incubated at 37°C for 30 min with stirring (900 rpm). The serum/zymosan mixture was placed in a microfuge tube and centrifuged at 14000 rpm for 1 min to pellet the complement-coated zymosan particles. The supernatant containing the complement-depleted serum was carefully removed, aliquoted and frozen. Aliquots were thawed at room temperature before addition to GFPs (20 % (v/v) final concentration) in platelet aggregation assays (section 2.14.3).

2.17.3 Serum depleted of specific complement proteins

Human serum samples depleted in specific complement factors were purchased from Calbiochem. These were used in platelet aggregation assays with GFPs at a final concentration of 20 % (v/v).

2.17.4 Depletion of specific complement factors from PRP samples using polyclonal antibodies

Antibodies against complement components C3, C4, C5 and Factor B were purchased from Calbiochem. These were raised in either goats (anti-C3, anti-C4, and anti-factor B) or

rabbits (anti-C5). Antibodies were diluted 1/100 into PRP samples, which were incubated at 37°C for 20 min prior to the addition of bacterial cells in platelet aggregation assays (section 2.14.2).

2.18 Detection of plasma proteins binding to bacteria by ELISA

2.18.1 Detection of complement formation on the bacterial surface

L. lactis (pNZ8037) and *L. lactis* (pNZ8037*clfA* PY) cells were induced overnight with 1.6 ng/ml nisin. Washed cells were adjusted to OD_{600} of 5.0 in PBS and 300 µl cell suspension was mixed with 300 µl human plasma in siliconized flat-bottom glass cuvettes (BioData) at 37°C with stirring (900 rpm). Control plasma samples, in which complement was inactivated by heating (56°C for 30 min) followed by centrifugation, were run in parallel with the test samples. At selected time points, 100 µl volumes of bacterial/plasma mixtures were removed into tubes containing 10 µl 100 mM EDTA and kept on ice to stop the complement reaction. Cells were washed extensively (4 washes) in PBS before resuspension in 200 µl 50 mM sodium carbonate, pH 9.6. Cells were coated on microtitre dishes (Nunc Maxisorb) for 16 h at 4°C. The wells were blocked with 2 % (w/v) BSA in PBS for 2 h at 37°C and washed with PBS. Rabbit-anti-C5b9 antibodies (Calbiochem) were diluted 1:2000 in BSA/Tween and incubated with the wells for 1.5 h at room temperature. Bound antibody was detected by incubating the washed wells with goat-anti-rabbit antibodies conjugated to horseradish peroxidase (Dako). After washing, binding was quantified using the substrate *p*-phenylenediamine dihydrochloride (Sigma) and measuring the absorbance at 450 nm in an ELISA plate reader.

2.18.2 Binding of plasma IgG to bacterial cells

The total IgG content in human plasma samples recognising *L. lactis* (pNZ8037) and *L. lactis* (pNZ8037*clfA* PY) was determined by ELISA. *L. lactis* strains were induced with 1.6 ng/ml nisin for 16 h. Cells were washed and adjusted to OD_{600} of 1.0 in 50 mM sodium carbonate, pH 9.5. Wells in microtitre dishes (Nunc Maxisorb) were coated with 100 µl cell suspension for 16 h at 4°C. Unbound cells were removed by washing and wells were then blocked with 2 % (w/v) BSA in PBS for 2 h at 37°C. Plasma samples were serially diluted (starting dilution $^1/_5$ in PBS) across a range of bacterial-coated wells and incubated for 1.5 h at

room temperature. The wells were washed five times with PBS and bound IgG was detected using goat-anti-human antibody conjugated to peroxidase (Dako). Binding was quantified as described above (section 2.18.1).

2.19 Platelet adhesion assays

The binding of resting, non-activated platelets to bacterial cells was measured as described previously (Kerrigan *et al.*, 2002). *L. lactis* cells expressing staphylococcal proteins were grown for 16 h in the presence of 1.6 ng/ml nisin, harvested by centrifugation and washed twice in PBS. *S. aureus* strains were grown to exponential phase (OD_{600} of 0.5 to 0.6) and washed in PBS. Bacteria were adjusted to an OD_{600} of 1.0 in PBS and 100 µl of bacterial suspension used to coat wells in 96-well flat bottomed plates (Nunc Maxisorb). Bacteria were allowed to stick to the wells for 2 h at 37°C. The wells were then blocked with a 2 % (w/v) solution of BSA that had been sterilized by filtration for a further 2 h at 37°C. During this incubation, washed GFPs were prepared as described in section 2.14.1. GFP samples were treated with GPIIb/IIIa inhibitors abciximab (20 µg/ml; Eli Lily) or tirofiban (20 µg/ml; Merck) as appropriate for 20 min at 37°C. Plasma (10 % final concentration), human fibrinogen (1 mg/ml), or human fibronectin (0.2 mg/ml) were added to the platelets where indicated just prior to the addition to the bacteria-coated wells. The blocking solution was aspirated off and the wells were washed three times with JNL buffer (150 µl/well). Platelet preparations (1 x 10^7 platelets) were added to the wells and incubated for 30 min at 37°C. Unbound platelets were washed off by washing the wells three times with JNL buffer. Adherent platelets were detected by using a lysis buffer containing a substrate for acid phospatase (100 mM Na acetate, 0.1 % (v/v) Triton-X-100, 10 mM *p*-nitrophenol phosphate (Sigma)). Plates were incubated in the dark at 37°C. Plates were then read after 2 h in an ELISA plate reader at 405 nm.

2.20 Calcium flux

Direct analysis of platelet activation was performed by measuring cytosolic increases in free Ca^{2+}. This increase (calcium flux) is one of the earliest markers of platelet activation.

Whole blood was drawn into ACD buffer and the PRP was isolated as described in section 2.14.1. The pH of the PRP was adjusted to 6.5 with ACD and prostaglandin E_1 was added at a final concentration of 1 μM. The platelets were pelleted by centrifugation and washed in 1 ml JNL buffer. The cell-permeable calcium ionophore FURA-2-AM (5 μM; Calbiochem) was incubated with the platelets for 30 min at 37°C in the dark. FURA-2-AM is a fluorescent calcium chelator (K_d = 224 nM) whose spectral properties differ in the presence of low calcium concentrations (excitation max: 362 nm, emission max: 512 nm) and high calcium concentrations (excitation max: 335 nm, emission max: 505 nm) allowing the monitoring of variations in the concentration of intracellular Ca^{2+}. Platelets loaded with FURA-2-AM were filtered on a column containing 10 ml packed sepharose 2B as described in section 2.14.1. GFPs were suspended in 50 % (v/v) plasma in JNL buffer to a concentration of 2 x 10^8 platelets/ml. For some experiments, GFPs were suspended in JNL buffer containing fibrinogen (1 mg/ml) and pooled human IgG (4 mg/ml). In other experiments, washed bacteria (10^9 cells) were incubated in plasma, washed in PBS, and added to FURA-2-labelled GFPs suspended in JNL buffer. Intracellular Ca^{2+} changes were detected after addition of 10^8 bacterial cells to 8 x 10^7 FURA-2-AM loaded GFPs using an LS-50 fluorimeter (Perkin Elmer, Norwalk, CT). Cells were stirred at 37°C in a quartz cuvette. FURA-2 fluorescence was measured following excitation at wavelengths 340 and 380 nm, with emission at 510 nm.

2.21 Statistical analysis

The statistical significance of differences between aggregation samples was determined by the Student t-test, using the online GraphPad software. Differences were considered significant if p values were less than 0.05.

2.22 Bioinformatic analysis of variation in FnBPA and FnBPB

Finished *S. aureus* genome sequences (strains COL, N315, Mu50, MW2, MSSA476 and MRSA252) were searched for proteins displaying homology to FnBPA or FnBPB from *S. aureus* 8325-4. All strains, except MRSA252, contained both an FnBPA-like and an FnBPB-like protein. Strain MRSA252 contained a single FnBP that was more similar to $FnBPA_{8325\text{-}4}$ than to $FnBPB_{8325\text{-}4}$, and was designated as FnBPA in this analysis. Subdomains of FnBPs

from the sequenced strains (A domain and BCD domains) were defined based on amino acid alignments with the FnBPs from strain 8325-4 (Signäs *et al.*, 1989; Jönsson *et al.*, 1991). Pairwise alignments of FnBPs were performed the ExPasy SIM-alignment tool (http://us.expasy.org/), and the percentage of identical amino acids between the aligned proteins was noted. Multiple alignments of FnBP proteins were performed using the ExPasy T-coffee program.

Regions of the *fnbA* and *fnbB* genes from *S. aureus* P1 were amplified by PCR using primer pairs (pFnBPA F4 / R4 and pFnBPB F2 / R2 respectively) and *S. aureus* P1 genomic DNA as the template. Primers were designed based on the *fnbA* and *fnbB* genes from strain 8325-4. The amplified fragments encompassed the entire coding region for the A domain of FnBPA or FnBPB plus some flanking sequence. DNA was cleaved with *Bam*HI and ligated to *Bam*HI-cleaved pBluescript KS+ DNA. Ligation mixtures were transformed into *E. coli* XL1-Blue cells, generating the recombinant plasmids pBluescript::*fnbA* P1 and pBluescript::*fnbB* P1. Plasmid DNA was isolated and the inserted fragment was sequenced by Lark Technologies. DNA sequences were translated into amino acid sequences and assembled to generate the entire A domain amino acid sequences of FnBPA or FnBPB from *S. aureus* P1. The similarity of the P1 A domains to the corresponding domains from the sequenced *S. aureus* strains was analysed as described above.

Chapter 3

Molecular analysis of human platelet activation promoted by *Staphylococcus aureus* clumping factor A

3.1 Introduction

S. aureus is now recognized as the leading cause of infective endocarditis (IE) (Moreillon and Que, 2004; Fowler *et al.*, 2005). Vegetations removed from infected heart valves are observed to contain clusters of bacteria, fibrin and aggregated platelets (Mylonakis and Calderwood, 2001). The interaction between *S. aureus* and human platelets is postulated to be an important aspect in the development of IE (Sullam *et al.*, 1996). Isogenic *S. aureus* mutants defective in binding directly to platelets have been shown to be less virulent in endocarditis models (Sullam *et al.*, 1996; Siboo *et al.*, 2005). Bacterial binding to platelets in developing sterile vegetations may facilitate colonization, thus establishing a focus of infection and subsequent bacterial proliferation. *In vitro*, the binding of *S. aureus* to platelets suspended in plasma results in rapid platelet activation, leading to aggregate formation (O'Brien *et al.*, 2002; Liu *et al.*, 2005). Also, perfusion of whole blood at physiological shear rates over immobilized *S. aureus* cells results in the development of stable platelet thrombi (Sjöbring *et al.*, 2002; S. Kerrigan, unpublished data). It has been observed that the ability of *Strep. sanguis* strains to aggregate platelets correlates with enhanced infectivity in experimental endocarditis models (Herzberg *et al.*, 1992). Platelet activation by *S. aureus* cells, either immobilized in developing thrombi or upon extracellular matrix components on the endocardial surface, could lead to thrombus formation, resulting in vegetation development and enhancement. *S. aureus* is an important cause of endocarditis in patients with no known cardiac problems (Moreillon and Que, 2004). The ability of *S. aureus* to directly activate platelets, forming bacterium-platelet micro-aggregates that may subsequently deposit on the valve surface, is postulated to be important in establishing vegetations on healthy heart valve tissue.

Although much research has focused on the interactions between *S. aureus* and platelets (reviewed in Section 1.5.3), there are many questions that remain unanswered. In particular, very little has been published on the specific *S. aureus* factors that mediate platelet activation and aggregation. The aggregation of platelets by bacteria is thought to be the result of a multi-step process (Clawson, 1973). Initially, receptors expressed by bacterial cells recognize receptors on the platelet surface, resulting in bacterial-platelet adhesion. Adhesion is then followed by activation; bacterial-platelet binding triggers signaling events and calcium oscillations in the platelet. Platelet activation results in conformational changes in the major platelet integrin GPIIb/IIIa (Nesbitt *et al.*, 2003). This conformational change

transforms GPIIb/IIIa from a low-affinity to a high-affinty state, exposing the binding site for fibrinogen (Xiao *et al.*, 2004). Binding of the γ-chain of bivalent plasma glycoprotein fibrinogen to activated GPIIb/IIIa results in cross-linking of adjacent platelets into aggregates. This is the process of platelet aggregation. Bacterial-mediated platelet aggregation is characterized by varying lag phases between the addition of bacteria to platelet samples and the onset of aggregation; this is known as the lag time. For *in vitro* analysis of platelet function, the aggregation of platelets is the most convenient measure of platelet activation.

The ability of *S. aureus* cells to induce platelet aggregation is thought to be mediated by surface-expressed proteins, as trypsin-treated *S. aureus* cells could not support aggregation (Hawiger *et al.*, 1979; Kessler *et al.*, 1991; Bayer *et al.*, 1995). The first report on platelet aggregation by a panel of surface protein-deficient isogenic mutants of a single *S. aureus* strain (strain Newman) demonstrated conclusively roles for clumping-factors (ClfA and ClfB) and the serine-aspartate repeat protein E (SdrE), while protein A played an auxiliary role (O'Brien *et al.*, 2002). Cells grown to stationary phase triggered faster aggregation (lag time of 1.5 min) than exponentially grown cells (lag time of 5 min). The rapid aggregation stimulated by stationary phase cells was due to the high-level of ClfA expression in this growth phase; a *clfA* mutant triggered aggregation with significantly longer lag times (> 8 min) whereas strains containing single mutations in either the protein A (*spa*) gene or an *sdrE* mutant were not altered in their ability to aggregate platelets. It should be noted that ClfB is only expressed in the exponential phase of growth, and therefore did not contribute to platelet aggregation by stationary phase *S. aureus*. Using double and triple surface protein-deficient mutants revealed that slow aggregation in the absence of ClfA was due to SdrE and protein A. Expression of candidate surface proteins in the non-aggregating surrogate host *L. lactis* demonstrated that ClfA, ClfB or SdrE could independently activate platelet aggregation, with lag times of 1.5, 7, and 13 min respectively. ClfA therefore appears to be the major pro-aggregatory protein expressed by *S. aureus* Newman, and can activate platelets in the absence of other *S. aureus* receptors. Likewise, ClfB and SdrE can independently activate platelet aggregation, although with longer lag times than ClfA indicating that these factors may play an accessory role. Due to the lack of selectable markers for creating multiple mutants, a similar approach was not conducted on exponentially growing cells, and roles for other factors (in addition to ClfA, ClfB, SdrE and Spa) could not be excluded. This clearly showed that platelet aggregation by *S. aureus* cells is a complex, multifactorial process. The interactions

between ClfA, ClfB and SdrE and the platelet, such as the platelet receptors that recognize the *S. aureus* antigens, were not elucidated in this study (O'Brien *et al.*, 2002).

The O'Brien *et al* study identified ClfA as the major pro-aggregatory molecule expressed by strain Newman. ClfA has been implicated as an important factor mediating the interaction of *S. aureus* and platelets (Sullam *et al.*, 1995; 1996; Siboo *et al.*, 2001; O'Brien *et al.*, 2002; Sjobring *et al.*, 2002), and in the pathogenesis of endocarditis (Moreillon *et al.*, 1995; Stutzmann-Meier *et al.*, 2001; Siboo *et al.*, 2001; Que *et al.*, 2001, 2005). Administration of human immunoglobulin containing elevated levels of antibodies recognizing ClfA has been shown to facilitate sterilization of valvular vegetations in a rabbit endocarditis model (Vernachio *et al.*, 2003). ClfA is expressed by all *S. aureus* strains examined (Hall *et al.*, 2003) and may be an attractive target for the development of novel therapeutics to treat endocarditis caused by *S. aureus*. The widespread distribution of antibiotic-resistant clinical *S. aureus* isolates highlights the need for new therapeutic approaches. A detailed understanding of the mechanisms of platelet activation by *S. aureus* is required if new treatments are to be development. Although ClfA was identified as a major mediator of platelet activation, the mechanism by which this occurs was not elucidated. This is the subject of the research described in this chapter.

An important issue to be addressed was the role for fibrinogen-binding by ClfA in stimulating platelet activation. ClfA binds avidly to fibrinogen, which is a very abundant blood glycoprotein (approximately 2.5 - 3 mg fibrinogen per ml plasma; Standeven *et al.*, 2005). It is likely that *S. aureus* cells in the bloodstream are covered to some extent by a fibrinogen-coat (O'Connell *et al.*, 1998; Massey *et al.*, 2002). It is widely presumed that the ability of ClfA to bind and activate platelets is due to fibrinogen-bridging between ClfA and GPIIb/IIIa (Pawar *et al.*, 2004; Liu *et al.*, 2005). However there is also evidence that ClfA binds directly to a 118 kDa platelet membrane protein (Siboo *et al.*, 2001). The identity of this membrane protein is not currently known. Issues such as the effect of fibrinogen binding to ClfA on this interaction or its biological significance in bacterial binding to platelets have not been addressed. Difficulties in establishing a role for fibrinogen binding in platelet activation arise from the requirement of exogenous fibrinogen for aggregation stimulated by various agonists. Strong agonists such as thrombin induce sufficient release of platelet granule contents, one of which is fibrinogen, such that aggregation can occur in platelets separated from plasma (Zucher, and Nachmias, 1985). Other weaker agonists, like ADP, can only stimulate full platelet aggregation if fibrinogen is added to purified platelet suspensions

(Hawiger *et al.*, 1982; Hettasch *et al.*, 1992). Bacteria are thought to be relatively weak agonists, and aggregation of gel-filtered-platelets (GFP) by *S. aureus* Newman required the addition of exogenous fibrinogen (O'Brien *et al.*, 2002). The precise role of fibrinogen in *S. aureus*-induced aggregation was not elucidated. One possibility was that *S. aureus* could bind directly to platelets resulting in activation, and fibrinogen was then required for aggregation through cross-linking activated GPIIb/IIIa receptors on adjacent platelets. Alternatively, fibrinogen acted as a bridging molecule between bacterium and the platelet, and this binding resulted in activation and aggregation.

It is thought that ClfA binds fibrinogen through the "dock, lock and latch" mechanism (see section 1.3.2.4.3) and the crystal-structure of the minimum ligand binding domain (N2N3 of the A domain) has been determined (Deivanayagam *et al.*, 2002; Figure 3.1). Residues lining the trench between the DEv-IgG folded N2 and N3 domains crucial for fibrinogen-binding were predicted based on this structure (Deivanayagam *et al.*, 2002). Recombinant ClfA proteins with substitutions in either residues Pro_{336} or Tyr_{338} were found to be completely defective in fibrinogen-binding (Deivanayagam *et al.*, 2002). These mutants, if expressed on the surface of a bacterial cell, could be used to analyze the requirement of ClfA-bound fibrinogen in the platelet activation process.

The aggregation of platelets by *S. aureus* is a complex and multifactorial process with at least 4 surface proteins (ClfA, ClfB, SdrE, SpA) playing a role (O'Brien *et al.*, 2002; Pawar *et al.*, 2004). Therefore difficulties occur in the analysis of the contribution of single staphylococcal factors to the platelet aggregation phenotype. Despite the fact that ClfA was shown to be the dominant factor for stationary phase *S. aureus* Newman, a *clfA* mutant still aggregated platelets, albeit with an extended lag time of 8 to 10 minutes (O'Brien *et al.*, 2002). This functional redundancy makes the use of surrogate host expression systems invaluable for the analysis of the staphylococcal-platelet interaction. *Lactococcus lactis* is a gram-positive bacterium widely used in the food industry as a starter culture, and is amenable to genetic manipulation. Staphylococcal surface proteins have been expressed successfully on the surface of *L. lactis* in fully-functional forms (Hartford *et al.*, 2001; Que *et al.*, 2001, 2005; O'Brien *et al.*, 2002). Since *L. lactis* does not naturally aggregate platelets *in vitro*, it is an attractive host for studying the role of *S. aureus* proteins involved in this process. Expression of ClfA on the surface of *L. lactis* results in rapid platelet aggregation, with a lag time of 1 to 2 minutes in platelet-rich-plasma (O'Brien *et al.*, 2002), whereas the *L. lactis* host strain did not stimulate platelet aggregation. These lag times were comparable to that observed for *S. aureus*

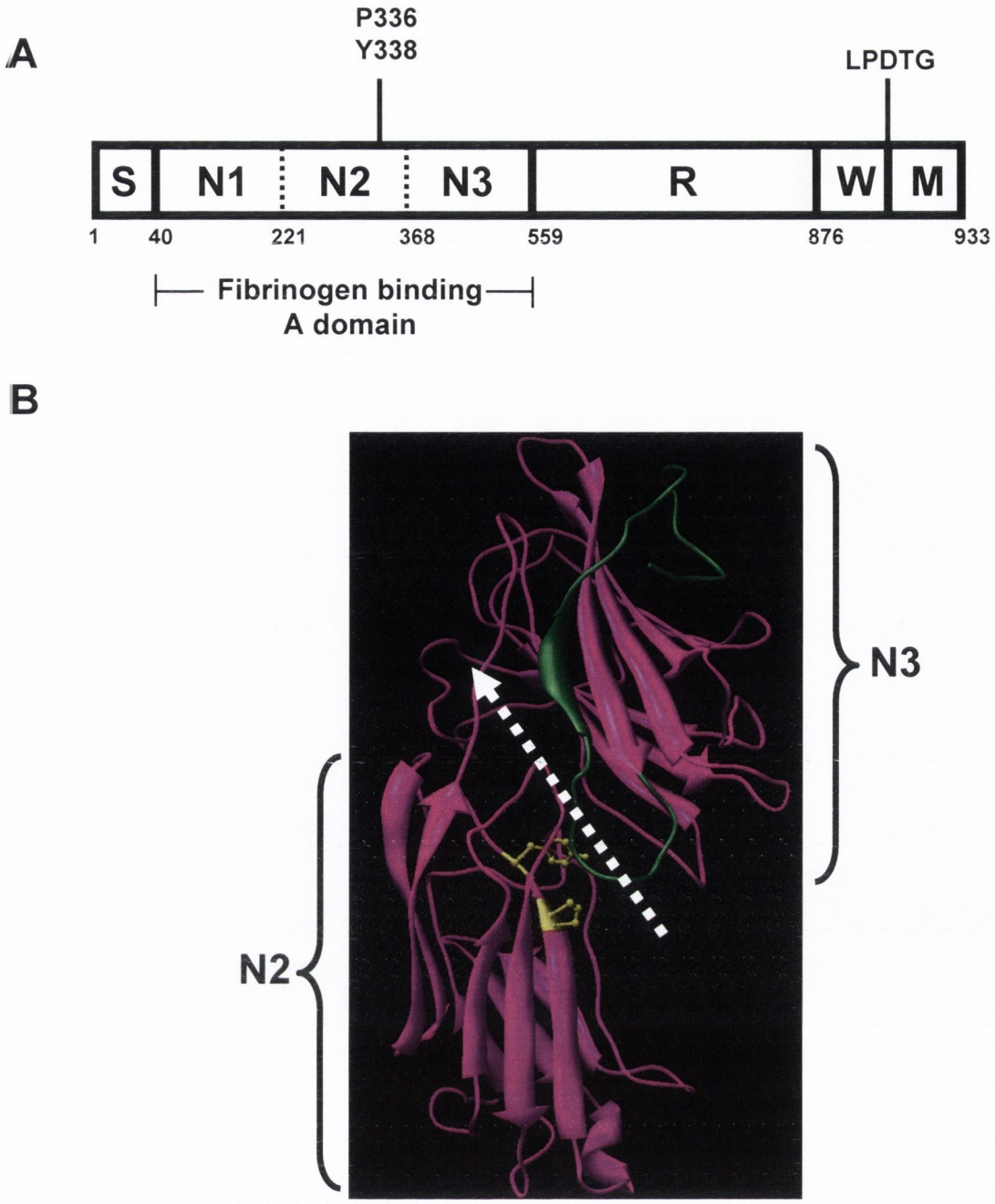


Figure 3.1. Schematic representation and structure of ClfA

A. The N-terminus of ClfA contains a signal sequence of 40 residues that targets the protein for secretion. The fibrinogen-binding domain (A domain) comprises subdomains N1, N2 and N3. The minimum ligand binding truncate of ClfA is N2N3 (residues 221-559). Amino acids P_{336} and Y_{338} 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.
B. Ribbon-diagram of the determined crystal structure of minimum ligand-binding domain of ClfA (Domain N2N3), kindly provided by F. Keane. Each domain consists of a variant-IgG fold. The trench formed between N2 and N3 (denoted by white dashed arrow) is the binding site for the fibrinogen γ-chain. Residues Pro_{336} and Tyr_{338} are shown in yellow.

Newman stationary phase cells, which are known to express high levels of clumping factor. Clearly, ClfA is a potent activator of platelets and requires no additional *S. aureus* factors in order to do so. A *L. lactis* expression system was employed in this study where expression of the gene of interest was controlled by the lantibiotic peptide nisin.

Most heterologous expression systems used to express staphylococcal surface proteins have utilized constitutive promoters driving gene expression, resulting in high-level expression of the protein-of-interest (Que *et al.*, 2000; Sinha *et al.*, 2000; Hartford *et al.*, 2001; O'Brien *et al.*, 2002). Expression levels achieved in these systems may not accurately reflect the expression levels achieved in the native host. The nisin-controlled gene expression system (NICE) in *L. lactis* is a widely used expression system for regulatable, heterologous protein production in gram-positive bacteria (Mierau and Kleerebezem, 2005). Nisin is a small, lantibiotic peptide produced and secreted by some strains of *L. lactis*. It binds to lipid II in the cytoplasmic membranes of bacteria; this is followed by rapid permeabilization of the membrane and subsequent cell death (Hasper *et al.*, 2004). Production of nisin by *L. lactis* may confer a survival advantage in competitive environments. The nisin structural gene (*nisA*) is contained on the conjugative transposon Tn5276 (Kuipers *et al.*, 1993). This element contains eleven genes involved in nisin biosynthesis, modification, immunity and export (Kuipers *et al.*, 1993; Siegers and Entian, 1995). The *nisA* promoter is regulated by the mature extracellular nisin peptide through the two-component NisRK system (Kuipers *et al.*, 1995). Extracellular nisin is recognized by the sensor histidine-kinase NisK, resulting in auto-phosphorylation of the response-regulator NisR. Phosphorylated NisR acts as a transcriptional activator, and activates transcription by binding specific sequences in the *nisA* promoter (Kuipers *et al.*, 1995). Using *L. lactis* strains that are defective in nisin-production, expression driven by the *nisA* promoter in expression plasmids can be regulated by the concentration of nisin added to the growth medium (de Ruyter *et al.*, 1996). This nisin-controlled expression system was used here to control the expression of ClfA on the surface of *L. lactis*. This allowed the relationship between the level of ClfA expression and the ability to activate platelets to be investigated.

Further questions that remained unanswered with respect to the nature of ClfA-mediated platelet activation were the identity of the platelet receptors involved in recognizing bacteria expressing ClfA, or the possible role of plasma factors other than fibrinogen in stimulating activation.

ClfA and a non-fibrinogen binding mutant ClfA (with substitutions $Pro_{336}Ser$ and $Tyr_{338}Ala$; called ClfA PY) were expressed on *S. aureus* and *L. lactis* to reveal if fibrinogen-binding by ClfA is essential for platelet activation. Controlled expression of ClfA using a nisin-inducible promoter allowed investigation of the expression level of ClfA required to activate platelets. A range of inhibitory antibodies against platelet receptors allowed identification of those receptors involved in recognizing ClfA-expressing cells. Reconstitution experiments with washed platelets allowed identification of plasma factors required for activation to occur. Taken together, the data presented here allow a model to be proposed for the activation of platelet aggregation by bacteria expressing ClfA. This model is compared to proposed mechanisms for platelet activation by other bacterial species.

3.2 Results

3.2.1 Expression of ClfA and a non-fibrinogen-binding ClfA mutant (ClfA PY) in *S. aureus*

S. aureus strain Newman is the archetypal clumping factor producing strain. The *clfA* gene was originally cloned from this strain, and an isogenic *clfA* mutant grown to stationary phase was defective in adherence to immobilized fibrinogen and cell-clumping in soluble fibrinogen (McDevitt *et al.*, 1994). Newman *clfA* mutants grown to exponential phase still possess fibrinogen adherence and clumping phenotypes, due to expression of another clumping factor (ClfB; Ni Eidhin *et al.*, 1998). ClfA is arguably one of the best characterized surface proteins of *S. aureus*. The crystal structure of the minimum ligand-binding truncate of ClfA (N2N3 of the A domain; Fig. 3.1) is known (Deivanayagam *et al.*, 2002). Based on this structure, it was predicted that residues Pro_{336} and Tyr_{338} were important for fibrinogen-binding by ClfA. Recombinant ClfA A domain mutants with the $Pro_{336}Ser$ or $Tyr_{338}Ala$ substitutions were defective in fibrinogen-binding (Deivanayagam *et al.*, 2002). To address the requirement for fibrinogen-binding by ClfA in stimulating platelet activation, it was necessary to express ClfA and the ClfA PY mutant on the surface of *S. aureus*. It was important to demonstrate that the ClfA PY mutant, when expressed on a bacterial cell, had the same fibrinogen-binding defect as observed for the recombinant protein. Multicopy plasmids containing the genes encoding ClfA (*clfA*; plasmid pCF77; Figure 3.2) and ClfA PY gene

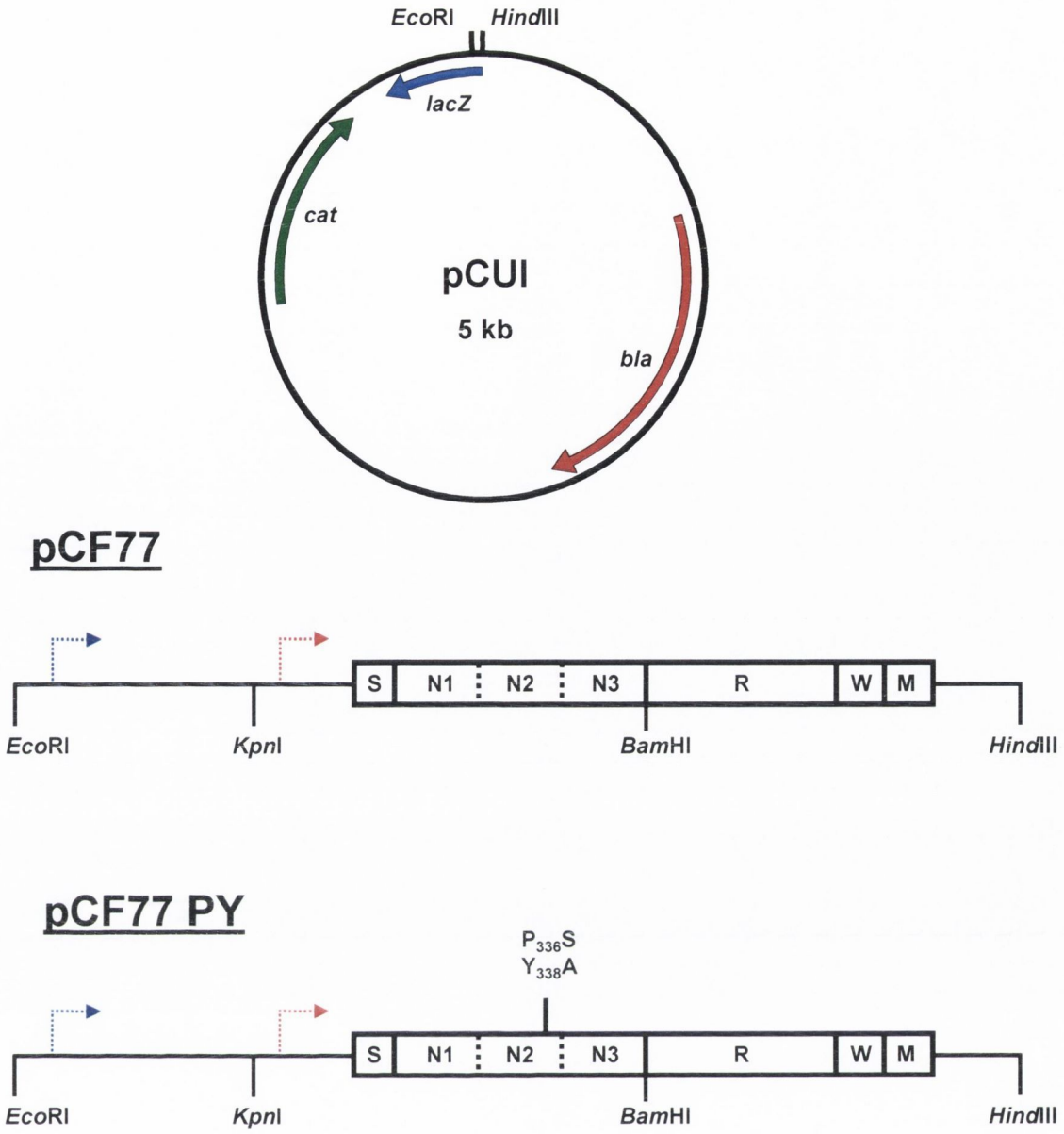


Figure 3.2. ClfA expression plasmids for *S. aureus*

Plasmid pCUI is an *E. coli-S. aureus* shuttle plasmid. DNA is cloned between the *Eco*RI and *Hind*III sites within the *lacZ* gene, facilitating detection of clones. The *bla* gene confers ampicillin resistance in *E. coli* and the *cat* gene confers chloramphenicol resistance in *S. aureus*. Plasmid pCF77 contains the full length *clfA* gene with an engineered *Bam*HI site between regions N3 and R (Hartford *et al.*, 1997). Approximately 1 kb upstream sequence is present to encompass the entire promoter region of *clfA*. A PCR-generated DNA fragment with mutations encoding the substitutions $Pro_{336}Ser$ and $Tyr_{338}Ala$ was cloned between the upstream *Kpn*I site and the downstream *Bam*HI site of pCF77 to generate plasmid pCF77 PY (J. Higgins, personal communication). The red arrow denotes a sigma factor 70-dependent promoter and the blue arrow denotes a sigma factor B-dependent promoter located approximately 1 kb upstream of the *clfA* ORF.

(*clfA* PY; plasmid pCF77 PY; Figure 3.2) were transformed into the restriction-deficient *S. aureus* strain RN4220 and subsequently transduced using phage 85 into a *S. aureus* Newman *clfAclfB* double mutant as described in section 2.4.

To demonstrate expression of ClfA and ClfA PY from the pCUI-derived multicopy plasmids, whole-cell immunoblotting was performed. For this experiment, it was necessary to introduce a mutation in the gene encoding the *S. aureus* IgG Fc receptor protein A (*spa*) to prevent non-specific IgG binding. This mutation (*spa*::kan) was transduced using phage 85 from a Newman *spa* strain into the Newman *clfAclfB* (pCF77) derivatives. The *clfA* and *clfA* PY genes in the multicopy plasmids are controlled by their own promoter. *S. aureus* Newman *clfAclfBspa* cells and its derivatives containing pCF77 and pCF77 PY were grown to stationary phase (where ClfA is maximally expressed). Serial dilutions of bacterial cells, starting at an OD_{600} of 1.0 were spotted onto nitrocellulose membranes and probed for ClfA expression using polyclonal rabbit antibodies raised against the A domain of ClfA. A representative dot-blot is shown in Figure 3.3. Expression of ClfA from pCF77 and ClfA PY from pCF77 PY in Newman *clfAclfBspa* was approximately the same as the level of wild-type ClfA expressed by the Newman *spa* strain (Figure 3.3). Antibodies did not bind to the Newman *clfAclfBspa* mutant. This demonstrates that equivalent levels of ClfA and ClfA PY were expressed on the bacterial cell-surface, allowing direct comparison in fibrinogen-binding assays.

The ability of washed cells to adhere to immobilized fibrinogen (section 2.8) and to clump in soluble fibrinogen (section 2.9) was tested. Human fibrinogen was coated onto 96-well dishes ranging from 2 μg/well to 4 ng/well. Wild-type *S. aureus* Newman adhered with high-affinity to the fibrinogen-coated wells in a dose-dependent and saturable manner (Figure 3.4). The Newman *clfAclfB* double mutant was defective in fibrinogen adherence (Figure 3.4). Expression of ClfA from pCF77 restored adhesion to levels comparable with the wild-type strain (Figure 3.4). Expression of ClfA PY on the surface of *S. aureus* Newman *clfAclfB* did not result in adherence of cells to fibrinogen (Figure 3.4).

The ability of the Newman derivatives expressing ClfA and ClfA PY to form clumps in soluble fibrinogen was tested, as this would be more representative of the conditions in platelet aggregation assays with plasma (approximately 3 mg/ml fibrinogen in plasma). Cell-clumping only occurred upon expression of the wild type ClfA protein (in the wild-type strain and the *clfAclfB* mutant complemented with pCF77; Figure 3.5). Neither the *clfAclfB* double mutant nor Newman *clfAclfB* expressing ClfA PY formed clumps in fibrinogen (Figure 3.5).

These results demonstrate that ClfA PY is defective in fibrinogen-binding when expressed on *S. aureus*, in agreement with recombinant protein studies (Deivanayagam *et al.*, 2002).

3.2.2 Activation of platelet aggregation by *S. aureus* expressing ClfA and ClfA PY

The *S. aureus clfAclfB* mutants expressing ClfA or ClfA PY were tested for their ability to activate platelet aggregation. Washed stationary phase cells were adjusted to OD_{600} of 1.6 in PBS. 50 µl of bacterial suspension (1 x 10^8 cells) was added to 450 µl platelet-rich-plasma (PRP). This resulted in a ratio of approximately one bacterium per platelet, which has been observed previously to produce maximum aggregation (D. Cox, personal communication). Platelet aggregation was measured by turbidometry in a platelet aggregometer. The 100 % light transmission was set using a 500 µl aliquot of platelet-poor-plasma (PPP). The lag time (i.e the time taken for the onset of aggregation after addition of bacteria to platelet samples) for each strain was measured. The wild-type Newman strain activated platelet aggregation with very short lag times (1.5 ± 0.25 min; n = 3; Figure 3.6). The *clfAclfB* mutant activated platelets with a significantly longer lag time (23 ± 4.5 min, n = 3, $p < 0.05$; Figure 3.6). Expression of ClfA from pCF77 resulted in lag times comparable to the wild-type (0.9 ± 0.1 min; n = 3; Figure 3.6). Newman *clfAclfB* expressing ClfA PY stimulated aggregation with a lag time of 9.6 ± 4.2 min (n = 3). A number of conclusions can be drawn from these results. Firstly, the ability of ClfA to stimulate rapid aggregation is associated with its ability to bind fibrinogen, suggesting a fibrinogen-dependent mechanism is involved. Furthermore, while the lag time for ClfA PY-expressing *S. aureus* is relatively long, it is significantly ($p < 0.05$) shorter than the parent *clfAclfB* strain. It would appear then that ClfA PY may participate in platelet activation when expressed on the surface of *S. aureus*. If so, this would be due to a fibrinogen-independent mechanism of activation. This is investigated further in section 3.2.4. The *clfAclfB* mutant activated aggregation with long lag times of > 20 min. This may be due to expression of SdrE, previously shown to activate platelets when expressed constitutively on the surface of *L. lactis* with long lag times (10 – 17 min; O'Brien *et al.*, 2002). To analyze the putative fibrinogen-independent mechanism of platelet activation by ClfA, it was necessary to express ClfA PY in a surrogate host that does not naturally activate platelets, such as *L. lactis*.

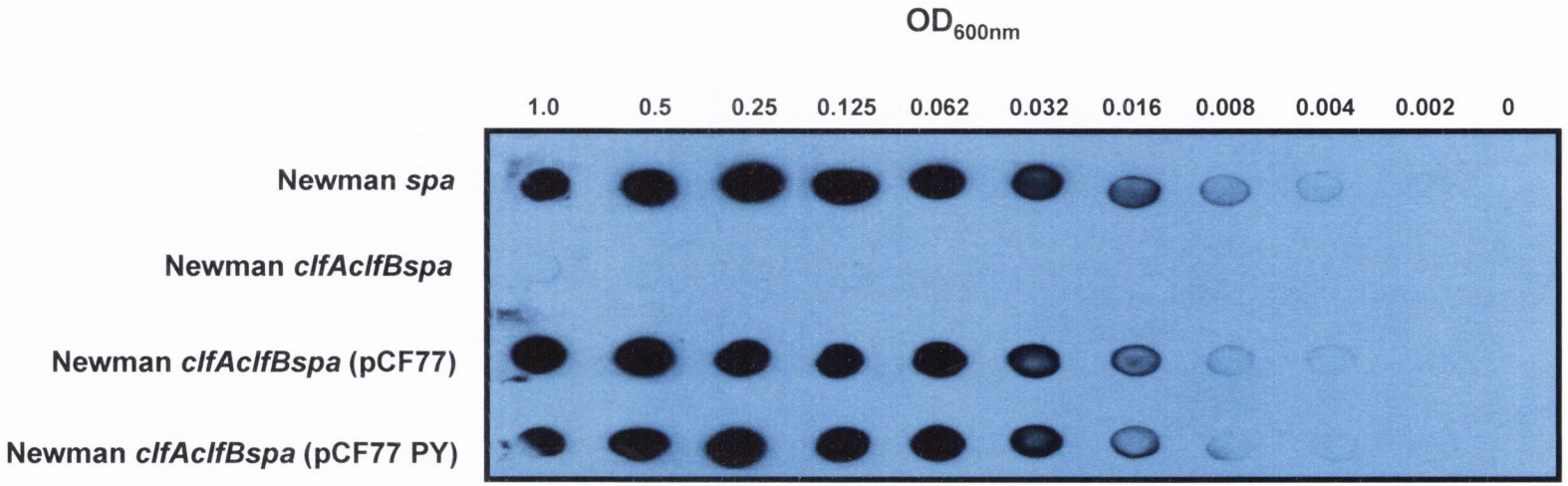


Figure 3.3. Whole-cell dot immunoblot of ClfA and ClfA PY expression on *S. aureus*

Cells were grown to stationary phase and washed twice in PBS. Cells were adjusted to OD_{600} of 1.0 in PBS and serially diluted in 96-well dishes. Five μl of each cell dilution was spotted onto nitrocellulose membranes and allowed to air-dry. Membranes were blocked with skimmed milk solution for 16 h and subsequently probed with rabbit-anti-ClfA antibodies (1:5000 dilution) followed by goat-anti-rabbit antibodies conjugated to horseradish peroxidase (1:2000 dilution). Membranes were developed by chemiluminescence and exposed to autoradiographic film.

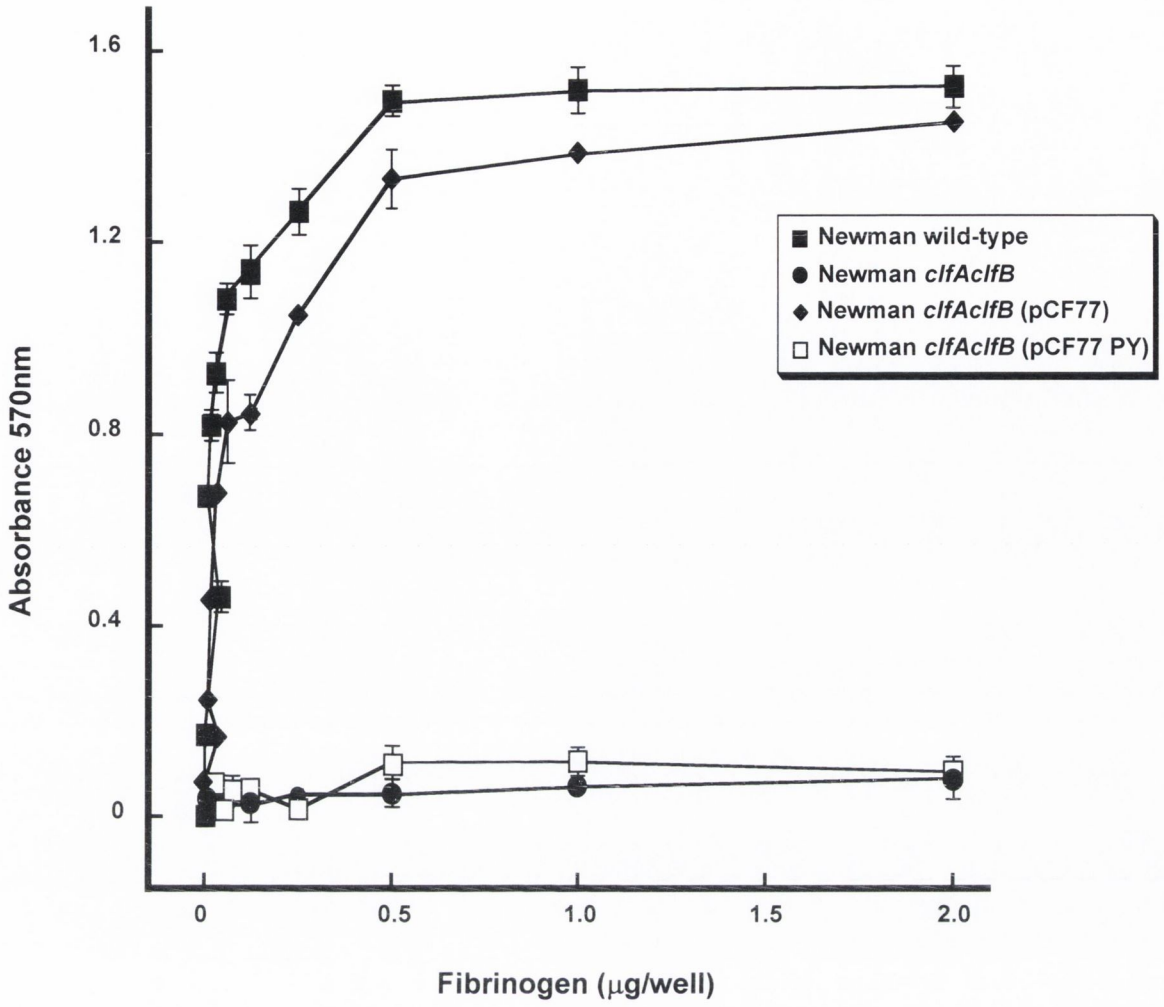


Figure 3.4. Adherence of *S. aureus* Newman derivatives expressing ClfA and ClfA PY to immobilized fibirinogen

The ability of *S. aureus* Newman *clfAclfB* and its derivatives expressing ClfA (pCF77) and ClfA PY (pCF77 PY) to adhere to wells coated with human fibrinogen (4 – 2000 ng/well) was tested. Adherent cells were stained with crystal violet and the absorbance of the wells at 570 nm was read.

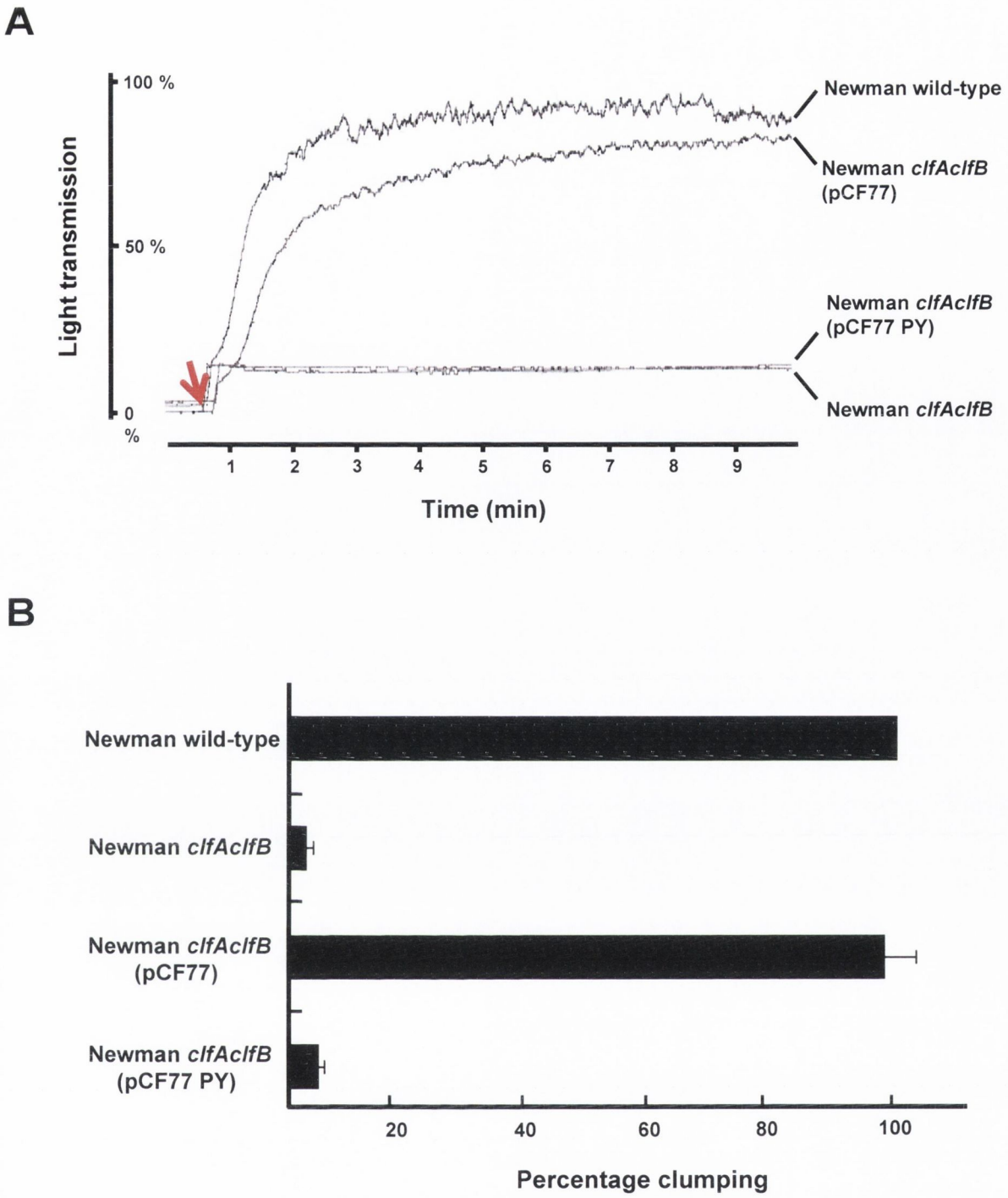


Figure 3.5. Clumping of *S. aureus* Newman derivatives expressing ClfA and ClfA PY in soluble fibrinogen

A. Cell-clumping was measured by turbidometery using a platelet aggregometer. Bacterial suspension (200 μl of OD_{600} 2.0) was incubated in siliconized glass tubes at 37°C with stirring (900 rpm). Fibrinogen (50 μl of 10 mg/ml solution) was added (red arrow) and clumping allowed to occur.

B. Data from 3 experiments. Results are presented as mean percentage clumping ± SD compared to the wild-type strain (100 % clumping).

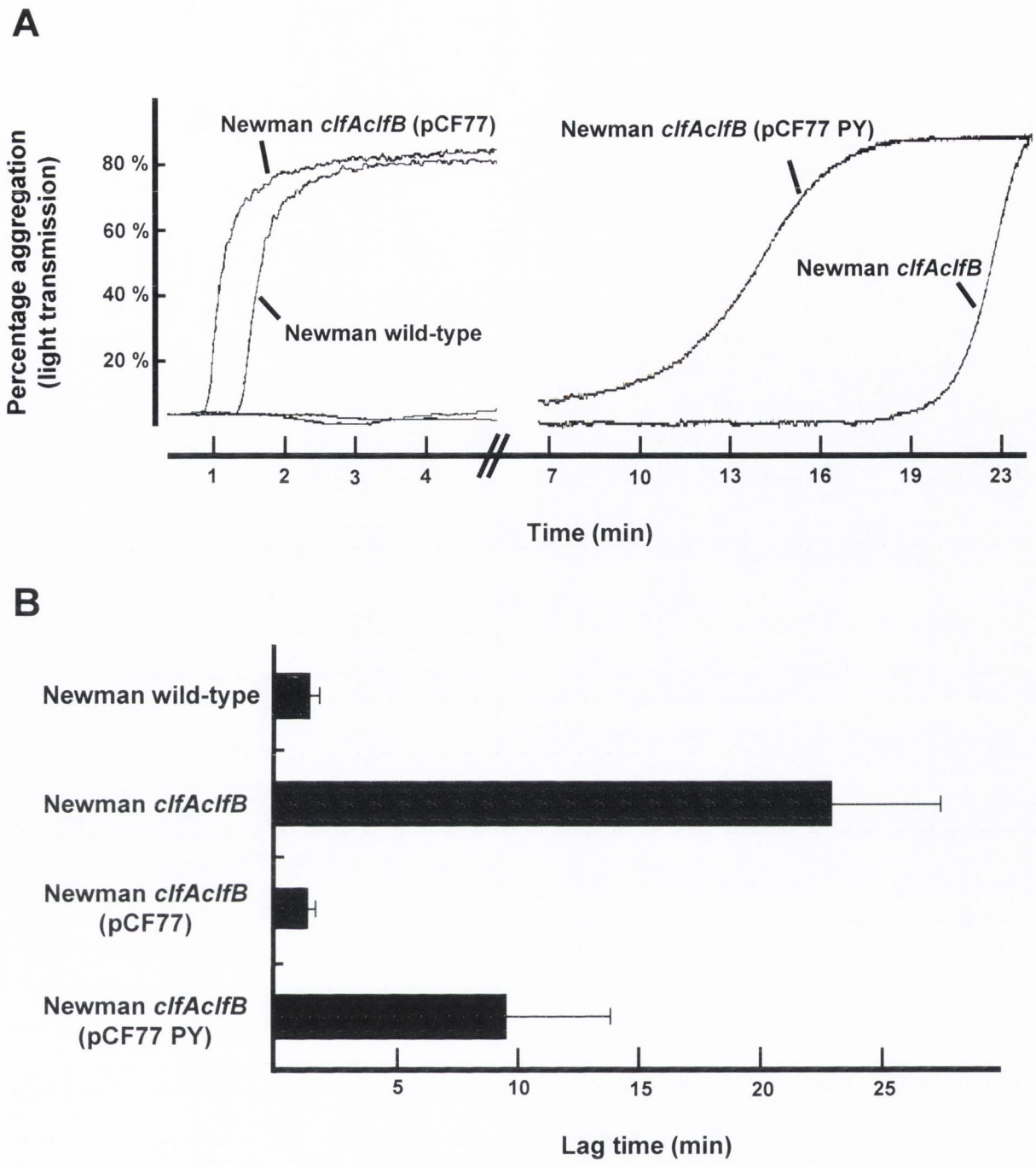


Figure 3.6. Activation of platelet aggregation by *S. aureus* Newman expressing ClfA and ClfA PY

A. A representative aggregometer trace obtained for *S. aureus* Newman wild-type, Newman *clfAclfB*, Newman *clfAclfB* (pCF77) and Newman *clfAclfB* (pCF77 PY) cells incubated in PRP. **B.** Mean lag times to platelet aggregation for *S. aureus* Newman derivatives in platelet-rich-plasma (PRP). This experiment was performed 3 times using platelets from 3 different donors.

3.2.3 Expression of ClfA and ClfA PY on the surface of *L. lactis*

The *clfA* and *clfA* PY genes were cloned into the nisin-controlled expression vector pNZ8037 (Figure 3.7) for expression on the surface of *L. lactis* strain NZ9800. This strain is a derivative of the commonly used laboratory strain MG1363 containing the nisin-sucrose conjugative transposon Tn*5276* (strain NZ9700; Rauch and de Vos, 1992). The 11 genes involved in nisin biosynthesis, modification and secretion are contained within this element. A silent mutation was introduced into the nisin structural gene (*nisA*) in this strain (Kuipers *et al.*, 1993), making it defective in nisin production. This allows nisin-controlled gene expression in this strain based on the concentration of exogenous nisin added to the growth medium. The level of gene expression from the *nisA* promoter in pNZ8037 is controlled by the level of available nisin (de Ruyter *et al.*, 1996), this control being mediated through the *nisRK* two-component system (Kupiers *et al.*, 1995).

The *clfA* and *clfA* PY genes were amplified by PCR. The forward primer used incorporated an *Nco*I site to allow translation fusion of the *clfA* genes to the *nisA* ATG start codon in pNZ8037. PCR products were ligated with pNZ8037 DNA cut with the appropriate enzymes. Ligation mixtures were electroporated into *L. lactis* NZ9800 cells as described in section 2.5.1.10. Transformants were screened for surface-ClfA expression by whole cell dot-immunoblotting (Section 2.7). Cultures of *L. lactis* NZ9800 (pNZ8037*clfA*) and *L. lactis* NZ9800 (pNZ8037*clfA* PY) were grown in GM17 broth with chloramphenicol to select for the plasmid. Nisin was added in doubling concentrations ranging from 0.025 to 3.2 ng/ml and cells grown for 16 h. To examine the effect of nisin on expression of ClfA and ClfA PY, whole-cell immunoblotting was performed. Serial dilutions of washed cells were applied to nitrocellulose membrane and probed with antibodies specific for ClfA. Representative blots of ClfA / ClfA PY expression are shown in Figure 3.8. Expression of ClfA and ClfA PY could be detected in uninduced cultures, indicating that repression is leaky. Increasing nisin concentrations resulted in increased ClfA expression, as seen by increasing dilutions needed to reach the endpoint. Maximal expression of ClfA and ClfA PY occurred at 1.6 ng/ml nisin. At nisin concentrations higher than 3.2 ng/ml, cell growth was inhibited by the lantibiotic and expression of ClfA and ClfA PY was variable (data not shown).

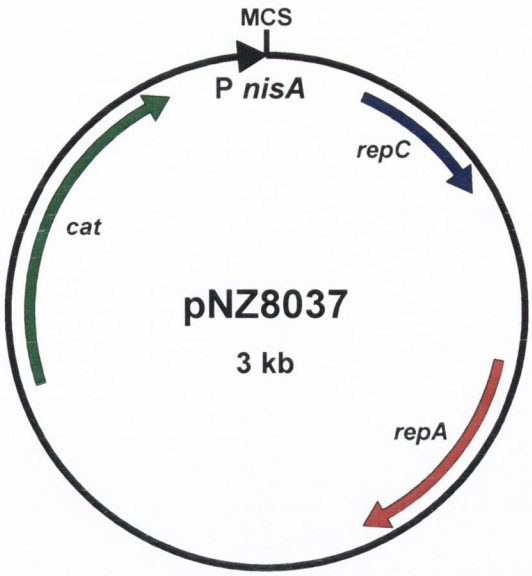
It was important to show that at the level of ClfA and ClfA PY expressed by pNZ8037 was the same at equivalent nisin concentrations. *L. lactis* NZ9800 (pNZ8037), *L. lactis* NZ9800 (pNZ8037*clfA*) and *L. lactis* NZ9800 (pNZ8037*clfA* PY) cells were grown overnight

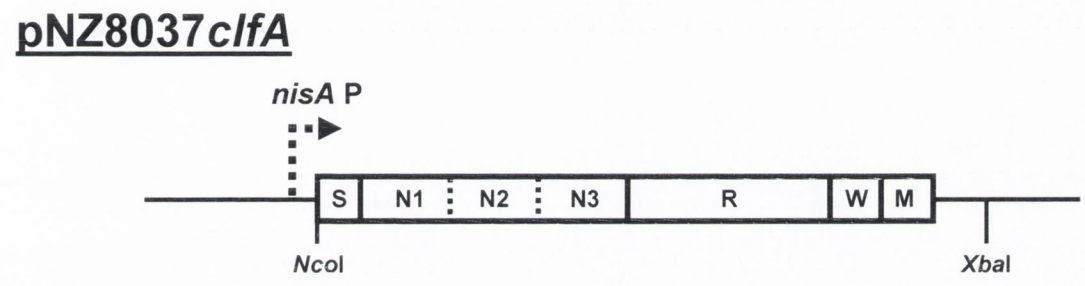
in the presence of 1.6 ng/ml nisin. Cells were washed, applied to membranes and probed with anti-ClfA antibodies as described above. A representative blot is shown in Figure 3.9 A. *L. lactis* expressed equivalent amounts of ClfA and ClfA PY when grown with 1.6 ng/ml nisin. *L. lactis* carrying the empty expression plasmid did not react with anti-ClfA antibodies.

Western blotting was performed to ensure that the full-length ClfA and ClfA PY molecules were expressed by *L. lactis*. It was previously shown that *L. lactis* constitutively expressing ClfA from the pKS80 expression vector had a 170 kDa cell wall-associated protein that reacted with anti-ClfA antibodies (O'Brien *et al.*, 2002a). This was the same size as ClfA expressed by *S. aureus* Newman (O'Brien *et al.*, 2002a). A protein of approximately 170 kDa that reacted with anti-ClfA antibodies was present in cell wall extracts of *L. lactis* ClfA+ (pNZ8037*clfA*) and *L. lactis* ClfA PY+ (pNZ8037*clfA* PY) (Figure 3.9 B). No immunoreaction was observed in cell wall extracts of *L. lactis* carrying the pNZ8037 vector (Figure 3.9 B). This shows that both ClfA and ClfA PY are expressed in their full-length forms from pNZ8037 in *L. lactis* NZ9800.

To ensure that the full-length ClfA and ClfA PY proteins expressed by *L. lactis* were functional, the ability of these strains to adhere to immobilized fibrinogen and to clump in soluble fibrinogen was tested. *L. lactis* ClfA+ adhered to fibrinogen coated wells (0.5 μg/well) in a nisin-dependent manner (Figure 3.10). There was significant adhesion of uninduced *L. lactis* ClfA+ cells to fibrinogen, correlating with ClfA expression seen in uninduced cells by dot-blotting (Figure 3.8). Increasing nisin concentrations resulted in increased binding, reaching saturation at 1.6 ng/ml nisin. This correlates well with the expression data presented in Figure 3.8. Neither the *L. lactis* host strain or *L. lactis* ClfA PY+ adhered to fibrinogen-coated wells when grown at any nisin concentration (Figure 3.10). The fact that the expression levels of ClfA PY and ClfA were similar over the induction range of nisin supports the conclusion that the bacterial-expressed ClfA PY mutant is defective in fibrinogen-binding.

L. lactis ClfA+ adhered in a dose-dependent, saturable manner (with very similar binding curves as seen for *S. aureus* Newman) to immobilized fibrinogen, reaching saturation at 1 μg/well (Figure 3.11). Neither *L. lactis* ClfA PY+ nor the plasmid-bearing host strain adhered to fibrinogen-coated wells (Figure 3.11). Fibrinogen concentrations up to 20 μg/well were tested, and no adhesion of *L. lactis* ClfA PY+ could be detected (data not shown). The ability of *L. lactis* expressing ClfA and ClfA PY to clump in the presence of soluble





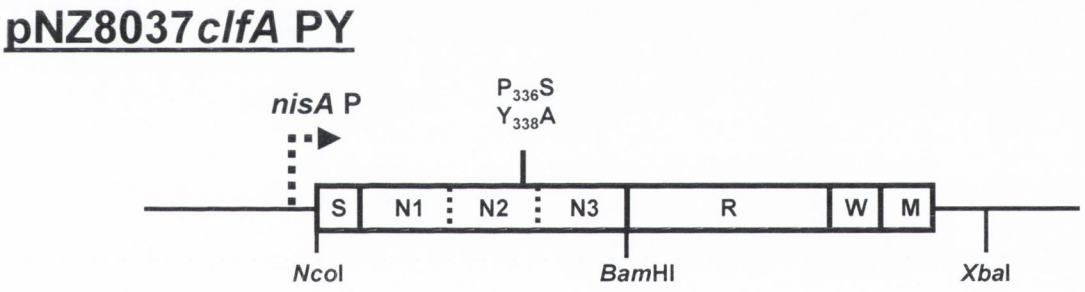


Figure 3.7. Plasmids used for expression of ClfA and ClfA PY in *L. lactis*

pNZ8037 is an *L. lactis* plasmid containing the nisin-inducible promoter *nisA* P. Target genes are cloned into an *Nco*I resulting in a translational fusion to the ATG codon in the inducible promoter (see Figure 2.1). A multiple cloning site (MCS) is located downstream of the *Nco*I to facilitate directional cloning of DNA. PCR amplified *clfA* (*S. aureus* Newman genomic DNA template) and *clfA* PY (pCF77 PY DNA template) genes with *Nco*I and *Xba*I sites at the 5' and 3' ends respectively were ligated with pNZ8037 DNA cleaved with the same enzymes. Ligated DNA was transformed into *L. lactis* NZ9800, generating plasmids pNZ8037*clfA* and pNZ8037*clfA* PY.

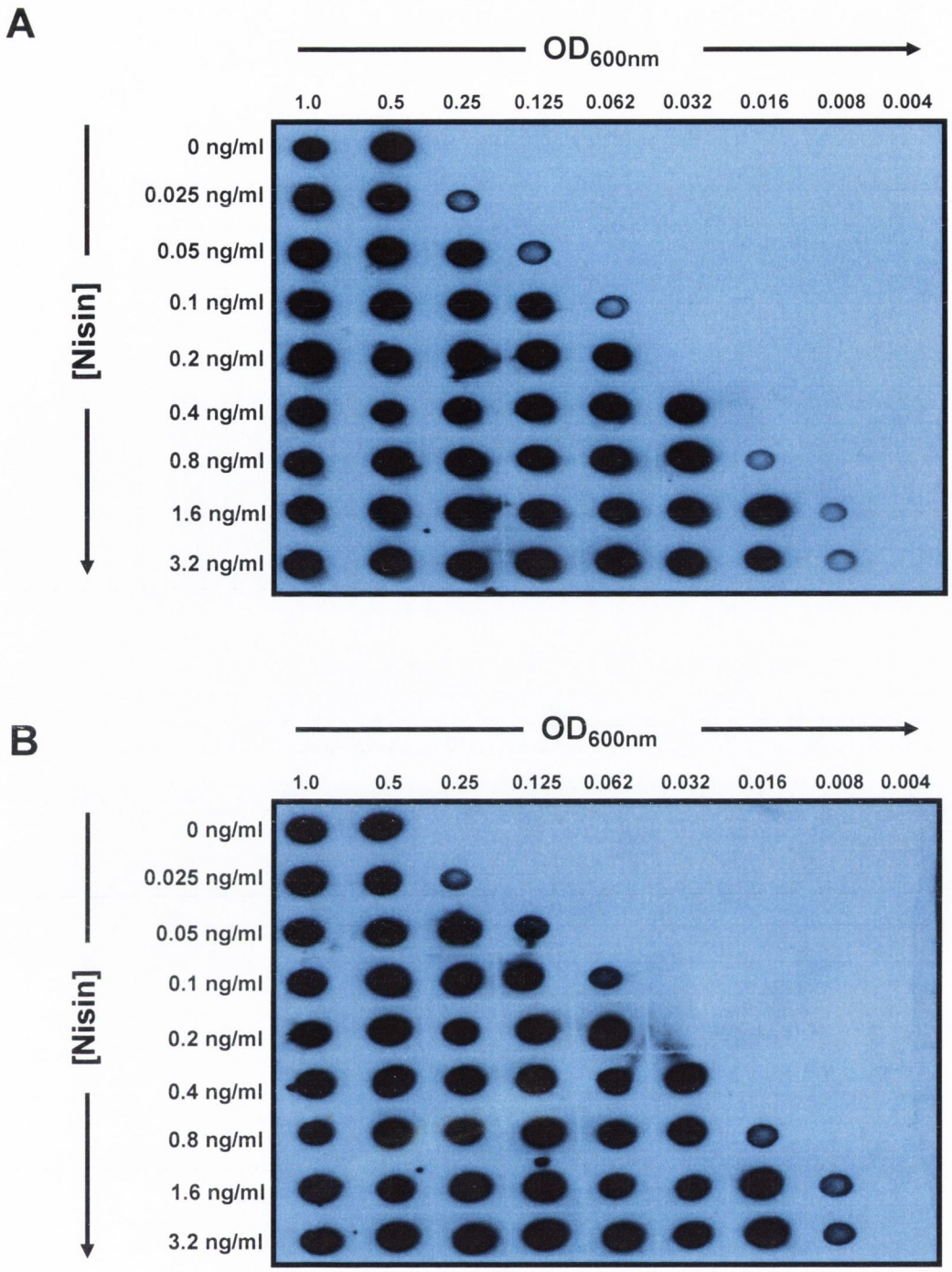


Figure 3.8. Nisin-controlled expression of ClfA and ClfA PY in *L. lactis*

L. lactis (pNZ8037*clfA*) (**A**) and *L. lactis* (pNZ8037*clfA* PY) (**B**) were grown in increasing concentrations of nisin as indicated. Cells were washed twice in PBS and suspended to OD_{600} of 1.0 in PBS. Serial dilutions were made in PBS in 96-well microtitre dishes. 5 μl of each dilution was applied to nitrocellulose membranes and blocked in 10 % skimmed milk solution for 16 h. Membranes were then incubated with rabbit-anti-ClfA antibodies (1:5000) and goat-anti-rabbit HRP conjugated antibodies (1:2000). Membranes were developed by chemiluminescence and exposed to autoradiographic film.

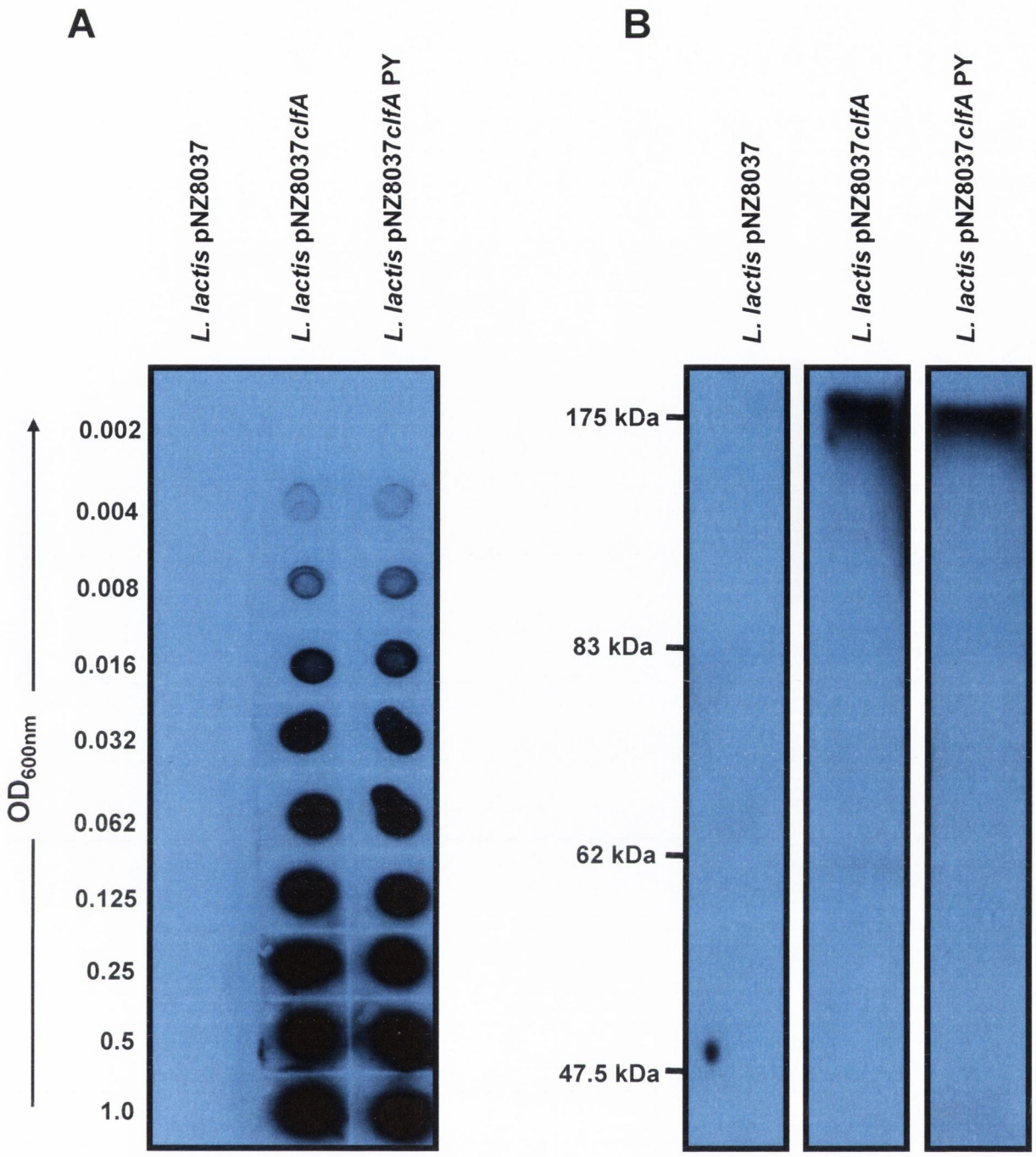


Figure 3.9. ClfA and ClfA PY expression at the same concentration of nisin

A. *L. lactis* NZ9800 (pNZ8037) and *L. lactis* NZ9800 expressing ClfA (pNZ8037*clfA*) and ClfA PY (pNZ8037*clfA* PY) were grown for 16 h in the presence of 1.6 ng/ml nisin. Membranes were prepared and probed with anti-ClfA antibodies as described for Figure 3.8.
B. Cell wall-associated proteins of *L. lactis* strains were solubilized by mutanolysin / lysozyme digestion and separated on 7.5 % SDS-PAGE gels. Separated proteins were electroblotted onto PVDF membranes and blocked in 10 % skimmed milk solution. Membranes were then incubated with anti-ClfA (1:5000) and goat-anti-rabbit HRP conjugated antibody (1:2000). Membranes were developed by chemiluminescence and exposed to autoradiographic film.

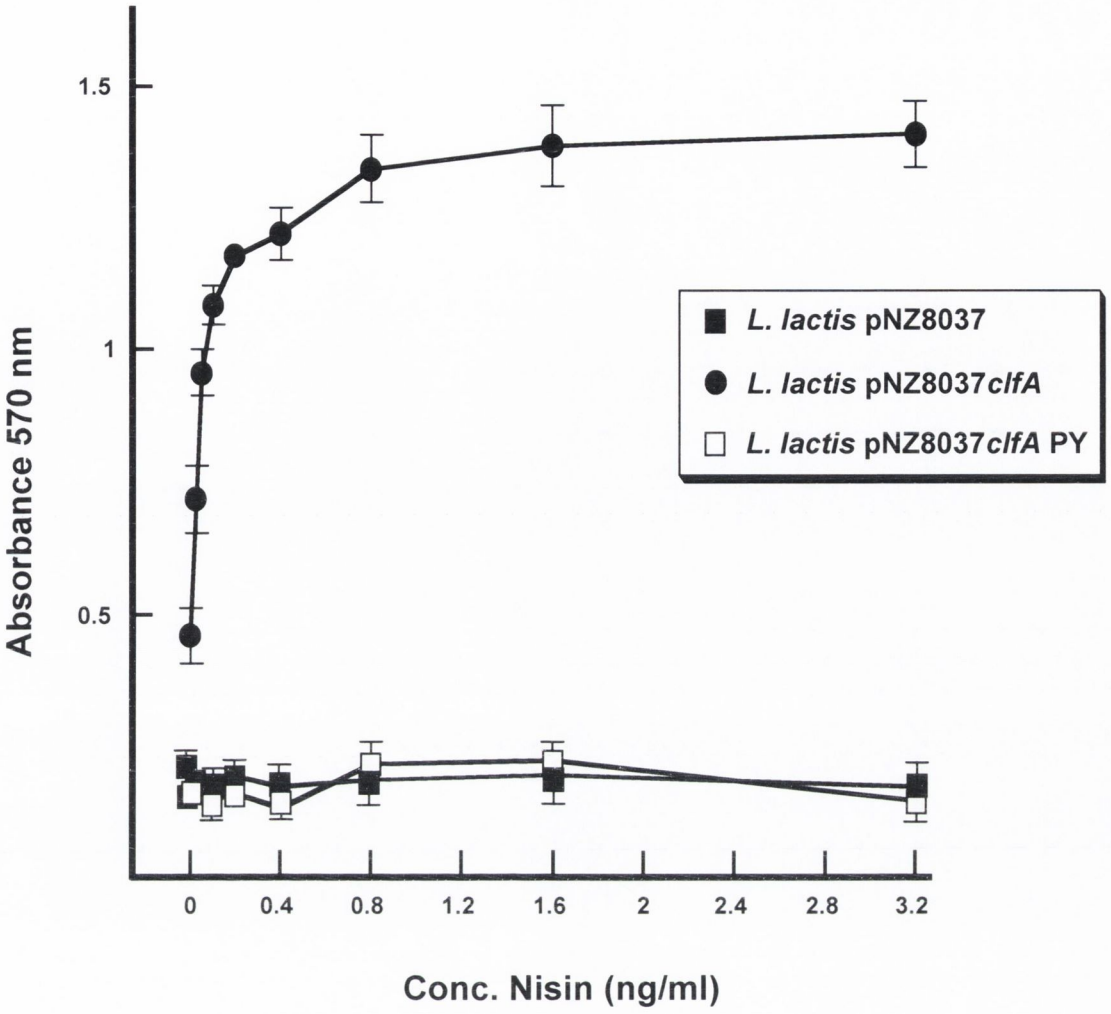


Figure 3.10. Nisin-dependent adherence of *L. lactis* ClfA+ to immobilized fibrinogen

L. lactis cultures were grown for 16 h in the presence of the nisin concentrations indicated (0.025 – 3.2 ng/ml). Washed cells were adjusted to OD_{600} of 1.0 in PBS and 100 μl bacterial suspension was added to fibrinogen-coated microtitre wells (0.5 μg fibrinogen/well). Cells were allowed to adhere for 2 h at 37°C. Adherent cells were stained with crystal violet and the absorbance of the wells at 570 nm determined using an ELISA plate reader.

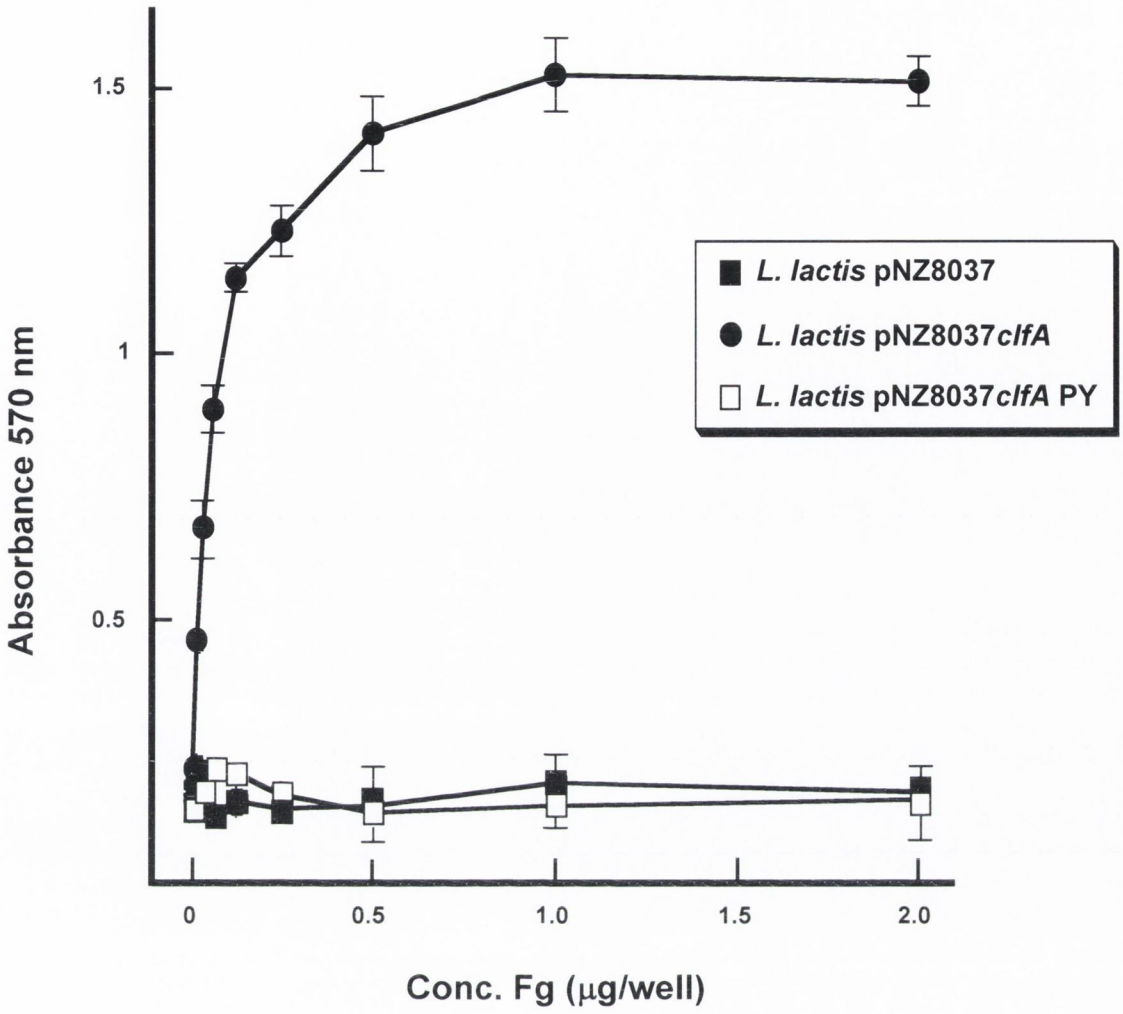


Figure 3.11. Adherence of *L. lactis* strains expressing ClfA and ClfA PY to immobilized fibrinogen

The ability of washed *L. lactis* (pNZ8037), *L. lactis* (pNZ8037*clfA*) and *L. lactis* (pNZ8037*clfA* PY) cells induced with 1.6 ng/ml nisin to adhere to immobilized fibrinogen (4 – 2000 ng/well) was tested. Adherent cells were stained with crystal violet and the absorbance at 570 nm read in an ELISA plate reader.

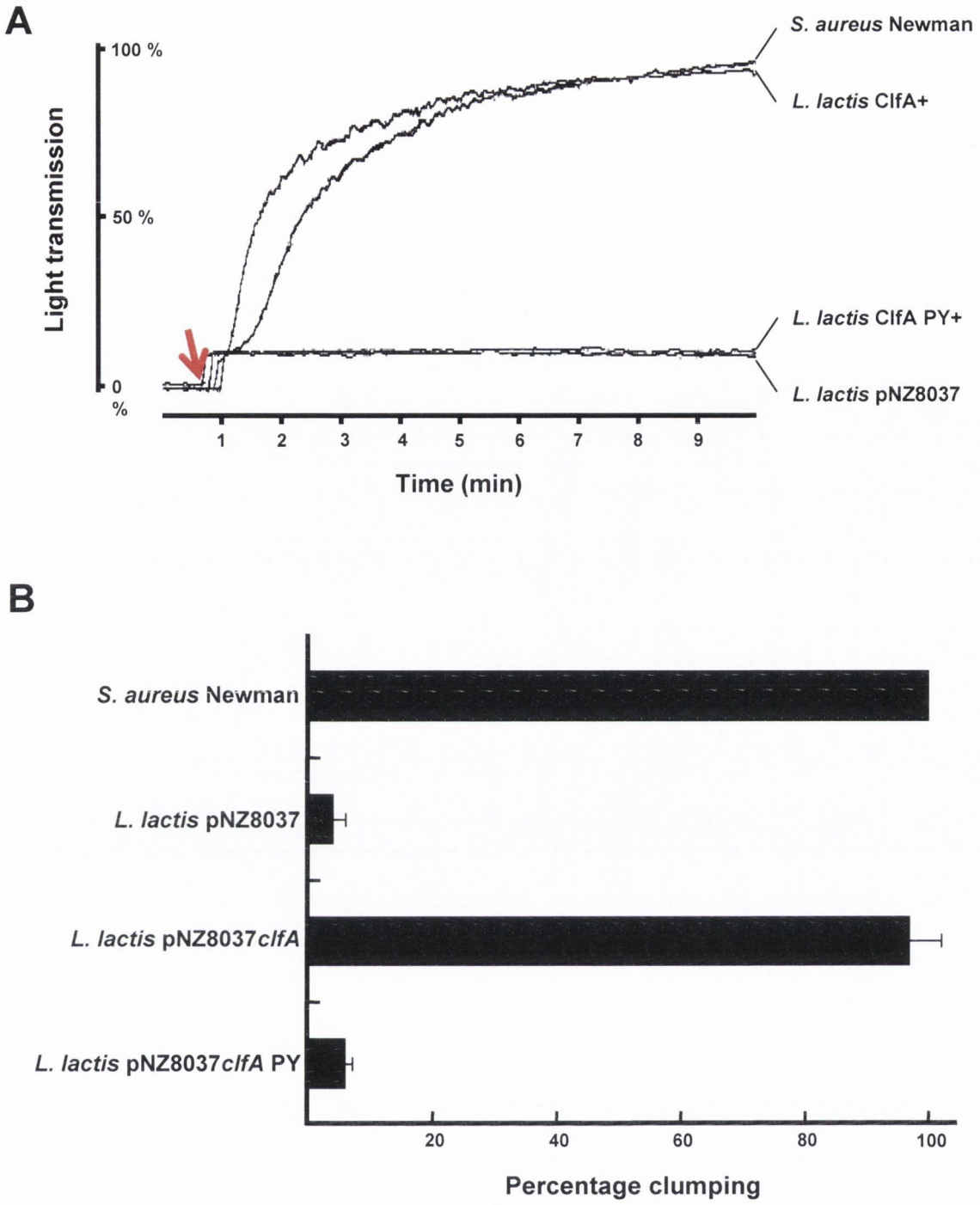


Figure 3.12. Clumping of *L. lactis* expressing ClfA and ClfA PY in soluble fibrinogen

A. Cell-clumping was measured by turbidometery using a platelet aggregometer. *L. lactis* strains were grown in the presence of 1.6 ng/ml nisin. Bacterial suspension (200 μl of OD_{600} 2.0 in PBS) was incubated in siliconized glass tubes at 37°C with stirring (900 rpm). Fibrinogen (50 μl of 10 mg/ml solution) was added (red arrow) and clumping allowed to occur.

B. Data from 3 cell clumping experiments. Results are presented as percentage clumping compared to *S. aureus* Newman (100 % clumping).

fibrinogen was tested. These conditions should mimic the binding ClfA / ClfA PY to soluble fibrinogen in platelet preparations such as PRP. As expected, *L. lactis* ClfA+ clumped avidly in 2 mg/ml fibrinogen. *L. lactis* ClfA PY+ and *L. lactis* (pNZ8037) failed to clump under these conditions (Figure 3.12).

3.2.4 Activation of platelet aggregation by *L. lactis* expressing ClfA and ClfA PY

3.2.4.1 Aggregation of platelet-rich-plasma (PRP)

L. lactis expressing ClfA and ClfA PY were tested for the ability to activate platelet aggregation in PRP. The lactococcal system is ideal for analyzing the contribution of single factors to the process of platelet aggregation, as *L. lactis* does not naturally stimulate platelet aggregation (O'Brien *et al.*, 2002). *L. lactis* (pNZ8037) and its derivatives expressing ClfA and ClfA PY were grown overnight in 1.6 ng/ml nisin. This concentration of nisin resulted in expression of ClfA and ClfA PY at levels comparable to strain Newman (Figures 3.3 and 3.8). Cells were washed and adjusted to OD_{600} of 1.6 in PBS. 50 µl of bacterial suspension was added to 450 µl platelet-rich-plasma (PRP) in siliconized glass cuvettes at 37°C with stirring. The lag time to aggregation for each strain was measured. *L. lactis* carrying the empty expression vector did not stimulate aggregation in this or any other aggregation experiments performed ($n > 20$). Results were considered negative if no aggregation had occurred after 25 min incubation. *L. lactis* ClfA+ caused aggregation with similar short lag times (1.5 ± 0.25 min; $n = 3$; Figure 3.13) to ClfA-expressing *S. aureus* Newman cells. *L. lactis* ClfA PY+ stimulated aggregation with longer lag times (7.8 ± 2.5 min, $n = 3$; $p < 0.05$; Figure 3.13) than that of *L. lactis* expressing the wild-type ClfA protein. These results confirm the previous finding that *L. lactis* expressing ClfA stimulates platelet aggregation with rapid lag times (between 1 and 2 min; O'Brien *et al.*, 2002). It appears that no other *S. aureus*-specific factor is required for ClfA-mediated platelet aggregation. *L. lactis* ClfA PY+ aggregated platelets with similar lag times to that seen in *S. aureus* Newman expressing ClfA PY. The ability of ClfA PY to stimulate the aggregation of platelets in plasma demonstrates that a fibrinogen-independent mechanism of platelet activation by ClfA exists. The extended lag time to aggregation for ClfA PY-expressing cells may reflect a lower-affinity binding to the platelet surface. It is also reasoned that the rapid aggregation of platelets by the wild-type protein reflects a high-affinity fibrinogen-dependent interaction with resting platelets.

3.2.4.2 Effect of ClfA-expression levels on platelet aggregation in PRP

The ability to control the level of ClfA and ClfA PY expression on the surface of *L. lactis* was utilized to examine the relationship between the ClfA expression level and the ability to stimulate platelet aggregation. *L. lactis* ClfA+ and *L. lactis* ClfA PY+ were grown in medium supplemented with nisin (0.1 – 3.2 ng/ml). The ability of washed cells (50 µl of OD_{600} 1.6 bacterial suspension) to stimulate aggregation in PRP (450 µl) was tested. Expression levels achieved at 0.4 ng/ml nisin were sufficient for *L. lactis* ClfA+ (n = 4) and *L. lactis* ClfA PY+ (n = 4) to stimulate aggregation (Figure 3.14). Expression levels achieved at 0.2 ng/ml nisin were not sufficient for aggregation by either *L. lactis* ClfA+ or *L. lactis* ClfA PY+ (after 25 min incubation; n = 4). *L. lactis* carrying the pNZ8037 vector did not aggregate platelets when grown at any inducer concentration (n = 4). It appeared that a threshold level of ClfA and ClfA PY expression was required to stimulate aggregation. Once the threshold level of expression was reached, the lag time to aggregation shortened with increasing ClfA expression (2.6 ± 0.4 min at 0.4 ng/ml nisin compared to 1.5 ± 0.1 min at 3.2 ng/ml nisin for *L. lactis* ClfA+; $p < 0.05$; n = 4). Similarly, increasing ClfA PY expression on *L. lactis* resulted in shorter lag times to aggregation (16.4 ± 4.5 min at 0.4 ng/ml nisin compared to 7.3 ± 1.9 min at 3.2 ng/ml nisin; $p < 0.05$; n = 4). The fastest aggregation occurred at inducer concentrations of 1.6 ng/ml and 3.2 ng/ml (Figure 3.14). The fact that the lag times at these two concentrations were the same for both ClfA and ClfA PY could be explained by these cells expressing the same level of ClfA protein (Figure 3.8). These results demonstrate that a critical level of ClfA must be expressed on *L. lactis* to stimulate aggregation. Expression levels below this threshold were not sufficient for aggregation to occur. This is consistent with the high-level ClfA expression and rapid platelet aggregation mediated by stationary phase *S. aureus* Newman cells.

Interestingly, *L. lactis* expressing ClfA at levels that could not support platelet aggregation (Figure 3.14) adhered substantially to immobilized fibrinogen (Figure 3.10) and formed clumps in soluble fibrinogen (data not shown). This might suggest that the fibrinogen-binding capacity of ClfA alone is not responsible for the rapid aggregation phenotype. Also of note is that the threshold expression level required for both ClfA-mediated and ClfA PY-mediated platelet aggregation is similar (Figure 3.14), although aggregation mediated by the

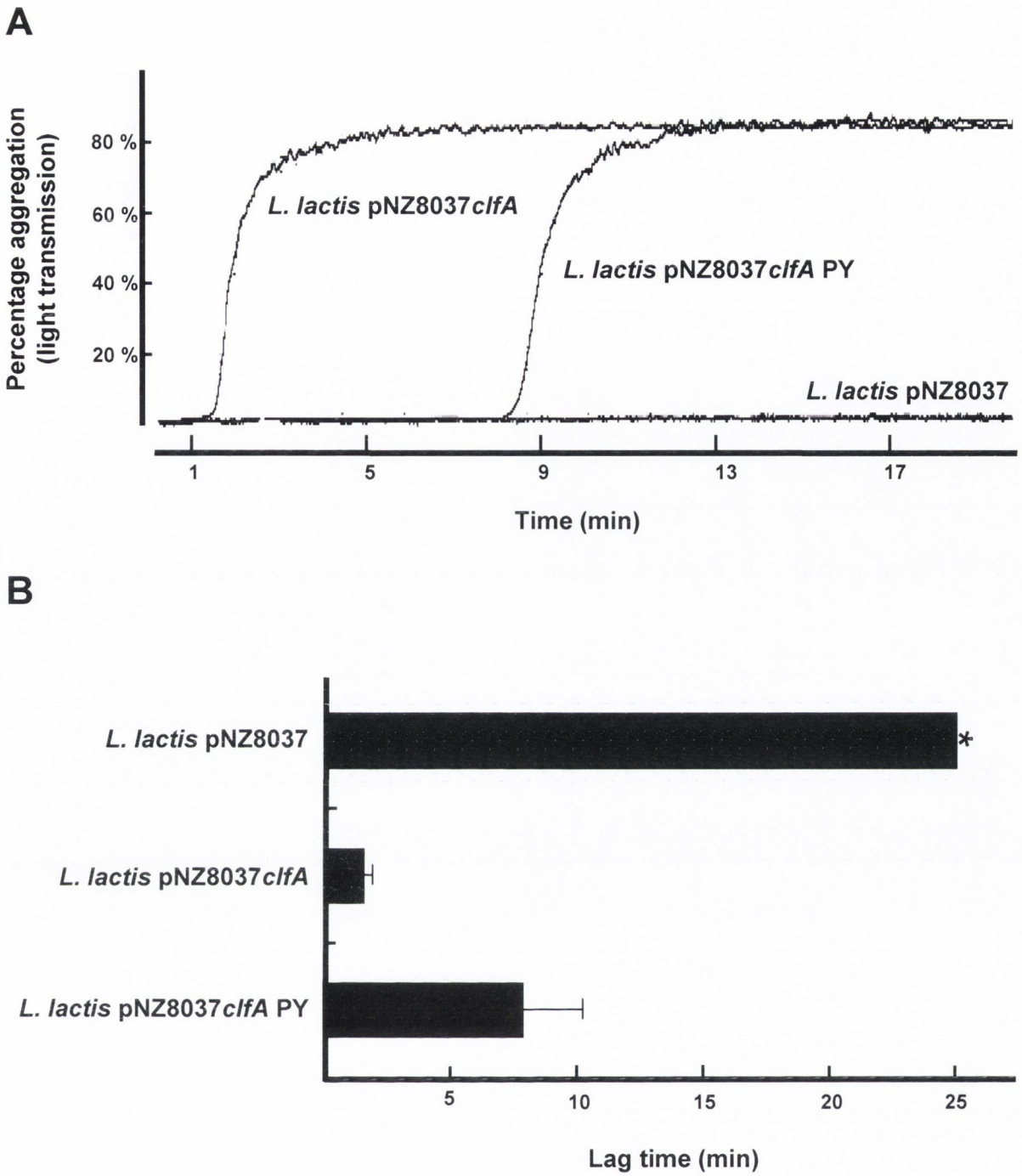


Figure 3.13. Activation of platelet aggregation by *L. lactis* strains expressing ClfA and ClfA PY.

A. A representative aggregometer trace obtained for *L. lactis* (pNZ8037), *L. lactis* (pNZ8037*clfA*) and *L. lactis* (pNZ8037*clfA* PY) cells grown in the presence of 1.6 ng/ml nisin.

B. Lag times to platelet aggregation for *L. lactis* strains in platelet-rich-plasma (PRP). This experiment was performed 3 times using platelets from 3 different donors. * indicates no aggregation occurred after 25 minutes incubation

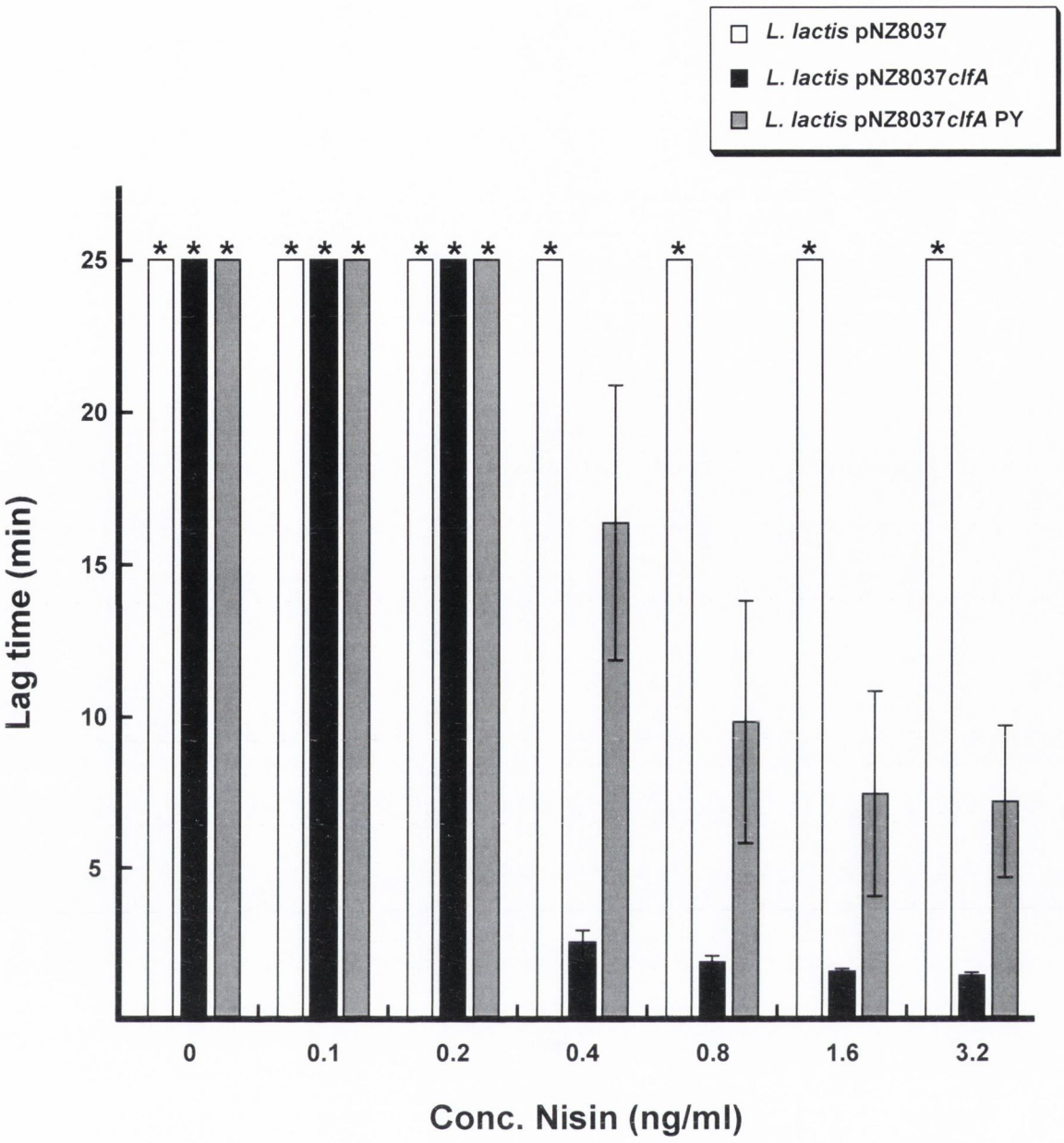


Figure 3.14. A threshold expression level of ClfA and ClfA PY is required to trigger aggregation

L. lactis (pNZ8037), *L. lactis* (pNZ8037*clfA*), and *L. lactis* (pNZ8037*clfA* PY) cells were induced with nisin concentrations as indicated. Cells were washed and adjusted to OD_{600} of 1.6 in PBS. Fifty μl cell suspension was added to 450 μl PRP prepared from sodium citrate anti-coagulated whole blood. Aggregation was monitored by light transmission with stirring at 37°C. Data is presented as mean lag times (in minutes) ± SD for 4 independent experiments using different platelet donors. The degree of aggregation (level of maximal light transmission) was comparable in samples where aggregation occurred. * indicates that no aggregation occurred after 25 minutes incubation.

wild-type protein always occurred with lag times of < 3 min and aggregation by the ClfA PY mutant occurred with lag times of > 6 min.

3.2.4.3 Effect of plasma calcium levels on ClfA-promoted platelet aggregation

The ability of ClfA to bind fibrinogen is progressively inhibited by increases in Ca^{2+} concentration (O'Connell *et al.*, 1998). In blood, free ionized calcium is maintained at a typical concentration of about 1.3 mM (Robertson and Marshall, 1981; May and Heptinstall, 2004). This may result in a proportion of ClfA molecules expressed on the cell-surface being unoccupied by soluble fibrinogen (O'Connell *et al.*, 1998; Figure 3.15). In preparing PRP for aggregation studies, sodium citrate is the most commonly used anticoagulant. However, chelation of plasma Ca^{2+} by citrate results in a low concentration of ionized calcium (0.1 mM) in platelet preparations (Mousa *et al.*, 2000; May and Heptinstall, 2004). At such a concentration, full saturation of ClfA receptors on a cell would be expected (Figure 3.15). To determine the effect of physiological calcium conditions on ClfA-promoted platelet aggregation, aggregation experiments were performed using PRP containing physiological levels of Ca^{2+}. Blood that had been treated with the anti-coagulant PPACK was used as the source of PRP. PPACK, a potent, irreversible thrombin inhibitor, does not deplete Ca^{2+} levels in plasma (May and Heptinstall, 2004).

L. lactis ClfA+ cells and *L. lactis* ClfA PY+ cells were induced with varying concentrations of nisin as described in section 3.2.4.2. Washed cells were tested in aggregation experiments using PPACK-PRP. The threshold expression levels of ClfA and ClfA PY required for aggregation were equivalent, achieved at 0.4 ng/ml nisin (Figure 3.16). Similar expression levels were needed for aggregation of low $Ca2^{+}$ (citrated) PRP (Figure 3.14). The lag times to aggregation for *L. lactis* ClfA PY+ were similar in low and physiogical $Ca2^{+}$ (7.5 ± 3.4 min in citrate-PRP versus 7.9 ± 1.4 min in PPACK-PRP; n = 3 at 1.6 ng/ml nisin). However, the lag times to aggregation for *L. lactis* expressing the wild-type ClfA protein were prolonged in PPACK-PRP compared to citrated PRP (1.5 ± 0.1 min in citrate-PRP versus 3.5 ± 0.6 min in PPACK-PRP; n = 3 at 1.6 ng/ml nisin; $p < 0.05$).

The longer lag times to aggregation promoted by *L. lactis* ClfA+ in PPACK-PRP compared to citrate-PRP was observed across the nisin induction range. The lag times in PPACK-PRP decreased from 5.1 ± 0.8 min at 0.4 ng/ml nisin to 2.8 ± 0.4 min at 3.2 ng/ml (Figure 3.16), whereas in citrate-PRP the lag times were significantly ($p < 0.05$) shorter (2.6 ± 0.4 min at 0.4 ng/ml nisin decreasing to 1.5 ± 0.1 at 3.2 ng/ml nisin; Figure 3.14). Stationary

phase *S. aureus* Newman cells caused aggregation of PPACK-PRP with prolonged lag times of 2.4 ± 0.2 min (n = 3; $p < 0.05$) compared to aggregation in citrate-PRP (1.5 ± 0.1 min; n = 3). This suggests that partial inhibition of fibrinogen-binding by ClfA in higher Ca^{2+} environments is associated with prolonged lag phases before aggregation. This is possibly due to impairment of the high-affinity fibrinogen-dependent interaction with resting platelets. Lag times for ClfA-promoted aggregation in physiological Ca^{2+} were still significantly shorter than that promoted by the ClfA PY-mutant protein (Figure 3.16), suggesting that fibrinogen-binding by ClfA still participates in the interaction with platelets in normal Ca^{2+} environments.

3.2.4.4 Inhibition of ClfA-mediated platelet aggregation by recombinant ClfA proteins

Despite the fact that *S. aureus* and *L. lactis* expressing ClfA stimulate rapid platelet aggregation, soluble recombinant ClfA does not stimulate aggregation when suspended in platelet preparations (McDevitt *et al.*, 1997). However, when it is immobilized on glass coverslips, recombinant ClfA can support thrombus formation when exposed to flowing blood (Sjöbring *et al.*, 2002). It is thought that bacterial proteins mediating platelet activation have to be presented to the platelet immobilized on a surface (for example, expressed on a bacterial cell) in order to elicit a pro-aggregatory effect. A recombinant ClfA protein (rClfA) encompassing the fibrinogen-binding domain has previously been shown to inhibit ADP-induced platelet aggregation (McDevitt *et al.*, 1997). Complete inhibition of aggregation occurred at concentrations of 8.5 μM rClfA, and the fibrinogen concentration in normal plasma is approximately 9 μM (3 mg/ml). It is thought that rClfA-mediated inhibition of ADP-induced aggregation is due to rClfA sequestering fibrinogen and as a result inhibiting binding of fibrinogen to activated GPIIb/III, thereby preventing aggregation. Recombinant proteins encompassing the entire A domains of wild-type ClfA (rClfA) and the A domain of the ClfA PY mutant (rClfA PY) were produced in *E. coli* as hexahistidine fusion proteins (Figure 3.17 A) and purified by Ni^{2+}-chelate chromatography. The effect of pre-incubation of these proteins with PRP before the addition of *L. lactis* ClfA+ or *L. lactis* ClfA PY+ was tested. The degree of light transmission (percentage aggregation) was measured, with high percentage aggregation values denoting complete aggregation had occured. *L. lactis* ClfA+ and *L. lactis* ClfA PY+ exhibited high level aggregation (> 70 % light transmission) in control untreated PRP samples (Figure 3.17 B). The lag times to aggregation were similar to those reported above (section 3.2.4.1). Incubation of PRP samples with rClfA (0.5 μM) for 20 min prior to the addition of bacterial cells completely inhibited aggregation by *L. lactis* ClfA+ (6 ±

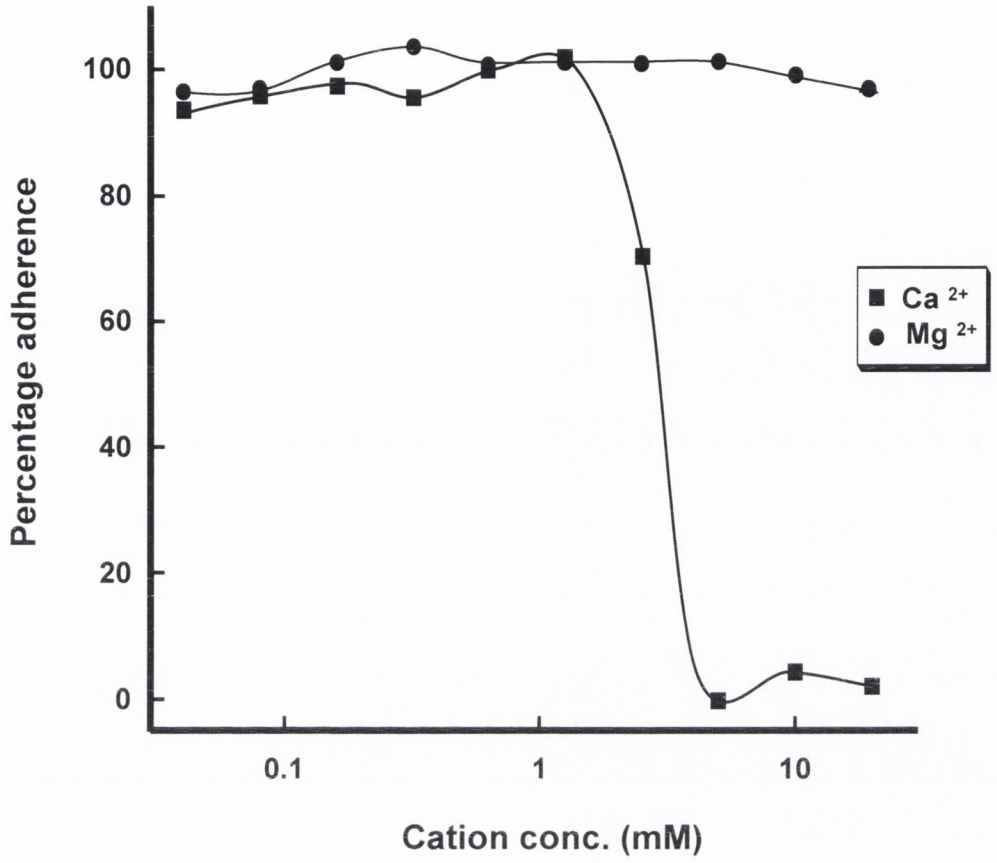


Figure 3.15. Inhibition of *L. lactis* ClfA+ adherence to fibrinogen by divalent cations

L. lactis (pNZ8037*clfA*) cells were induced with 1.6 ng/ml nisin, washed and adjusted to OD_{600} of 1.0 in saline. Aliquots of cell suspension (100 μl) were incubated with cation concentrations as indicated for 30 minutes prior to the addition of cells to wells coated with 250 ng of fibrinogen. Adherent cells were stained with crystal violet and the absorbance of the wells at 570 nm was read. Results are presented as percentage binding relative to control samples in which cations were not added.

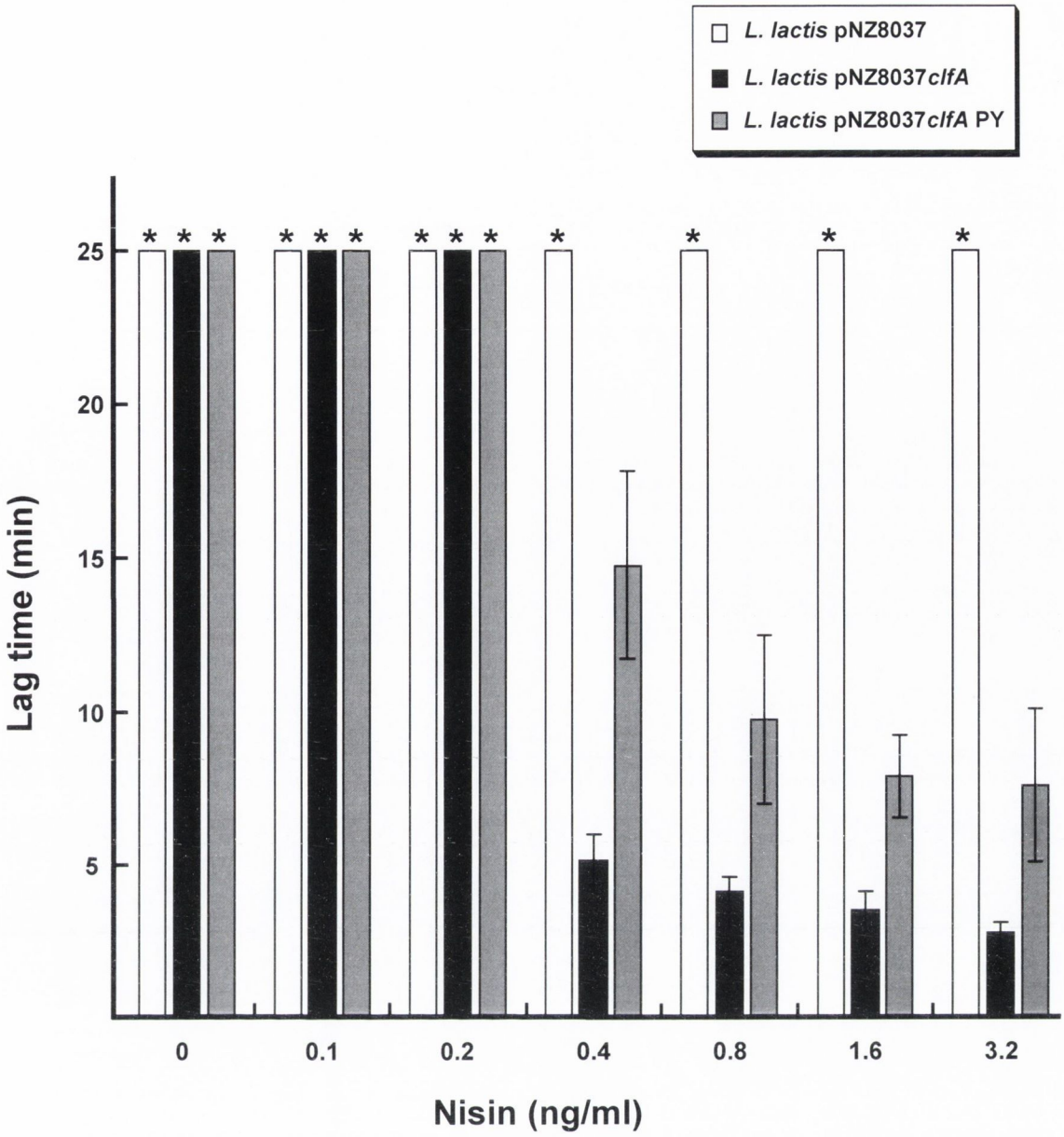


Figure 3.16. Aggregation of platelets by ClfA and ClfA PY in physiological calcium concentrations

L. lactis (pNZ8037), *L. lactis* (pNZ8037*clfA*), and *L. lactis* (pNZ8037*clfA* PY) cells were induced with nisin concentrations as indicated. Cells were washed and adjusted to OD_{600} of 1.6 in PBS. Fifty μl of the cell suspensions were added to 450 μl PRP. Blood used to prepare PRP was treated with the thrombin inhibitor PPACK to prevent coagulation while maintaining normal ionized calcium levels. Aggregation was monitored by light transmission with stirring at 37°C. Data is presented as mean lag times (in minutes) ± SD for 3 independent experiments using different platelet donors. * indicates that no aggregation occurred after 25 minutes incubation.

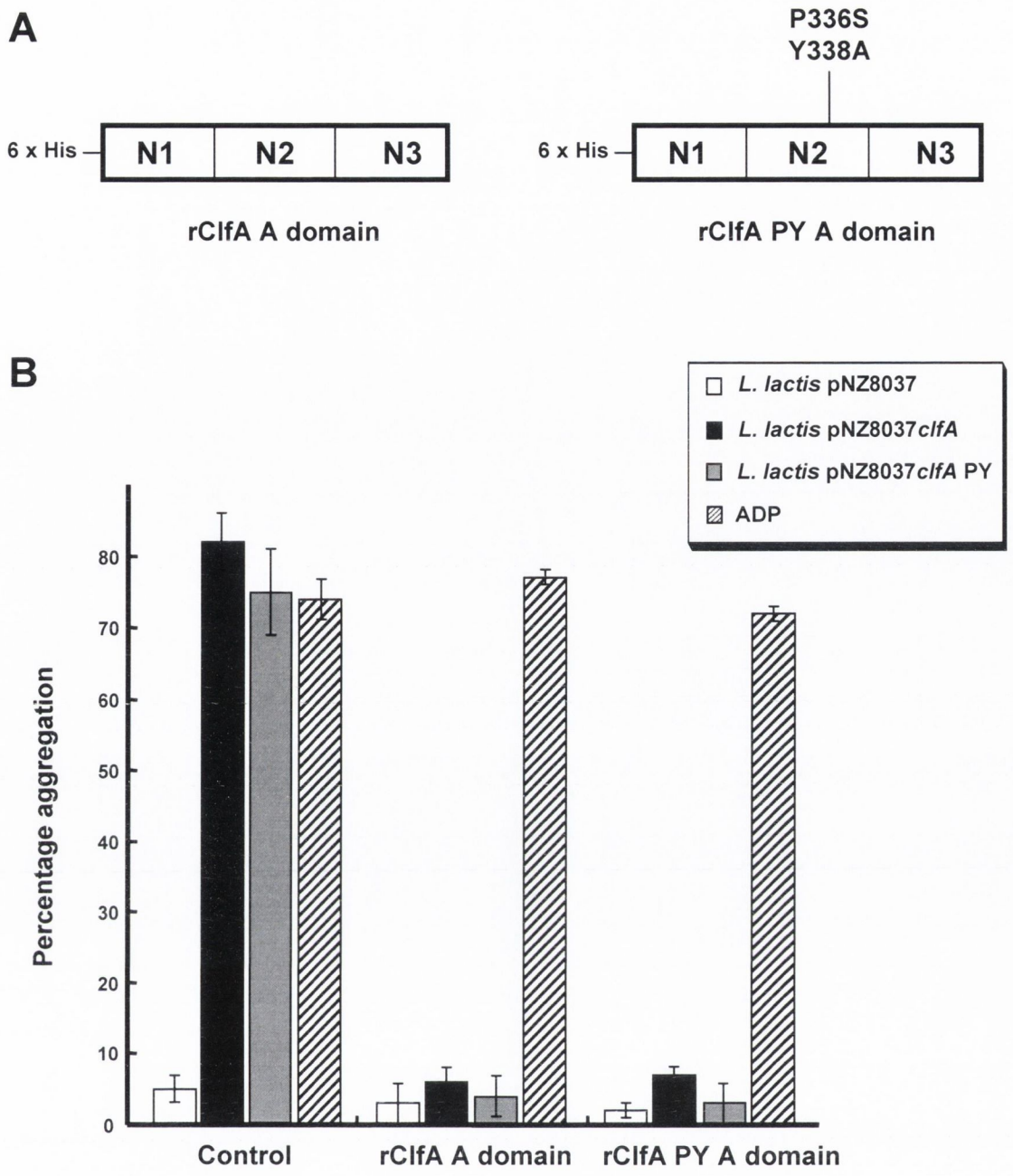


Figure 3.17. Inhibition of ClfA-mediated aggregation by recombinant ClfA proteins

A. Schematic representation of the recombinant ClfA proteins used in this experiment. Proteins encompass the entire A domains of ClfA / ClfA PY (residues 40 – 559).
B. Effect of rClfA A domain and rClfA PY A domain proteins on platelet aggregation by *L. lactis* strains. PRP samples (450 μl) were incubated with 0.5 μM recombinant protein for 20 min before addition of washed bacterial cells (50 μl). *L. lactis* (pNZ8037), *L. lactis* (pNZ8037*clfA*) and *L. lactis* (pNZ8037*clfA* PY) cells were grown in the presence of 1.6 ng/ml nisin. Aggregation in untreated PRP samples (control) is also shown. Aggregation induced by ADP (20 μM) is also shown. Results are presented as percentage aggregation (light transmission) observed after 25 min incubation of bacterial cells with PRP. This experiment was performed 3 times using platelets from 3 different donors

2 % aggregation; n = 3; $p < 0.05$) and *L. lactis* ClfA PY (4 ± 3 % aggregation; n = 3; $p < 0.05$) (Figure 3.17 B). Similar inhibition was seen with 0.5 μM rClfA PY for *L. lactis* ClfA+ (7 ± 2 % aggregation; n = 3) and *L. lactis* ClfA PY+ (3 ± 3 % aggregation; n = 3). Treatment of PRP with either rClfA (0.5 μM) or rClfA PY (0.5 μM) had no effect on ADP-induced aggregation compared to control samples (> 70 % aggregation; n = 3), indicating the inhibition seen for *L. lactis* ClfA+- and *L. lactis* ClfA PY+-induced aggregation was specific to these (Figure 3.17 B). ADP-induced aggregation was not affected by rClfA wild-type protein under these experimental conditions, presumably because the low concentration of recombinant protein used could not sufficiently deplete plasma fibrinogen to the level required to block aggregation. This then suggests that rClfA at 0.5 μM does not inhibit aggregation by *L. lactis* ClfA+ by blocking binding of fibrinogen to the bacterial cell. Furthermore, cross-inhibition is observed (i.e. rClfA PY inhibits *L. lactis* ClfA+ and vice-versa). Possible scenarios that explain these results are: (1) rClfA and rClfA PY binds to and occupies a platelet receptor involved in recognizing ClfA-expressing bacteria, preventing bacterial binding and activation of aggregation or (2) rClfA and rClfA PY bind to and sequester a plasma protein (that is not fibrinogen) involved in ClfA-mediated platelet activation and thereby inhibit aggregation.

3.2.4.5 ClfA stimulates platelet activation

Platelet aggregation by *S. aureus* Newman cells has previously been shown to be inhibited by aspirin (O'Brien *et al.*, 2002), which blocks a cyclo-oxygenase signaling enzyme involved in platelet activation. Aggregation was also inhibited by prostaglandin (PG) E_1, which inhibits platelet activation by increasing intracellular cAMP levels. This suggests that *S. aureus* aggregates platelets by initially stimulating platelet activation. Activation of platelets by *S. aureus* would result in GPIIb/IIIa activation allowing fibrinogen-binding and aggregation. It was important to show platelet aggregation by *L. lactis* expressing ClfA and ClfA PY was the result of a genuine platelet activation event. PGE_1 treatment of platelet samples completely blocked aggregation by *L. lactis* ClfA+ (83 ± 6 % aggregation in normal PRP compared to 1.5 ± 0.5 % in PRP treated with 2 μM PGE_1; n = 3; $p < 0.01$) and by *L. lactis* ClfA PY+ (84 ± 3 % aggregation in normal PRP compared to 2 ± 1 % with PRP treated with PGE_1; n = 3; $p < 0.01$), implying that *L. lactis* ClfA+ and *L. lactis* ClfA PY+ stimulate activation.

One of the earliest detectable signs of platelet activation is a rise in intracellular calcium (calcium flux). Binding of an agonist to its receptor on the platelet surface, such as platelet adhesion to collagen, stimulates intracellular signaling, leading to platelet granule secretion and upregulation of integrin affinity (Gibbins, 2004). Rises in intracellular calcium occur through release from intracellular stores or influx of calcium across the plasma membrane, and regulates events such as modulation of integrin conformation (Jackson *et al.*, 2003). The ability of *L. lactis* expressing ClfA and ClfA PY to stimulate calcium flux in platelets was assessed. Platelets were separated from plasma proteins by gel-filtration (GFP) and loaded with the fluorescent dye FURA-2-AM as described in section 2.20. FURA-2 displays different excitation properties in the presence of low and high calcium concentrations. *L. lactis* ClfA+ or *L. lactis* ClfA PY+ cells that were incubated in plasma for 30 min, washed and added to FURA-2 loaded platelets stimulated calcium flux (Figure 3.18). ClfA- or ClfA PY-expressing *L. lactis* cells incubated in PBS buffer did not stimulate calcium increases in FURA-2 loaded platelets (Figure 3.18). The *L. lactis* host strain (pNZ8037) did not stimulate calcium flux when incubated either in plasma or PBS (Figure 3.18). This indicates that ClfA and ClfA PY stimulate platelet activation, and this appears to be dependent on bacterial-bound plasma proteins.

3.2.4.6 Inhibition of ClfA-mediated platelet activation using antibodies to platelet receptors

The platelet receptors involved in recognizing ClfA-expressing bacteria and mediating activation have not been fully characterized. A panel of antibodies directed against various platelet receptors were tested for their ability to inhibit platelet activation by *L. lactis* expressing ClfA or ClfA PY and *S. aureus* Newman. The anti-GPIIb/IIIa monoclonal antibody abciximab inhibited aggregation by *L. lactis* expressing ClfA and ClfA PY (Figure 3.19). Various GPIIb/IIIa inhibitors (including abciximab) were shown to block aggregation by *S. aureus* Newman (O'Brien *et al.*, 2002 and this study; Figure 3.19), indicating an important role GPIIb/IIIa in the aggregation process. This is further evidence (in addition to calcium flux) that *S. aureus* ClfA stimulates true platelet aggregation (GPIIb/IIIa-dependent) and that aggregation was not the result of agglutination (which does not require GPIIb/IIIa).

The role of the platelet low-affinity IgG Fc receptor (FcγRIIa) in platelet activation by ClfA was examined. A function-blocking monoclonal antibody (IV-3) against FcγRIIa

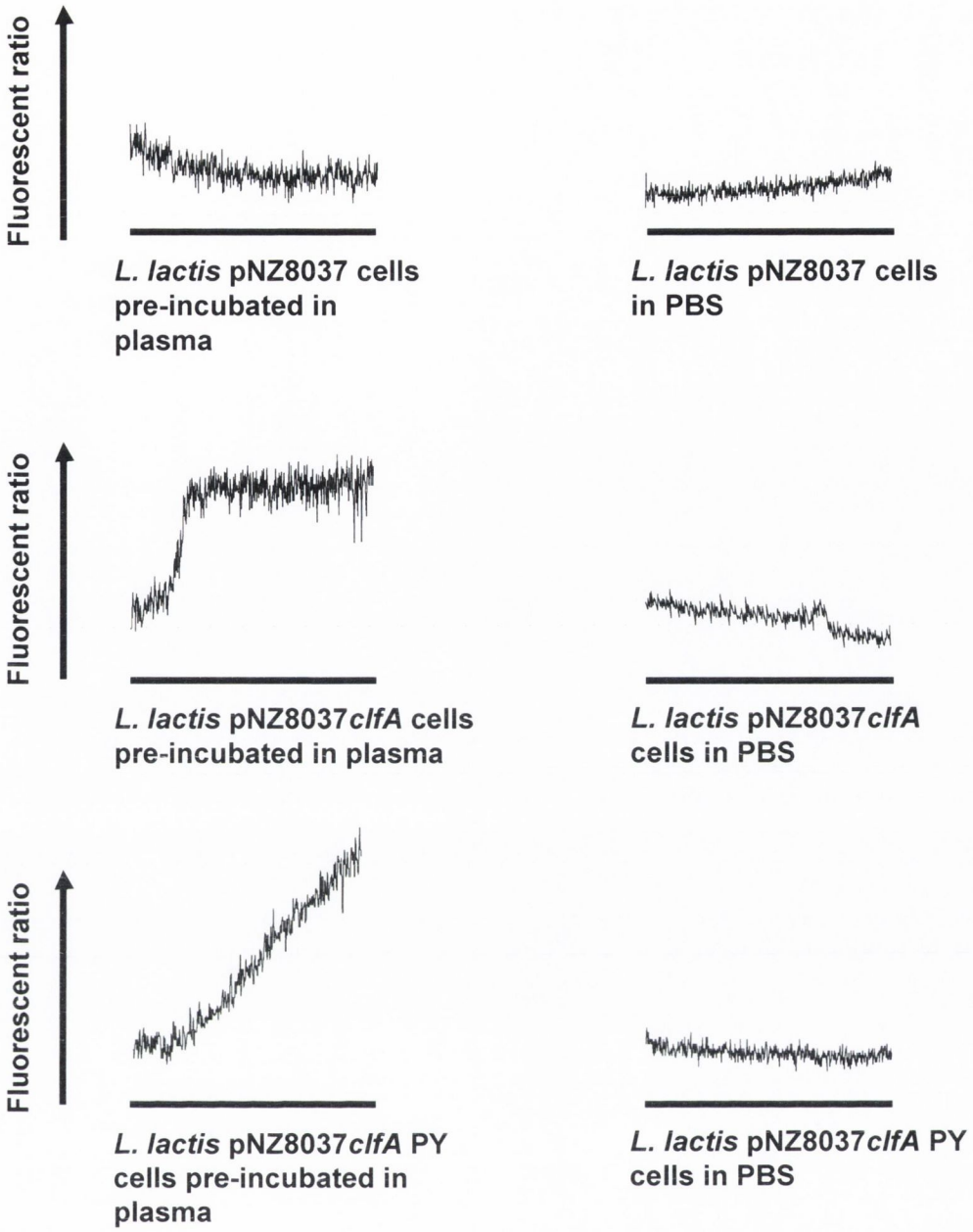


Figure 3.18. Direct measurement of platelet activation by *L. lactis* ClfA and *L. lactis* ClfA PY using calcium fluorimetry

Bacterial cells were either incubated in plasma samples or in PBS as indicated before washing in PBS. Bacterial suspension (50 μl OD_{600} 1.6 in PBS) was added to FURA-2-AM-loaded GFPs (400 μl of 2 x 10^8 platelets/ml) in a quartz cuvette with stirring at 37°C. Intracellular Ca^{2+} changes were detected using an LS-50 fluorimeter. FURA-2 fluorescence was measured following excitation at wavelengths 340 and 360 nm, with emission at 510 nm. Increases in the $^{Ex360}/_{Ex340}$ ratio (fluorescent ratio) represent increases in intracellular calcium (calcium flux), indicative of platelet activation. This experiment was performed on 3 separate occasions using 3 different blood donors with similar results.

completely inhibited activation by *L. lactis* ClfA+ (1 ± 0.5 % aggregation in IV-3 treated PRP; n = 3; p <0.05), *L. lactis* ClfA PY+ (1 ± 1 % aggregation; n = 3; p <0.05) and *S. aureus* Newman (1 ± 0.5 % aggregation; n = 3; p <0.05), suggesting a crucial role for this receptor in activation by ClfA (Figure 3.19). This shows that FcγRIIa is crucial in the response of the platelet to ClfA-expressing bacteria, and might suggest a role for bacterial-bound immunoglobulin in triggering activation. This is examined further in section 3.2.4.8.

The glycoprotein (GP) Ib complex is a major surface component of platelets, and has been implicated in bacterial-platelet interactions by other bacterial species (*Streptococcus sanguis*, *Streptococcus gordonii*, *Helicobacter pylori*). A monoclonal antibody against the α subunit of GPIb (antibody AN51) substantially inhibited aggregation by *L. lactis* ClfA PY+ (Figure 3.19). AN51 partially inhibited aggregation by *L. lactis* expressing the wild-type ClfA protein (83 ± 6 % for the ClfA+ control compared to 32 ± 6 % aggregation in AN51 treated PRP, n = 3, p <0.05). Aggregation by *S. aureus* Newman was reduced in AN51 treated platelets (90 ± 3 % aggregation in PRP compared to 68 ± 12 % aggregation in AN51 treated PRP, n = 3, p <0.05). Aggregation by ClfA PY+ cells was completely blocked by AN51, whereas aggregation by cells expressing the wild-type protein was only partially inhibited. This suggests that ClfA wild-type and ClfA PY cause activation by different mechanisms. These data suggest a role for GPIb in activation by ClfA, but the failure of AN51 to completely inhibit aggregation by *L. lactis* ClfA+ and *S. aureus* Newman may indicate that it plays only a minor role or that inhibition is indirect.

A function-blocking antibody against the platelet integrin α5β1, a fibronectin receptor on platelets which plays a supplementary role in platelet adhesion at sites of vascular damage, had no effect on activation by *L. lactis* ClfA+, *L. lactis* ClfA PY+ or *S. aureus* Newman (Figure 3.19). This receptor apparently is not involved in activation by ClfA, and suggests the inhibition seen with the anti-GPIIb/IIIa, anti-FcγRIIa, and anti-GPIb antibodies is specific, implying a role for each of these receptors in the platelet response to ClfA-expressing bacteria.

3.2.4.7 Role of plasma factors in ClfA-promoted platelet activation

Platelet aggregation by bacteria often requires the presence of specific plasma factors such as bacterial-specific IgG (Sullam *et al.*, 1988; Ford *et al.*, 1997; Sjöbring *et al.*, 2002; Pietrocola *et al.*, 2004), or complement proteins (Ford *et al.*, 1996). The requirement for specific plasma factors in platelet activation by *L. lactis* expressing ClfA and ClfA PY was

investigated. Platelets were prepared by gel-filtration to separate platelets from plasma as described in Section 2.14.1. Aggregation experiments were performed with gel-filtered platelets (GFPs) supplemented with purified fibrinogen (1 mg/ml final concentration) and 1 mM $CaCl_2$. The presence of fibrinogen is absolutely necessary for the aggregation of activated platelets by cross-linking activated GPIIb/IIIa receptors (Hettasch *et al.*, 1992). Fibrinogen was purified by protein A-sepharose (section 2.13.1) followed by anion-exchange chromatography (section 2.13.2) to remove contaminants such as IgG, fibronectin and vitronectin that are often present in commercially available fibrinogen preparations (Figure 3.20 A). Supplementation of GFPs with purified fibrinogen was not sufficient for *L. lactis* ClfA+ or *L. lactis* ClfA PY+ to cause high-level aggregation that is observed in PRP (Figure 3.21), indicating that one or more plasma proteins are involved in activation by ClfA. To demonstrate this, *L. lactis* ClfA+ and *L. lactis* ClfA PY+ were tested for aggregation in GFP/fibrinogen mixtures supplemented with 20 % normal human serum. Both *L. lactis* ClfA+ and *L. lactis* ClfA PY+ stimulated aggregation of GFPs in the presence of both fibrinogen and serum (70 ± 6 % aggregation and 66 ± 5 % aggregation respectively; n = 3), similar to the levels observed in PRP (Figure 3.21). The lag times to aggregation in GFP/fibrinogen/serum mixtures (1.2 ± 0.2 min for *L. lactis* ClfA+ and 6.5 ± 1.8 min for *L. lactis* ClfA PY+; n = 3) were similar to that observed in PRP (Figure 3.13). This confirms that a plasma factor (that is not fibrinogen) is required for ClfA-mediated platelet activation. Based on the crucial role of FcγRIIa seen by inhibition studies (Figure 3.19), the role of IgG in platelet activation by ClfA was investigated. Human serum was depleted of IgG by repeated passage over a protein A-sepharose column (Figure 3.20 B), and tested for its ability to support aggregation by *L. lactis* ClfA+ and *L. lactis* ClfA PY+. GFP/fibrinogen mixtures supplemented with 20 % IgG-depleted serum did not aggregate in response to *L. lactis* ClfA+ (11 ± 3 % aggregation; n =3; $p < 0.05$) or *L. lactis* ClfA PY+ (5 ± 1 % aggregation; n =3; $p < 0.05$; Figure 3.21). Supplementing GFP/fibrinogen/IgG-depleted serum mixtures with purified pooled human IgG (2 mg/ml) restored the ability of *L. lactis* ClfA+ and *L. lactis* ClfA PY+ to cause aggregation (72 ± 4 % and 68 ± 5 % aggregation respectively; n = 3; $p < 0.05$; Figure 3.21). This demonstrates that IgG is a crucial co-factor for platelet activation by ClfA. The involvement of IgG in platelet activation by ClfA and ClfA PY is discussed further in Section 3.2.4.8.

3.2.4.8 Requirement for IgG in ClfA-promoted platelet activation

The results presented above suggest that the presence of human IgG is crucial for

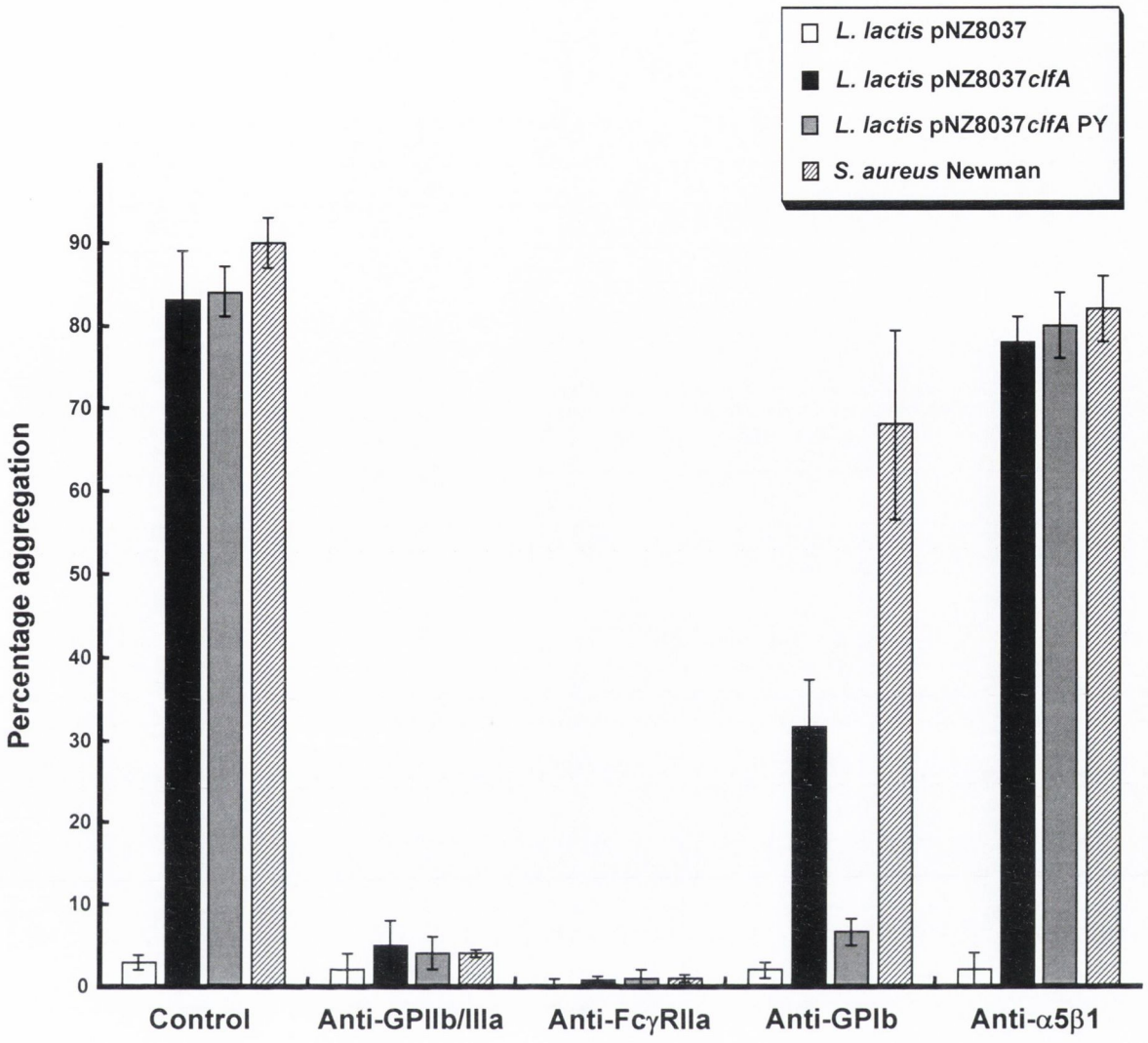


Figure 3.19. Effect of platelet inhibitors on ClfA-mediated platelet activation

PRP samples (450 μl) were incubated with antibodies to platelet receptors as indicated for 20 min at 37°C. Antibodies to GPIIb/IIIa (abciximab), FcγRIIa (IV-3), GPIb (AN51) and α5β1 were used at a final concentration of 20 μg/ml. Washed bacterial cells (50 μl OD_{600} 1.6) were added to PRP samples as indicated and the level of aggregation after 20 min incubation was measured. This experiment was performed on 3 separate occasions using 3 different blood donors.

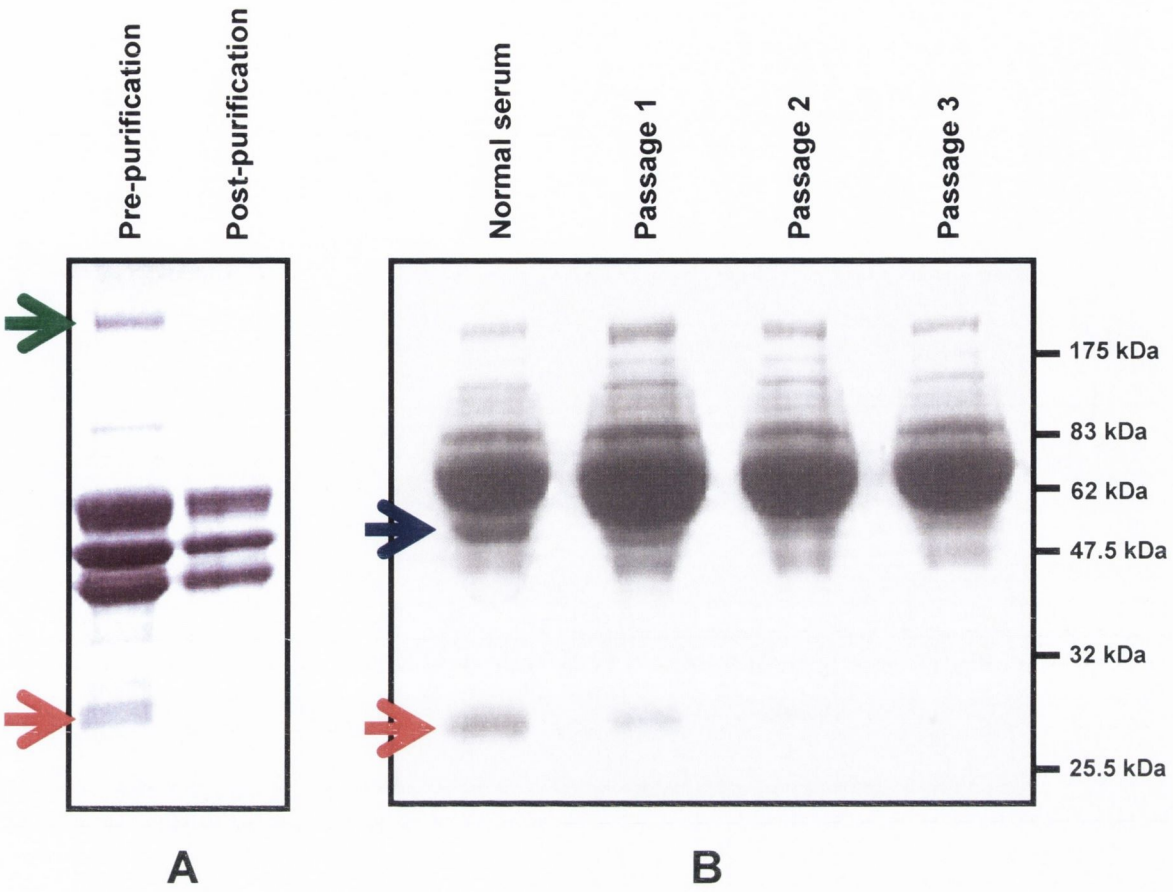


Figure 3.20. Preparation of purified plasma components for platelet aggregation assays

A. A commercial fibrinogen preparation was purified using protein A-sepharose and anion-exchange chromatography. Aliquots of pre- and post-purified fibrinogen samples were separated on 10 % SDS-PAGE gels and stained with Coomassie brilliant blue. Arrows denote bands corresponding to fibronectin (green) and the IgG light chain (red).
B. Human serum was repeatedly passaged over a protein A-sepharose column to remove IgG. Aliquots were taken after each passage, seperated on 10 % SDS-PAGE gels and stained with Coomassie brilliant blue. Arrows highlight the bands corresponding to the IgG heavy chain (blue) and light chain (red) that were depleted by this procedure.

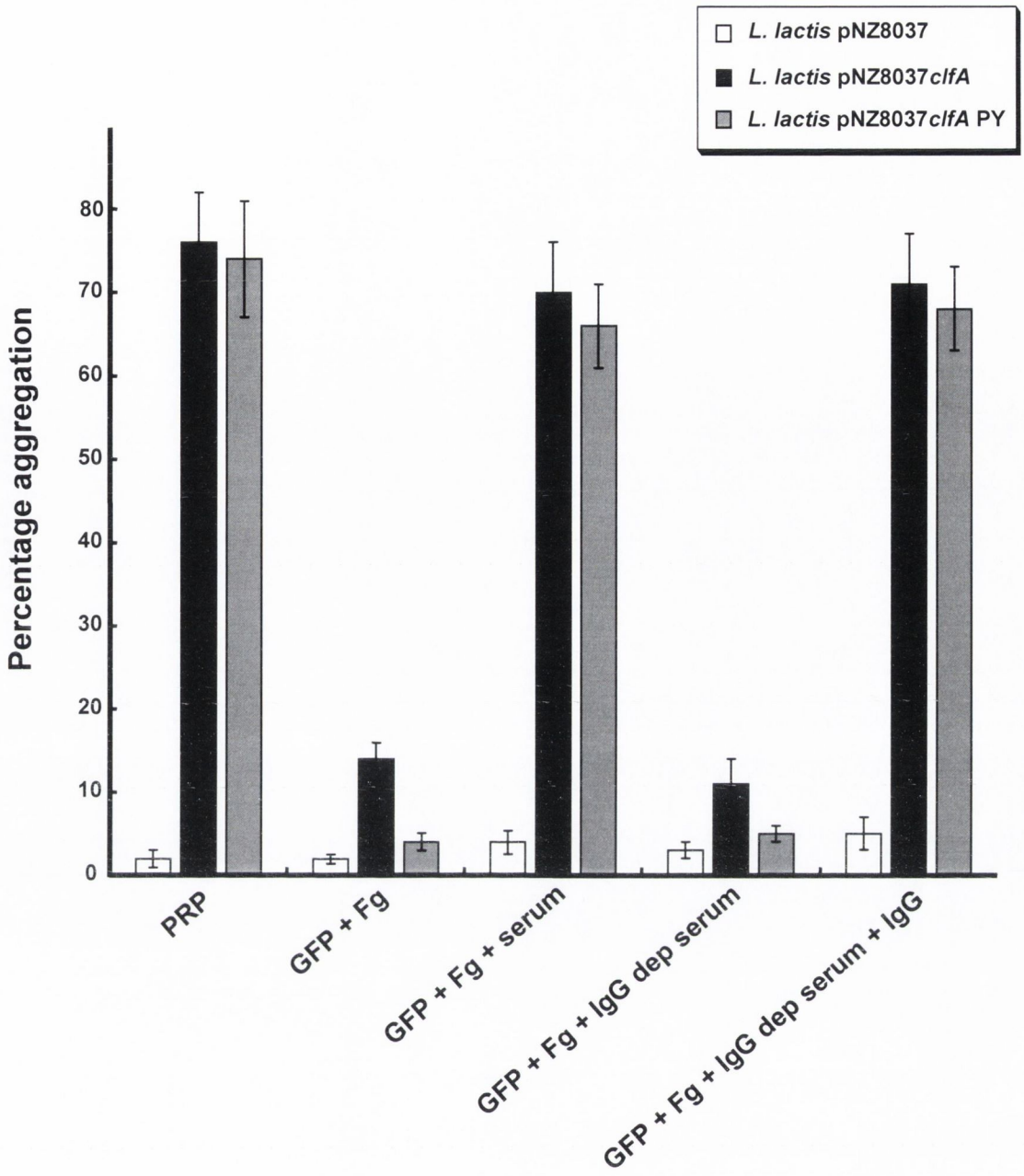


Figure 3.21. Role of plasma proteins in ClfA-promoted platelet activation

L. lactis (pNZ8037), *L. lactis* (pNZ8037*clfA*) and *L. lactis* (pNZ8037*clfA* PY) were grown overnight in the presence of 1.6 ng/ml nisin. Cells were washed and adjusted to OD_{600} 1.6 in PBS. Bacterial cells (25 μl) were added to PRP (225 μl) or GFP samples supplemented with purified fibrinogen (Fg; 1 mg/ml) and human serum samples (20 % serum) as indicated (5 x 10^7 GFPs in 225 μl final volume). Purifed human IgG was added at 2 mg/ml where indicated. This experiment was performed 3 times using 3 different blood donors. Results are presented as mean ± SD of light transmission (percentage aggregation) observed after 20 min.

platelet aggregation by *L. lactis* expressing ClfA and ClfA PY. This is consistent with the potent inhibition of aggregation by the anti-FcγRIIa monoclonal antibody IV-3 (Figure 3.19) and strongly suggests that bacteria-bound IgG recognizing FcγRIIa on platelets is crucial for activation. To determine if IgG is the only serum factor required for full aggregation of GFP/fibrinogen mixtures by *L. lactis* ClfA and *L. lactis* ClfA PY, experiments using GFPs supplemented with purified fibrinogen (1 mg/ml) and purified pooled human IgG (2 mg/ml) were performed. *L. lactis* ClfA+ stimulated aggregation of GFP/purified fibrinogen/human IgG mixtures (72 ± 4 % aggregation; n = 3), similar to the degree of aggregation observed in PRP samples (78 ± 3 % aggregation; n = 3; Figure 3.22). Interestingly, *L. lactis* ClfA PY+ consistently failed (n = 8) to aggregate GFPs supplemented with fibrinogen and IgG (8 ± 2 % aggregation; n = 3; $p < 0.05$), despite the fact that full aggregation occurred in autologous PRP samples (76 ± 5 % aggregation; n = 3; Figure 3.22).

To investigate the possibility that antibodies specific for ClfA in human IgG were required for activation, a series of adsorption experiments were performed to deplete specific antibodies from human IgG samples. IgG samples were incubated with washed, fully induced *L. lactis* (pNZ8037) cells or *L. lactis* ClfA+ cells (section 2.15.1). In addition, a sample of IgG was repeatedly passaged over a column containing immobilized rClfA protein to specifically deplete antibodies recognizing the A domain of ClfA (section 2.15.2). The presence of anti-ClfA antibodies in the treated IgG samples were determined by ELISA (Figure 3.23). Treatment of IgG samples with *L. lactis* expressing ClfA or by passage over a column of immobilized ClfA protein greatly reduced the amount of ClfA-specific antibodies present, whereas treatment with the host *L .lactis* strain did not affect the presence of anti-ClfA antibodies present compared to control samples (Figure 3.23). Aggregation experiments using GFPs supplemented with fibrinogen and adsorbed IgG samples were performed (Figure 3.22). *L. lactis* ClfA+ fully aggregated GFP/fibrinogen supplemented with *L. lactis* (pNZ8037)-adsorbed IgG (67 ± 8 % aggregation; n =3), whereas significantly reduced levels of aggregation were achieved in the presence of *L. lactis* ClfA+-adsorbed IgG (22 ± 3 % aggregation; n = 3; $p < 0.05$) or with rClfA-adsorbed IgG (20 ± 5 % aggregation; n = 3; $p < 0.05$; Figure 3.22). The degree of aggregation achieved by *L. lactis* ClfA+ directly correlated with the presence of anti-ClfA IgG present (Figure 3.23). Neither the *L. lactis* host strain (pNZ8037) nor *L. lactis* ClfA PY+ stimulated aggregation under these experimental conditions (Figure 3.22).

A number of conclusions can be drawn from this experiment:

(1) *L. lactis* ClfA+ only requires fibrinogen and IgG to stimulate aggregation. The IgG must contain antibodies specific for ClfA. Furthermore, the failure of *L. lactis* ClfA PY+ to activate platelets under the same conditions implies that fibrinogen-binding by ClfA is necessary for activation mediated by *L. lactis* expressing the wild-type protein. The role of fibrinogen-binding by ClfA in binding to platelets will be discussed further in Section 3.2.4.9.

(2) *L. lactis* ClfA PY+ requires an additional plasma factor (in addition to IgG) to stimulate activation. As *L. lactis* ClfA PY+ cannot bind fibrinogen, it cannot interact with resting platelets in a fibrinogen-dependent manner, as appears to occur with *L. lactis* expressing the wild-type protein. In the absence of fibrinogen-binding by ClfA, a fibrinogen-independent interaction with platelets requiring one or more plasma factors is postulated, leading to activation and aggregation. The role of other plasma factors in activation by *L. lactis* ClfA PY+ will be investigated further in Chapter 5.

The above experimental setup revealed that ClfA-specific antibodies are required for platelet activation by *L. lactis* expressing the wild-type protein. To determine if IgG specific for ClfA is also required for activation by the ClfA PY mutant protein, similar reconstitution experiments with GFPs supplemented with fibrinogen, IgG-depleted serum, and adsorbed IgG samples were performed (Figure 3.24). As previously seen, *L. lactis* ClfA PY+ could not aggregate GFPs in the presence of fibrinogen and IgG-depleted serum (10 ± 5 % aggregation; n = 3), but full aggregation occurred upon the addition of exogenous IgG to the depleted serum (74 ± 3 % aggregation; n = 3). Addition of *L. lactis* (pNZ8037)-adsorbed IgG to GFP / fibrinogen / IgG-depleted serum supported full aggregation by *L. lactis* ClfA PY+ (82 ± 9 % aggregation; n = 3), whereas rClfA-adsorbed IgG did not support aggregation by *L. lactis* ClfA PY+ (6 ± 4 % aggregation; n = 3; $p < 0.05$; Figure 3.24). This shows that ClfA-specific IgG is required for activation by ClfA PY.

3.2.4.9 Adhesion of *L. lactis* expressing ClfA and ClfA PY to resting platelets

Rapid activation and aggregation promoted by *L. lactis* ClfA+ apparently involves a fibrinogen-dependent interaction with resting platelets in conjunction with adhesin-specific IgG binding to FcγRIIa. To demonstrate fibrinogen-dependent binding of ClfA to platelets, adhesion experiments were performed. The ability of GFPs to adhere to bacterial cells immobilized in 96-well dishes was tested. GFPs alone did not adhere significantly to *L. lactis* expressing ClfA or ClfA PY (Figure 3.25). Supplementation of GFPs with fibrinogen resulted

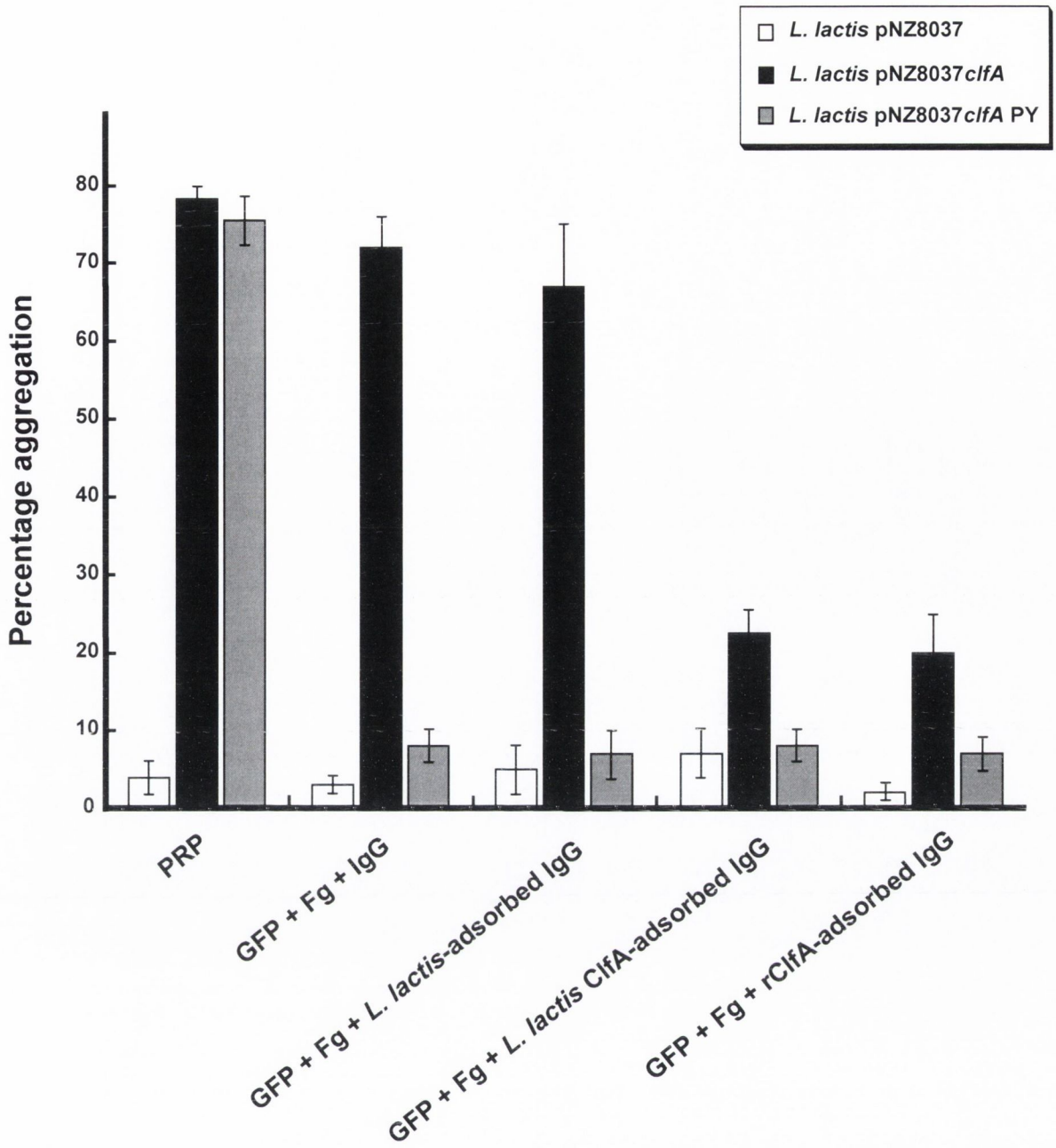


Figure 3.22. Role of ClfA-specific IgG in ClfA-promoted platelet activation

L. lactis (pNZ8037), *L. lactis* (pNZ8037*clfA*) and *L. lactis* (pNZ8037*clfA* PY) were grown overnight in the presence of 1.6 ng/ml nisin. Cells were washed and adjusted to OD_{600} 1.6 in PBS. Bacterial cells (25 μl) were added to PRP (225 μl) or GFP samples (5 x 10^7 GFPs in 225 μl final volume) supplemented with purified fibrinogen (Fg; 1 mg/ml) and purified human IgG samples (2 mg/ml) as indicated. IgG samples adsorbed with bacterial cells or immobilized recombinant ClfA protein were prepared as described in Section 2.15. This experiment was performed 3 times using 3 different blood donors. Results are presented as mean ± SD of light transmission (percentage aggregation) observed after 25 min.

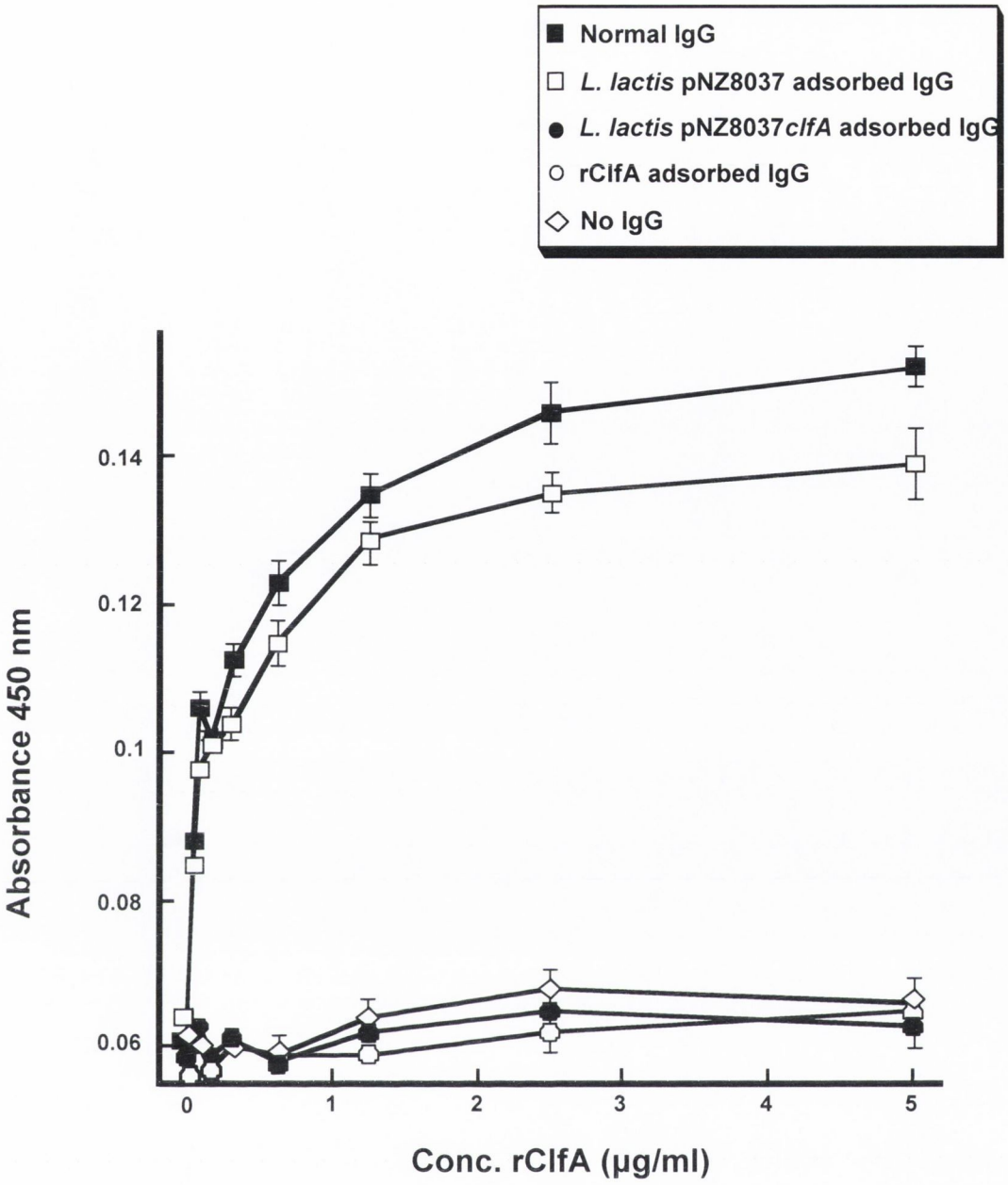


Figure 3.23. Depletion of ClfA-specific IgG from pooled human IgG

Anti-ClfA specific IgG levels in human pooled IgG samples adsorbed against bacterial cells and recombinant ClfA A domain were measured. *L. lactis* strains were induced with 1.6 ng/ml nisin for IgG adsorbtion experiments. The levels of anti-ClfA IgG in different adsorbed IgG samples or an unadsorbed control sample were measured by ELISA. Recombinant ClfA A domain protein was immobilized in microtitre wells at the indicated concentrations. IgG samples (2 µg in 100 µl buffer) were added to each well and incubated for 1 hour. Wells were washed and bound antibodies detected using a goat-anti-human antibody congugated to peroxidase.

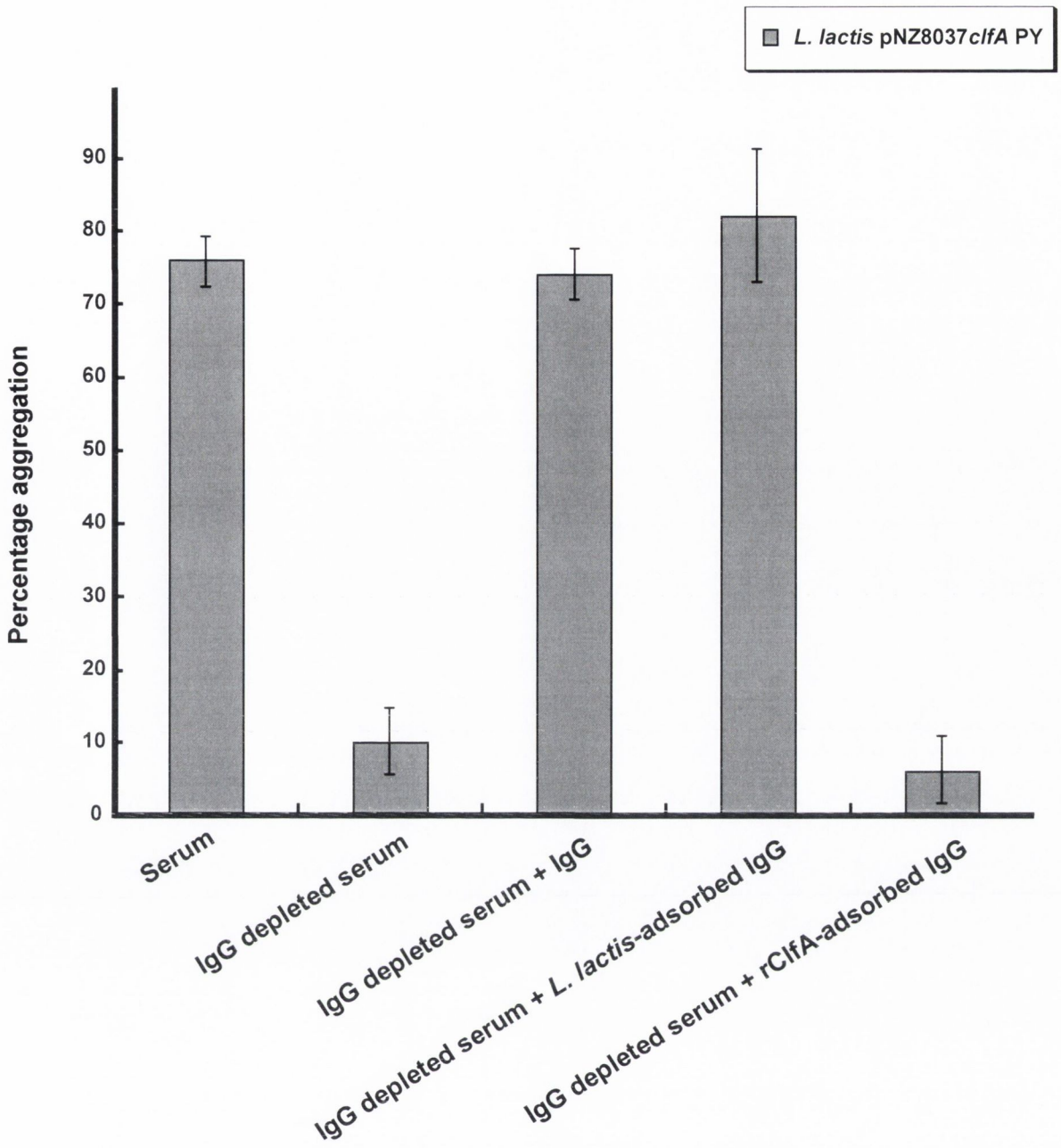


Figure 3.24. ClfA-specific IgG is required for ClfA PY-promoted platelet activation

L. lactis (pNZ8037*clfA* PY) cells were grown overnight in the presence of 1.6 ng/ml nisin. Cells were washed and adjusted to OD_{600} 1.6 in PBS. Bacterial cells (25 μl) were added to GFP samples (5 x 10^7 GFPs in 225 μl final volume) supplemented with purified fibrinogen (Fg; 1 mg/ml), serum or IgG-depleted serum (20% final concentration) and purified human IgG samples (2 mg/ml) as indicated. This experiment was performed 3 times using 3 different blood donors. Results are presented as mean ± SD of light transmission (percentage aggregation) observed after 25 min.

in significantly enhanced adhesion of platelets to *L. lactis* ClfA+ cells, whereas no difference in adhesion to *L. lactis* ClfA PY+ cells was observed (Figure 3.25). Fibrinogen-dependent adhesion of platelets to *L. lactis* ClfA+ cells was completely blocked ($p < 0.05$) by pretreatment of GFP samples with the GPIIb/IIIa inhibitors abciximab and tirofiban (Figure 3.25). These results indicate that adhesion of platelets to ClfA-expressing bacteria is mediated by ClfA-bound fibrinogen bridging the bacterium to GPIIb/IIIa on the platelet surface.

3.2.5 IgG is required for platelet activation by *S. aureus* Newman

Platelet aggregation by *S. aureus* Newman was completely inhibited by blockade of the FcγRIIa receptor (Figure 3.19), indicating that IgG was also required for platelet activation by *S. aureus* Newman cells. The requirement for ClfA-specific IgG for aggregation by *S. aureus* Newman cells was investigated. GFPs were supplemented with purified fibrinogen (1 mg/ml) and various adsorbed human IgG samples (2 mg/ml); the ability of *S. aureus* Newman cells to aggregate these platelet suspensions was tested (Figure 3.26). Low-levels of aggregation were observed in GFP/fibrinogen (10 ± 1 % aggregation; $n = 3$; $p < 0.05$) compared to high-level aggregation in control PRP samples (85 ± 5 % aggregation; $n = 3$). Addition of human IgG to GFP/fibrinogen restored aggregation to levels comparable with PRP (84 ± 10 % aggregation; $n = 3$) indicating that IgG is also crucial for platelet activation by *S. aureus* cells. Human IgG depleted of ClfA-specific antibodies supported reduced aggregation (42 ± 8 % aggregation; $n = 3$; $p < 0.05$), whereas IgG adsorbed with control *L. lactis* cells fully supported aggregation (75 ± 8 % aggregation; $n = 3$). IgG samples adsorbed against whole *S. aureus* Newman cells supported significantly reduced aggregation (12 ± 4 % aggregation; $n = 3$; $p < 0.05$), comparable to the level observed in the absence of IgG (Figure 3.26). These results indicate that ClfA-specific antibodies are required for maximal aggregation by stationary phase *S. aureus* Newman cells, similar to the requirement seen for *L. lactis* strains expressing ClfA or ClfA PY. However, the removal of ClfA-specific IgG did not abolish aggregation, indicating that IgG targeted against other *S. aureus* surface components may play a role. The fact that IgG samples adsorbed against *S. aureus* cells could not support aggregation by Newman cells supports this conclusion.

3.3 Discussion

The experiments performed here were designed to investigate the mechanism by which bacterial cells displaying ClfA on their surface could trigger the activation and subsequent aggregation of platelets, a factor which may be important in thrombus formation in the development of IE. The ability of ClfA and a non-fibrinogen binding ClfA mutant (ClfA PY) to stimulate platelet aggregation was studied by expression in *S. aureus* and the non-activating surrogate host *L. lactis*.

It was demonstrated that bacterial cells expressing sufficient levels of ClfA on their surface produced a reproducible platelet aggregation response with human PRP, with lag times of between 1 and 2 minutes in all donors examined. Bacteria expressing similar levels of the ClfA PY mutant protein, which cannot mediate bacterial adhesion to immobilized fibrinogen or cell-clumping in soluble fibrinogen, aggregated PRP samples with longer and more variable lag times (5 – 12 min). This demonstrated that the ability of ClfA to bind fibrinogen is associated with rapid platelet aggregation. Accordingly, when experiments were performed in elevated calcium conditions, where the ability of ClfA to bind fibrinogen is somewhat impaired, the lag times to aggregation were increased for the wild-type protein, but not for the ClfA PY mutant protein. These experiments also demonstrated a less efficient fibrinogen-independent mechanism of activation by ClfA PY. Platelet aggregation by bacteria expressing ClfA or ClfA PY was inhibited by PGE_1, indicating that aggregation triggered by ClfA and ClfA PY was the result of platelet activation. It was shown that *L. lactis* expressing either ClfA or ClfA PY induced intracellular calcium increases, a marker for activation. Furthermore, calcium flux induced by *L. lactis* ClfA or ClfA PY was dependent on one or more plasma factors.

Experiments performed with platelets separated from plasma (GFPs) allowed identification of plasma factors required in the ClfA-platelet activation process. The addition of exogenous fibrinogen and human IgG to GFP was both necessary and sufficient for *L. lactis* expressing ClfA to promote rapid and complete platelet aggregation. *L. lactis* ClfA PY+ did not aggregate platelets in the presence of these factors, indicating a requirement for another plasma factor(s). Therefore, it is reasoned that fibrinogen bound to ClfA is involved in the platelet activation process. The most likely scenario, as has been previously suggested (Herrmann *et al.*, 1993; O'Brien *et al.*, 2002; Pawar *et al.*, 2004; Liu *et al.*, 2005), is that fibrinogen acts as a bridge between ClfA on the bacterium and GPIIb/IIIa on the platelet

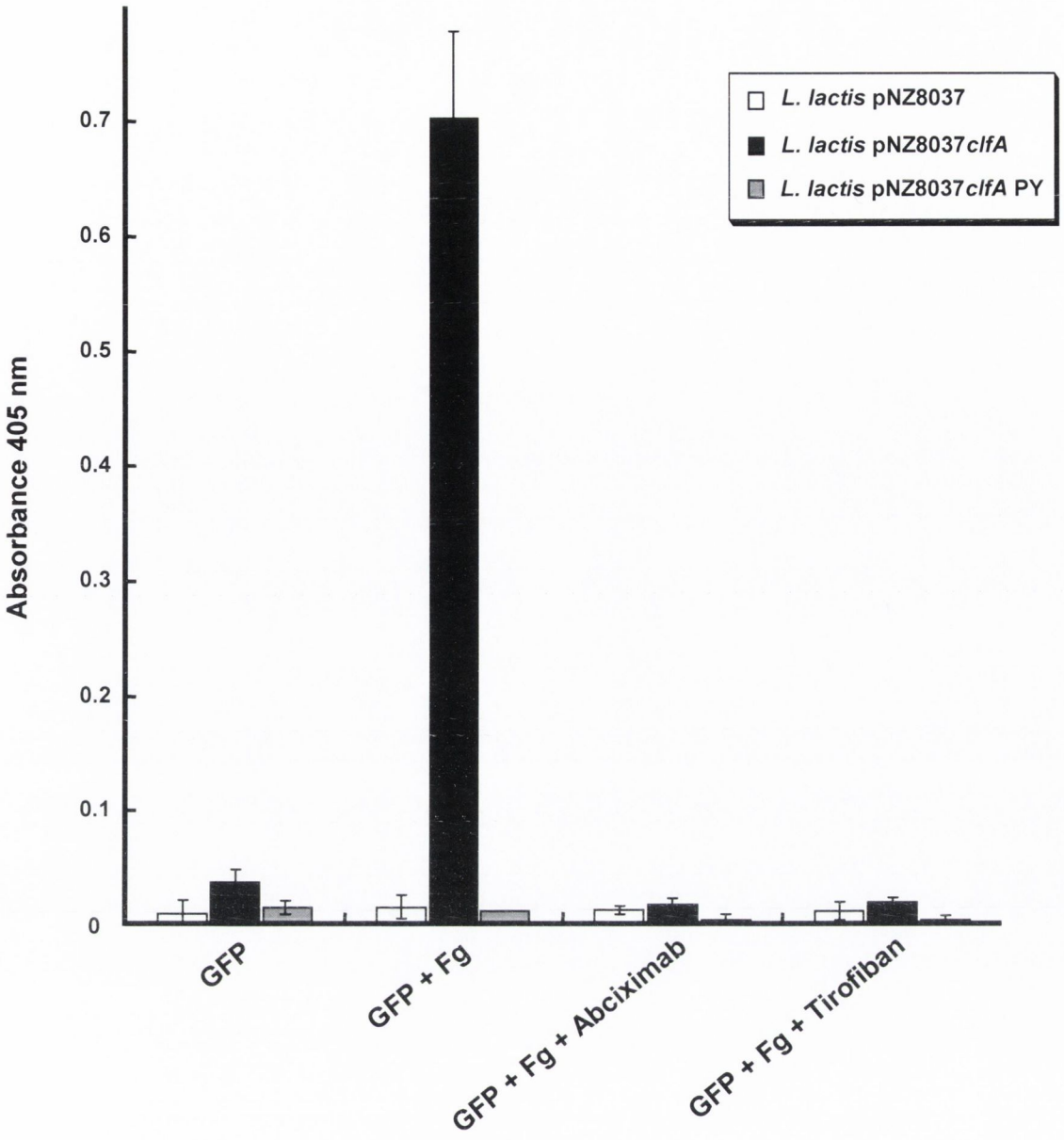


Figure 3.25. Adhesion of platelets to immobilized *L. lactis* cells expressing ClfA and ClfA PY

L. lactis (pNZ8037), *L. lactis* (pNZ8037*clfA*) and *L. lactis* (pNZ8037*clfA* PY) were grown in the presence of 1.6 ng/ml nisin. Cells were washed and adjusted to OD_{600} 1.0 in PBS and used to coat wells of 96-well dishes. GFPs were incubated with the GPIIb/IIIa antagonists abciximab and tirofiban (20 μg/ml) for 30 min at 37°C. Fibrinogen (1 mg/ml final concentration) was added to GFP samples before addition to the bacterial-coated wells (1 x 10^7 platelets/well) for 30 min as indicated. Non-adherent platelets were removed by washing and adherent platelets were lysed with 100 μl lysis buffer (0.1 M Na acetate, 0.1 % (v/v) Triton X-100) containing a substrate for acid phosphatase (10 mM *p*-nitrophenol phosphate) per well. The absorbance of the wells at 405nm was determined using an ELISA plate reader after 1 h incubation at 37°C. This experiment was performed 4 times using 4 different blood donors.

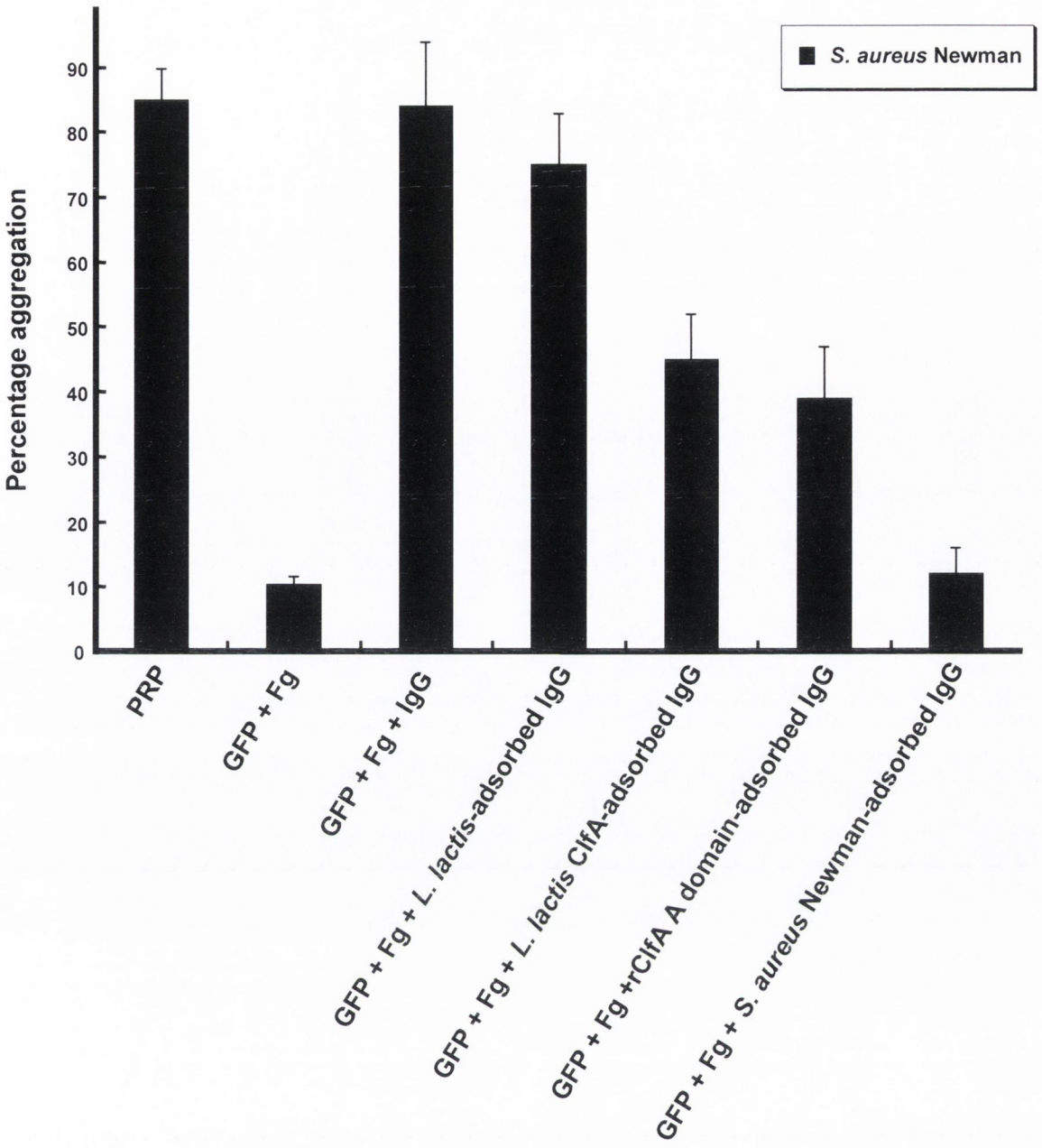


Figure 3.26. Bacterial-specific IgG is required for platelet activation by *S. aureus* Newman

S. aureus Newman cells were grown to stationary phase (16 h), washed in PBS, and adjusted to OD_{600} 1.6 in PBS. Bacterial cells (25 μl) were added to PRP (225 μl) or GFP samples (5 x 10^7 GFPs in 225 μl final volume) supplemented with purified fibrinogen (Fg; 1 mg/ml) and purified human IgG samples (2 mg/ml) as indicated. This experiment was performed 3 times using 3 different blood donors. Results are presented as mean ± SD of light transmission (percentage aggregation) observed after 25 min.

surface. It was demonstrated here that adhesion of platelets to immobilized *L. lactis* expressing ClfA was greatly enhanced in the presence of fibrinogen, and that this adhesion was GPIIb/IIIa dependent. Collectively, these data point to fibrinogen playing an essential role in the adhesion of ClfA-expressing bacteria to GPIIb/IIIa on resting platelets, and that this binding is in part required for rapid activation and aggregation. Although GPIIb/IIIa is in the low-affinity "non-adhesive" state on resting platelets and cannot bind soluble fibrinogen, it can bind to fibrinogen immobilized on surfaces (Savage *et al.*, 1996). ClfA-expressing cells in the presence of plasma will presumably be coated with fibrinogen to some extent (Massey *et al.*, 2002), and act as a fibrinogen-coated surface supporting platelet adhesion.

However, fibrinogen-dependent binding of ClfA-expressing cells to platelet GPIIb/IIIa is itself insufficient to trigger a complete aggregation response. Aggregation of GFPs by *L. lactis* ClfA+ or *S. aureus* Newman did not occur when highly purified human fibrinogen was added. It was shown here that, in addition to ClfA-bound fibrinogen, IgG was absolutely required for ClfA to aggregate plasma-depleted platelets. This apparently contradicts previous observations showing that aggregation of washed platelets by *S. aureus* only requires exogenous fibrinogen (O'Brien *et al.*, 2002; Liu *et al.*, 2005). However, these studies were performed using commercially available fibrinogen preparations, which are contaminated to some extent with other plasma proteins, including IgG. It was observed here that impure fibrinogen preparations supported aggregation of GFPs by *S. aureus* Newman (data not shown). Removal of contaminating IgG by passage over a protein A-sepharose column abolished aggregation, and full aggregation was restored upon addition of exogenous IgG.

Numerous experiments demonstrated that the IgG supporting aggregation must contain antibodies recognizing ClfA. IgG samples that had been depleted of anti-ClfA antibodies, either by incubation with bacteria expressing ClfA or by passage over a column containing the recombinant A domain, did not support aggregation by *L. lactis* ClfA+ in the presence of purified fibrinogen. A requirement for ClfA-specific IgG was also demonstrated for the fibrinogen-independent (ClfA PY) mechanism. For *S. aureus* Newman, anti-ClfA depleted IgG supported aggregation of platelets suspended in fibrinogen, but to a much reduced extent compared to that seen with platelets suspended in fibrinogen and normal IgG. *S. aureus* did not aggregate platelets suspended in fibrinogen and IgG that had been adsorbed against whole *S. aureus* Newman cells. This suggests that antibodies recognizing other *S. aureus* components could participate to some extent in activation. The presence of a functional FcγRIIa receptor on platelets was critical for aggregation by ClfA-expressing *S. aureus* and *L.*

lactis strains, indicating that this receptor recognizes the Fc region of ClfA-bound IgG. A model is proposed whereby bacteria armed with a sufficient number of surface-bound fibrinogen molecules adhere to platelet GPIIb/IIIa, allowing ClfA-bound IgG molecules to engage FcγRIIa receptors and stimulate receptor clustering (Figure 3.27). This can trigger activation leading to GPIIb/IIIa activation, fibrinogen-binding by GPIIb/IIIa and finally platelet aggregation. In the case of activation stimulated by ClfA PY, it appears that specific IgG engaging FcγRIIa is required, along with another plasma factor linking ClfA PY and the platelet surface.

The precise role of GPIIb/IIIa in the platelet response to *S. aureus* was established in these studies. GPIIb/IIIa has been demonstrated to be essential for platelet aggregation by *S. aureus* (O'Brien *et al.*, 2002; Pawar *et al.*, 2004; Liu *et al.*, 2005). It was possible that *S. aureus* activated platelets independently of GPIIb/IIIa, this activation leading to inside-out GPIIb/IIIa activation, conformational changes and finally fibrinogen-binding resulting in aggregation. Alternatively, a fibrinogen-bridging mechanism between *S. aureus* ClfA and GPIIb/IIIa could possibly occur, this adhesion event then leading to activation and aggregation. Support for the latter model recently emerged, where it was shown that platelet shape change (indicative of activation) stimulated by *S. aureus* was inhibited by GPIIb/IIIa antagonists (Liu *et al.*, 2005). It was shown here that fibrinogen promotes GPIIb/IIIa-dependent platelet adhesion to ClfA, and that rapid activation by bacterial cells expressing ClfA requires fibrinogen-binding to the adhesin. These data support the notion that GPIIb/IIIa / fibrinogen / ClfA interactions are crucial for fast activation.

The absolute requirement for IgG specific for the bacterial cell in platelet activation by *S. aureus* has not been previously demonstrated. This is perhaps surprising, given the wealth of information on the role of FcγRIIa and specific IgG in platelet activation by numerous other bacteria, especially *Streptococcus* species (discussed in section 1.5.4). Many strains of *Strep. sanguis* utilize specific IgG and FcγRIIa to stimulate activation (Sullam *et al.*, 1988; Ford *et al.*, 1997). This is generally accompanied by another interaction between bacterial receptors and the platelet, which may or may not require a plasma bridging factor. *Strep. sanguis* strains that express the glycoprotein adhesin SrpA adhere directly to platelet GPIb (Kerrigan *et al.*, 2002; Plummer *et al.*, 2005), but activation also requires FcγRIIa (Kerrigan *et al.*, 2002). Other *Strep. sanguis* strains stimulate activation through surface-bound IgG and complement proteins interacting with FcγRIIa and a complement receptor(s) respectively (Ford *et al.*, 1996,

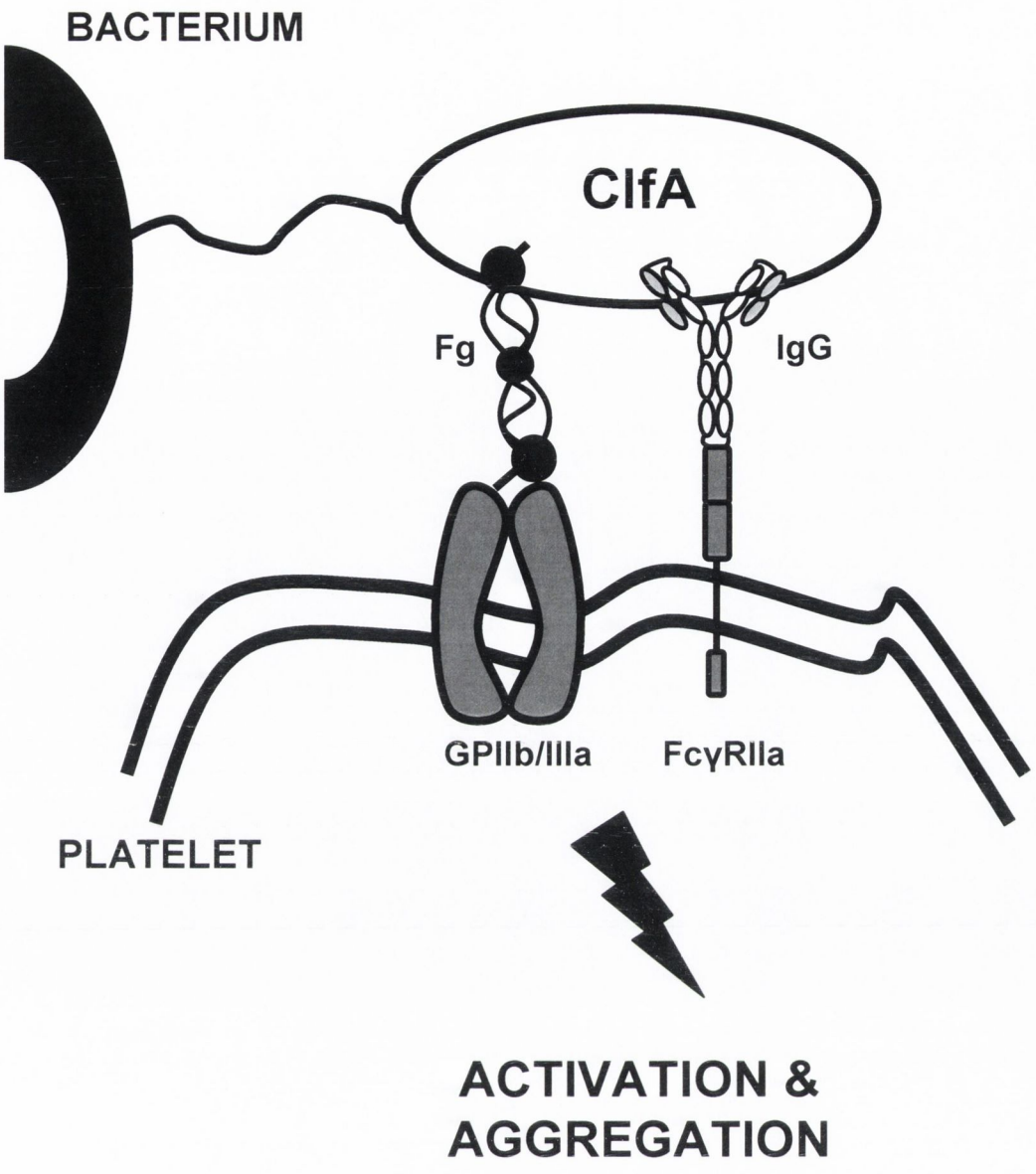


Figure 3.27. Proposed model for platelet activation by ClfA-expressing bacteria

ClfA-expressing cells in plasma bind the extreme C-terminus γ-chain of fibrinogen (Fg), promoting bacterial adhesion to GPIIb/IIIa. In addition, antibodies bound to ClfA are required for an interaction with FcγRIIa. This stimulates receptor clustering and signaling events in the platelet, resulting in calcium oscillations and GPIIb/IIIa activation. This results in cross-linking of platelets into aggregates.

1997). Platelet activation by *Strep. pyogenes* fibrinogen-binding M proteins involves fibrinogen-bridging between the M protein and GPIIb/IIIa along with M protein-specific IgG bound to the streptococcal surface cross-linking FcγRIIa receptors (Sjöbring *et al.*, 2002). The FbsA fibrinogen-binding protein expressed by *Strep. agalactiae* activated platelets through anti-FbsA antibodies binding FcγRIIa in conjunction with fibrinogen-bridging to GPIIb/IIIa (Pietrocola *et al.*, 2004). A similar mechanism was proposed for *H. pylori*, where *H. pylori*-bound vonWillebrand factor links the bacterial cell to GPIb, and IgG bound to the bacterial surface engages FcγRIIa (Byrne *et al.*, 2003). The model proposed here for platelet activation by ClfA closely parallels these models, especially that seen for streptococcal M proteins and the FbsA protein of *Strep. agalactiae* (Sjöbring *et al.*, 2002; Pietrocola *et al.*, 2004). FcγRIIa has been shown to be involved in thrombus formation by *S. aureus* under flow conditions (Sjöbring *et al.*, 2002), suggesting a role for IgG, although this was not demonstrated directly. The finding that platelet activation stimulated by *S. aureus* ClfA requires adhesin-specific IgG suggests that *S. aureus* and other bacteria share certain similarities with respect to their mechanism of inducing platelet activation.

This requirement for anti-ClfA specific IgG indicates that antibodies to bacterial surface components are present in donors' plasma. Due to the commensal nature of *S. aureus*, one would expect that exposure would generate low-level antibody responses in the population. Anti-staphylococcal antibodies have been detected in sera from healthy individuals (Dryla *et al.*, 2005), with the presence of low-levels of anti-ClfA antibodies in all donors tested (Colque-Navarro *et al.*, 2000; Dryla *et al.*, 2005). The immunoglobulin source used for this study, consisting of human Ig preparations pooled from a wide range of plasma donors, was shown to contain antibodies recognizing ClfA, indicating that anti-ClfA specific antibodies are present in the normal population. This may explain the fact that very little variation was observed in platelet preparations from a variety of donors tested in this study. Platelets from all donors tested aggregated rapidly (lag time < 2 minutes) in response to either *S. aureus* or *L. lactis* ClfA+. Platelets always responded to *L. lactis* ClfA PY+, although some variability was observed in the lag times to aggregation, ranging from 5 to 12 minutes at the same high expression levels of the bacterial protein. To induce aggregation, both ClfA and ClfA PY had to be expressed at a sufficiently high level on the surface of *L. lactis*. Moreover, the threshold expression levels of ClfA and ClfA PY required were the same. Specific IgG to ClfA is the common factor required for activation by ClfA and ClfA PY. The inhibition of

aggregation that was observed upon incubation of PRP samples with recombinant A domains of ClfA or ClfA PY is likely due to sequestering the low-level specific IgG present in donors plasma. It is tempting to speculate that a high expression level of ClfA or ClfA PY is required to stimulate sufficient binding of adhesin-specific IgG present in donors plasma to the bacterial cell surface, allowing productive interactions with platelet FcγRIIa to trigger activation. This is supported by the finding that aggregation by wild-type ClfA was prolonged in elevated Ca^{2+} conditions (associated with reduced fibrinogen-binding by ClfA), but that the threshold expression level was the same as in lower Ca^{2+} environments. *L lactis* ClfA PY+ stimulated aggregation with similar lag times and threshold expression levels in both low and physiological Ca^{2+} platelet preparations.

As ClfA has been reported to bind directly to an unidentified 118 kDa platelet membrane protein (Siboo *et al.*, 2001), it is possible that this interaction participated in the fibrinogen-independent activation mechanism by ClfA PY. Another possibility is that GPIb functioned as a receptor for ClfA PY, as it was observed here that a monoclonal anti-GPIb antibody completely inhibited aggregation by *L. lactis* ClfA PY+, but only partially inhibited aggregation by the wild-type protein. Based on a number of observations, it is unlikely that a direct binding mechanism in addition to IgG-FcγRIIa is responsible. (1) Washed platelets did not directly adhere to *L. lactis* ClfA PY+. (2) A plasma factor in addition to IgG was required for activation. This excluded the possibility that only a ClfA PY-p118 interaction (in addition to IgG and FcγRIIa) was required. However, a possible plasma-protein bridging interaction between ClfA and GPIb could not be excluded. The primary plasma ligand for GPIb is von Willebrand factor, and a vonWillebrand factor bridge linking *H. pylori* to platelet GPIb has been shown to participate in activation (Byrne *et al.*, 2003). Again, the possiblilty that von Willebrand factor links ClfA PY and GPIb to aid activation is unlikely. *L. lactis* ClfA+ and *L. lactis* ClfA PY+ failed to adhere to purified von Willebrand factor immobilized in microtitre wells (data not shown). The addition of purified von Willebrand factor to platelets suspended in fibrinogen and IgG was not sufficient for aggregation by *L. lactis* ClfA PY+ (data not shown). Finally, incubation of PRP samples with polyclonal anti-von Willebrand factor sera did not block ClfA PY-promoted aggregation (data not shown).

One possibility for the observed inhibition of aggregation by the anti-GPIb antibody is the close physical proximity of GPIb and FcγRIIa in the platelet membrane (Sullam *et al.*, 1998). It is possible that antibodies bound to GPIb sterically blocked the crucial interaction

between bacterial-bound IgG and FcγRIIa (Sullam *et al.*, 1998). The anti-GPIb antibody substantially inhibited aggregation by wild-type ClfA (61 % inhibition), and it completely blocked aggregation by ClfA PY (92 % inhibition). With the lack of any obvious participation of GPIb in the platelet response to wild-type ClfA, it is suggested that partial inhibition by anti-GPIb antibodies may be a result of impairment of signaling mediated by FcγRIIa. The greater inhibition of aggregation seen for ClfA PY may simply be the result of the reduced efficiency of this activation mechanism.

Platelet activation by ClfA PY apparently involves an unidentified plasma factor mediating adhesion to platelets and ClfA-specific immunoglobulin binding FcγRIIa. The ability of ClfA to activate platelets through both fibrinogen-dependent and fibrinogen-independent mechanisms may serve to maximize the interaction with platelets *in vivo*, where the ability of ClfA to bind fibrinogen may be impaired by prevailing Ca^{2+} concentrations. The identification of the participating plasma component is discussed in chapter 5.

In conclusion, it has been shown that ClfA expressed on the surface of *S. aureus* or the surrogate host *L. lactis* can promote rapid platelet activation by engaging the low affinity platelet integrin GPIIb/IIIa through a fibrinogen bridge and the platelet low-affniity Fc receptor FcγRIIa through an IgG bridge requiring specific antibodies. A fibrinogen-independent interaction involving specific IgG and one or more plasma factors was demonstrated. In triggering platelet activation, bacteria expressing ClfA use host adhesive proteins (fibrinogen), and exploit the adaptive immune response by using antibodies intended to protect the host as a pathogenic mechanism, leading to thrombus formation and disease progression.

Chapter 4

Staphylococcus aureus fibronectin-binding proteins are the major mediators of platelet activation in the exponential growth phase

4.1 Introduction

Platelet activation by *S. aureus* strain Newman cells grown to stationary phase has been shown to be predominantly mediated by ClfA (Chapter 3). Activation is rapid, with a lag time to the onset of aggregation ranging between 1 and 1.5 minutes. It was noted that exponentially growing cells of strain Newman stimulated slower aggregation, with lag times of approximately 4 minutes (O'Brien *et al.*, 2002). The surface components of *S. aureus* Newman that contribute to platelet activation in the exponential growth phase were not determined in that study. Experiments were performed more recently in this laboratory to determine what factors contribute to platelet activation by exponentially growing *S. aureus* cells. This is the *in vitro* growth phase that is most likely to represent bacteria growing in the bloodstream during endovascular infection.

S. aureus contains two closely linked genes, *fnbA* (Signäs *et al.*, 1989) and *fnbB* (Jönsson *et al.*, 1991), encoding fibronectin-binding proteins A and B. These are present in most isolates, but some strains contain only one *fnb* gene (Peacock *et al.*, 2000). Both FnBPA and FnBPB are predominantly expressed in the exponential growth phase (Saravia-Otten *et al.*, 1997). The N-terminal A domains of FnBPA and FnBPB mediate binding to the C-terminus of the fibrinogen γ-chain and to elastin peptides (Wann *et al.*, 2000; Roche *et al.*, 2004; Figure 1.4). Located distal to the A domain is the BCD domains in FnBPA or the CD domains in FnBPB (Jönsson *et al.*, 1991). These regions are unfolded and do not contain any discernable secondary structure (House-Pompeo *et al.*, 1996). Multiple, tandemly arranged motifs mediate binding to the N-terminal type I modules of fibronectin by a tandem β-zipper mechanism (Sottile *et al.*, 1991; Schwarz-Linek *et al.*, 2003; Figure 1.4). Both fibrinogen and fibronectin serve as adhesive ligands for platelets (Bowditch *et al.*, 1991; Savage *et al.*, 1996). This raises the possibility that *S. aureus* interacts with platelets via fibronectin or fibrinogen bridging between its surface-expressed FnBPs and the platelet receptors for these ligands, which may result in platelet activation and aggregation. Mutants of *S. aureus* defective in fibronectin-binding have been shown to be less virulent in some experimental endocarditis studies (Kuypers and Proctor, 1989) but not in others (Flock *et al.*, 1996). The contradictory results regarding the role of FnBPs in virulence is likely due to inappropriate animal models or the use of *S. aureus* strains that express FnBPs at low levels. Heterologous expression of FnBPA in *L. lactis* increased infectivity 100-fold in a rat endocarditis model (Que *et al.*, 2001). FnBPA expression by *L. lactis* correlated with increased bacterial densities in

vegetations and enhanced valvular destruction (Que *et al.*, 2005). FnBPs may be important contributing factor to the destructive nature and severe clinical course associated with endocarditis caused by *S. aureus*.

The *fnbA* and *fnbB* genes in *S. aureus* strain Newman each contain an internal stop codon (Grundmeier *et al.*, 2004), resulting in expression of truncated FnBPA and FnBPB proteins that are secreted from the cell instead of being anchored to the cell wall. *S. aureus* Newman is defective in phenotypes associated with FnBP-expression, such as adhesion to immobilized ligands specific for FnBPs (fibronectin and elastin) and *in vitro* invasion of host cells (Vaudaux *et al.*, 1998; Roche *et al.*, 2004). Therefore previous studies using strain Newman would have failed to detect any contribution of FnBPs to platelet activation.

The *fnbA* and *fnbB* genes were originally cloned from *S. aureus* strain 8325-4 (Signäs *et al.*, 1989; Jönsson *et al.*, 1991). However, low expression of FnBPs due to a defective sigma factor (SigB) means that this strain is not a suitable host for *in vitro* studies of these proteins (Bischoff *et al.*, 2004; Roche *et al.*, 2004). *S. aureus* strain P1 expresses high-levels of FnBPs, as indicated by the high-level adherence of this strain to immobilized elastin peptides (Roche *et al.*, 2004). It was therefore selected for studies examining the role of FnBPs in promoting platelet activation. Phage transduction was performed by Dr. J.R. Fitzgerald to construct derivatives of strains Newman and P1 that were deficient in one or more surface proteins implicated in platelet activation by *S. aureus* cells grown to stationary phase (ClfA, ClfB, SdrE and protein A; O'Brien *et al.*, 2002). Systematic elimination of ClfA, ClfB, SdrE and protein A from strain Newman resulted in a progressive lengthening of the lag time from 3.6 min in the wild-type to 9 min in the *clfAclfBsdrEspa* quadruple mutant. However, in strain P1, none of these factors seemed to contribute to the rapid aggregation caused by this strain when it was grown to exponential phase, because even the *clfAclfBsdrEspa* mutant aggregated platelets with lag times of 1 to 1.5 min. However, a derivative of strain P1 defective in FnBP-expression exhibited a substantially prolonged lag time (> 8 minutes) suggesting a major role for fibronectin-binding proteins in promoting platelet activation. Complementation of this strain with multicopy plasmids expressing either FnBPA or FnBPB restored the lag times back to levels achieved in the wild-type (1 – 1.5 minutes). This demonstrated that expression of FnBPA and/or FnBPB by *S. aureus* confers a potent ability to aggregate platelets. This information was obtained by Dr. J.R. Fitzgerald and the data has been published (Fitzgerald *et al.*, 2006).

The experiments described in this chapter were performed to investigate the molecular mechanisms by which bacteria expressing FnBPs activate platelet aggregation. The results presented here indicate that FnBPs activate platelets by a mechanism that closely parallels that suggested for ClfA in Chapter 3. A general mechanism by which *S. aureus* cells induce platelet activation is proposed. These data have broad implications for our understanding of the pathogenesis of infective endocarditis and for the development of novel therapeutics against vascular infections.

4.2 Results

4.2.1 Expression of fibronectin-binding proteins by *S. aureus* strain P1

S. aureus strain P1 adheres strongly to immobilized elastin peptides, which is indicative of high-level FnBP expression (Roche *et al.*, 2004). To examine the functional expression of FnBP, adherence assays to immobilized human fibronectin were performed. An *S. aureus* P1 mutant defective in FnBP production (P1 *fnbAfnbB*), and the mutant expressing FnBPA and FnBPB from multicopy plasmids (pFnBA4 and pFnBB4 respectively) were tested in parallel with the parental strain. *S. aureus* strains, grown to exponential phase for optimum FnBP expression, were tested for the ability to adhere to the fibronectin-coated wells. *S. aureus* P1 adhered with high affinity to the ligand in a dose-dependent, saturable manner (Figure 4.1). In contrast, strain P1 *fnbAfnbB* adhered poorly (Figure 4.1). The high-level adherence to fibronectin characteristic of *S. aureus* P1 was restored in the P1 *fnbAfnbB* mutant by complementation with expression plasmids containing either the *fnbA* gene (pFnBA4) or the *fnbB* gene (pFnBB4) from strain 8325-4 (Figure 4.1).

Cell wall-associated proteins of these strains were solubilized by lysostaphin, separated by SDS-PAGE and probed for the presence of FnBPs by Western ligand affinity blotting and Western immunoblotting (section 2.10). This was performed to establish the levels of FnBPA and FnBPB expression in strain P1. Strains P1 *fnbAfnbB* and P1 overexpressing FnBPA and FnBPB were included as controls. To detect the presence of both FnBPA and FnBPB, membranes were probed with fibronectin that had been conjugated to biotin, followed by detection with peroxidase-conjugated streptavidin. *S. aureus* P1 expressed a high-molecular weight protein (> 175 kDa) with fibronectin-binding activity which was absent in the

fnbAfnbB mutant (Figure 4.2), indicating that this protein is the product of either the *fnbA* or *fnbB* genes, or both genes. P1 *fnbAfnbB* containing either plasmid pFnBA4 or pFnBB4 expressed fibronectin-binding proteins of > 175 kDa (Figure 4.2) which corresponds to the previously reported sizes of FnBPA and FnBPB (Signäs *et al.*, 1989; Jönsson *et al.*, 1991). Extensive breakdown of FnBPs was observed, both in the wild-type strain and in the FnBPA and FnBPB complemented strains (Figure 4.2). Degradation of FnBPs upon extraction from the cell wall has been previously observed (Signäs *et al.*, 1989; Jönsson *et al.*, 1991), and likely reflects the presence of proteases in the lysostaphin lysates. The large proteins in each sample are probably the full length intact protein with the A domain present. The smaller, strongly reactive band is most likely a degradation product with the majority of the A domain cleaved off. It was judged by densitometry analysis that the wild-type strain expressed approximately 4-fold lower levels of FnBPs than the *fnbAfnbB* strain either expressing FnBPA or FnBPB from plasmids.

To identify the FnBPs expressed by *S. aureus* P1, membranes were probed with polyclonal rabbit antibodies recognizing the A domains of either FnBPA or FnBPB that were originally cloned from *S. aureus* 8325-4. Antibodies recognizing the A domain of FnBPA bound to a high-molecular weight (> 175 kDa) band present in the cell-wall extract of strain P1 *fnbAfnbB* (pFnBA4) (Figure 4.2). Similarly, anti-FnBPB A domain antibodies recognized a high molecular weight protein in strain P1 *fnbAfnbB* (pFnBB4) (Figure 4.2). Antibodies recognizing FnBPA did not cross-react with FnBPB and vice-versa, unless used at < 1:1000 dilution (data not shown). No immunoreactive proteins were observed in extracts of the FnBP-deficient strain (Figure 4.2). Interestingly, antibodies against FnBPA or FnBPB did not recognize the high-molecular weight FnBPs expressed by the wild-type P1 strain (Figure 4.2). A faint reaction was observed with antibodies against the FnBPB A domain in comparison to P1 *fnbAfnbB* (pFnBB4) (Figure 4.2). It appears that polyclonal antibodies raised against the recombinant A domains of FnBPA and FnBPB cloned from *S. aureus* 8325-4 do not recognize FnBPA or FnBPB from strain P1. The possibility that FnBPs are subject to antigenic diversity is discussed later in section 4.2.10.

4.2.2 Platelet activation and aggregation by *S. aureus* P1

It was observed by Dr. J.R. Fitzgerald that *S. aureus* P1 stimulated rapid platelet aggregation when grown to exponential phase and that the FnBP-defective strain stimulated slower aggregation after a lag time of approximately 10 minutes. Rapid aggregation is likely a

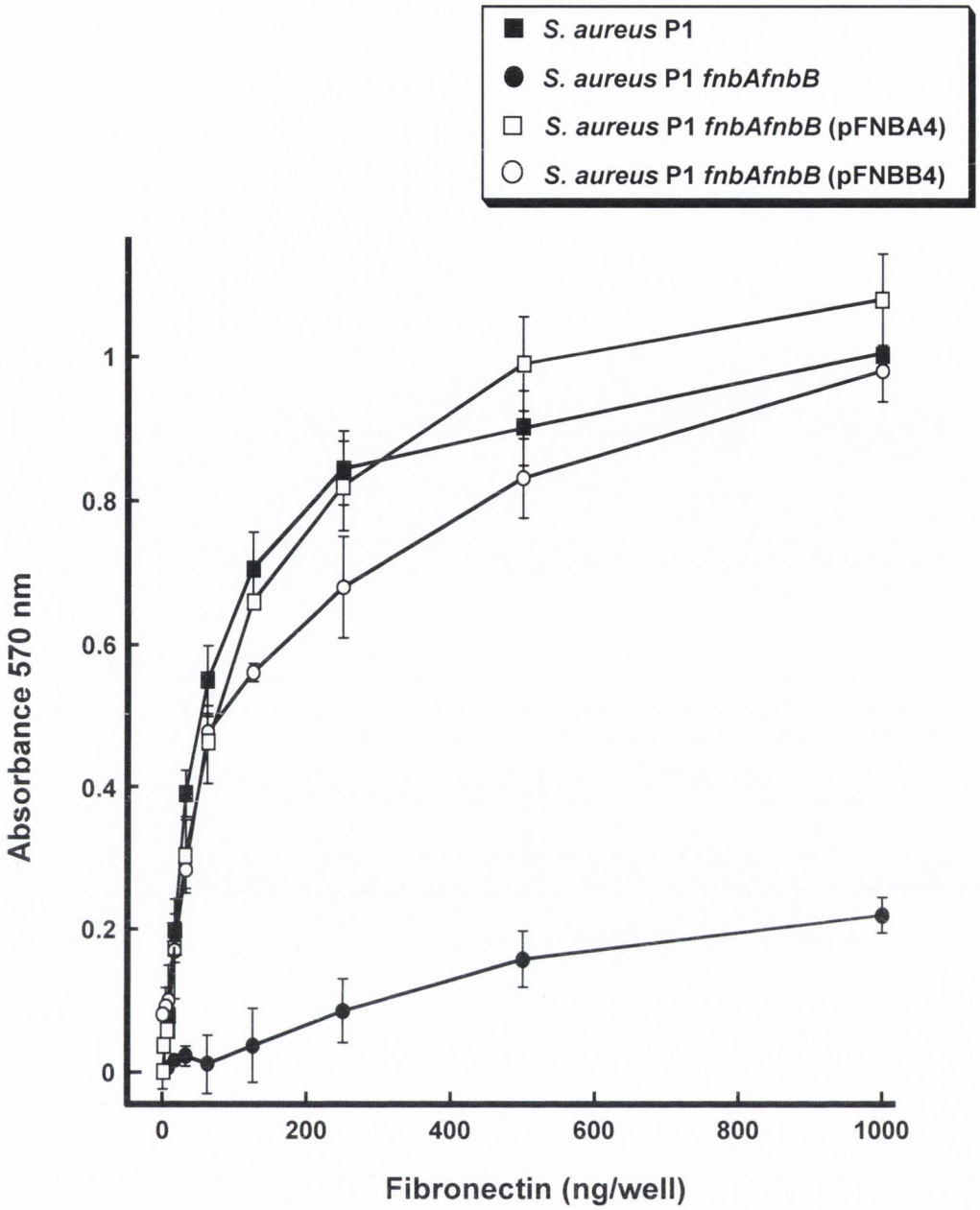


Figure 4.1. Adhesion of S. aureus P1 derivatives to immobilized fibronectin

S. aureus strains were grown to early exponential phase and washed in PBS. The ability of cell suspensions to adhere to fibronectin immobilized in microtitre wells (4 – 1000 ng/ml) was tested. Adherent cells were stained with crystal violet and the absorbance of the wells at 570 nm was determined using an ELISA plate reader.

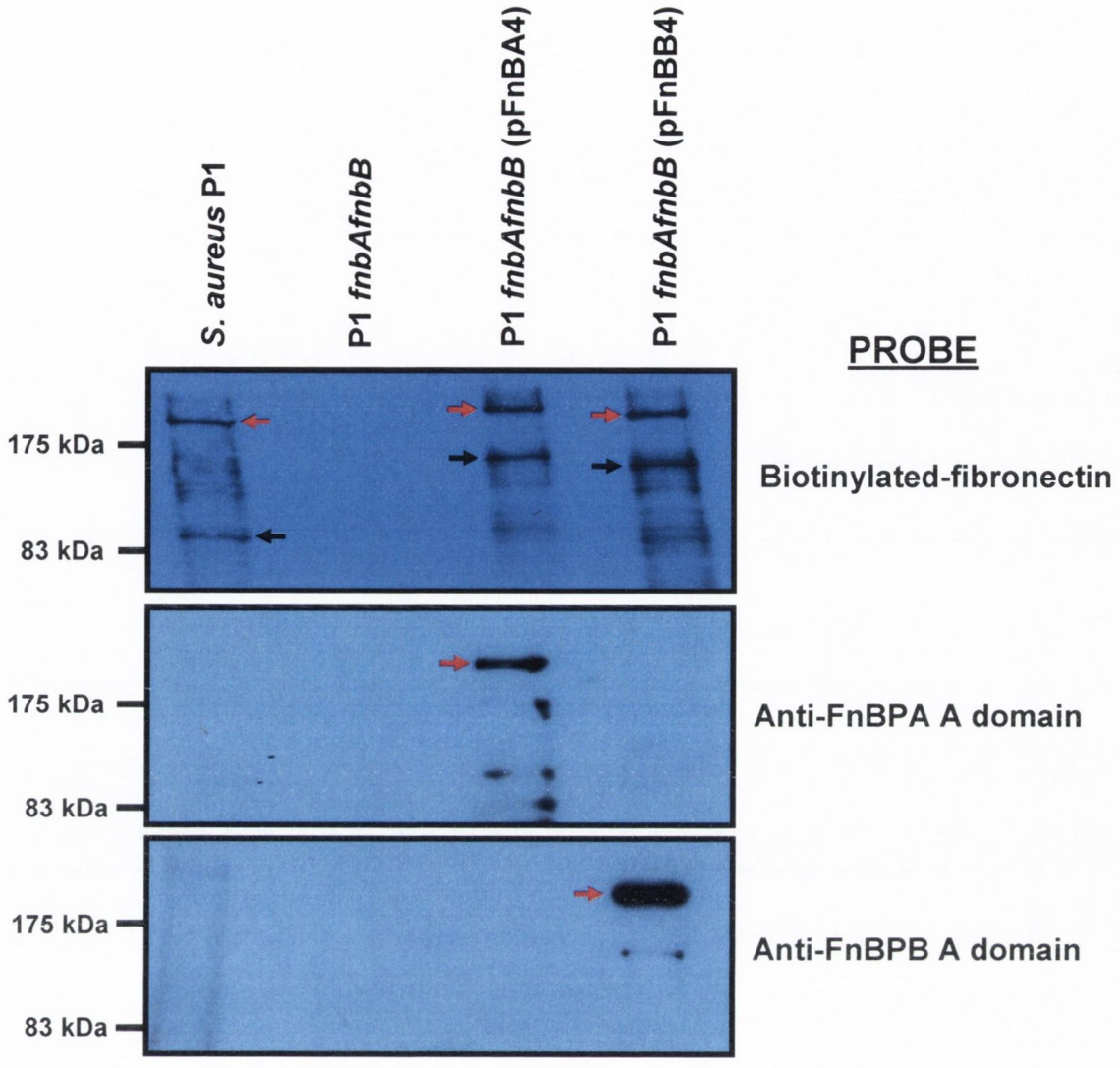


Figure 4.2. Detection of FnBPs in cell wall extracts of *S. aureus* P1 and its derivative strains

Total cell wall proteins of (1) *S. aureus* P1 wild-type (2) *S. aureus* P1 *fnbAfnbB* (3) *S. aureus* P1 *fnbAfnbB* (pFnBA4) and (4) *S. aureus* P1 *fnbAfnbB* (pFnBB4) were seperated on 7.5 % acrylamide gels and electroblotted onto PVDF membranes. Membranes were probed with a solution of biotinylated fibronectin (30 μg/ml) or polyclonal antibodies recognising the A domains of FnBPA or FnBPB. Bound probe was detected with the appropriate conjugated substrate and the membranes developed by chemiluminescence. Red arrows denote the position of full-length FnBPs, with lower molecular weight bands (black arrows) reflecting breakdown products of the full-length protein.

result of FnBP(s) expressed by *S. aureus* P1 interacting with resting platelets leading to activation, conformational modulation of GPIIb/IIIa into its high-affinity state and finally fibrinogen-dependent aggregation. Further experiments were performed here to measure FnBP-mediated platelet activation, either by measurement of aggregation or direct measurement of calcium increases in platelets. In human platelet-rich-plasma (PRP), *S. aureus* P1 caused aggregation after a lag time of 1.4 ± 0.3 min; n = 3. The FnBP-deficient P1 derivative caused aggregation with a much extended lag time (10.2 ± 0.8 min; n = 3; $p < 0.05$). Times to aggregation comparable to the wild-type were observed upon expression of FnBPA (1.6 ± 0.1 min; n =3) and FnBPB (1.3 ± 0.2 min; n = 3) from multicopy plasmids in strain P1 *fnbAfnbB*. A typical aggregation trace is shown in Figure 4.3. This confirms the original findings of Dr. J.R. Fitzgerald that FnBPs are major mediators of platelet aggregation by *S. aureus* P1 in the exponential growth phase. It also demonstrates that both FnBPA and FnBPB can independently stimulate aggregation.

To confirm that the observed aggregation response was the result of genuine platelet activation, experiments were conducted to measure calcium increases (flux) in platelets. Gel-filtered platelets (GFP) were isolated from plasma and loaded with the calcium ionophore FURA-2-AM as described in section 2.20. GFPs were adjusted to physiological concentrations (2×10^8 platelets/ml) in JNL buffer supplemented with 1 mM Ca^{2+} and 50 % v/v plasma. Washed cell suspensions of *S. aureus* P1 and its derivative strains were added to the labeled platelets. Calcium increases were monitored by measuring the fluorescence of intracellular FURA-2. In the presence of high calcium, FURA-2 fluoresces at 362 nm, whereas in low calcium the wavelength at which fluorescence occurs is 335 nm. Calcium flux therefore results in an increase in fluorescence at 360 nm, with a concomitant decrease in fluorescence at 340 nm. By monitoring the Ex $^{360}/_{340}$ fluorescent ratio over a time course, the dynamic changes in platelet intracelular calcium that occurs in response to bacteria can be observed. *S. aureus* P1 wild-type cells stimulated rapid calcium flux (activation) in FURA-2 loaded platelets (Figure 4.4). *S. aureus* P1 *fnbAfnbB* did not stimulate calcium flux (Figure 4.4), and no activation was observed after 5 minutes of monitoring (n = 3). Expression of FnBPA or FnBPB in *S. aureus* P1 *fnbAfnbB* was sufficient to cause rapid activation (Figure 4.4). Together, these data demonstrate that *S. aureus* expressing FnBPs stimulates rapid aggregation of platelets in plasma, and this is a result of FnBP-mediated activation of resting platelets.

4.2.3 Expression of fibronectin-binding proteins in *Lactococcus lactis*

To analyze the molecular mechanisms by which FnBPs promote platelet activation, the genes encoding FnBPA and FnBPB were cloned into the nisin-inducible expression vector pNZ8037 for expression on the surface of *L. lactis*. Genomic DNA was isolated from *S. aureus* 8325-4 as described in section 2.3. PCR reactions were performed with 8325-4 genomic DNA as the template and the appropriate primers (section 2.5). PCR products were cleaved with *Nco*I/*Xho*I (*fnbA*) or *Nco*I/*Xba*I (*fnbB*), ligated with pNZ8037 cleaved with the same enzymes, and transformed into *L. lactis* NZ9800 (section 2.5). Transformant colonies were purified, grown for 16 h in the presence of 1.6 ng/ml nisin, and screened by testing adhesion to immobilized fibronectin and immobilized fibrinogen.

Cultures of *L. lactis* (pNZ8037), *L. lactis* (pNZ8037*fnbA*) and *L. lactis* (pNZ8037*fnbB*) were induced overnight with 1.6 ng/ml nisin. Cells were washed in PBS and tested for adhesion to microtitre wells coated with fibrinogen or fibronectin (4 – 2000 ng protein/well). *L. lactis* (pNZ8037) did not adhere to wells coated with either ligand (Figure 4.5). Expression of FnBPA or FnBPB by *L. lactis* supported dose-dependent saturable binding to both fibrinogen and fibronectin (Figure 4.5), indicating that both proteins were expressed by *L. lactis* in a fully-functional form.

L. lactis strains expressing FnBPA and FnBPB were grown overnight in varying nisin concentrations to induce FnBP expression. The ability of cells to adhere to immobilized fibrinogen (0.5 μg/well) was tested. *L. lactis* (pNZ8037*fnbA*) and *L. lactis* (pNZ8037*fnbB*) adhered strongly to fibrinogen, the degree of adherence being relative to the concentration of nisin in which the cells were grown (Figure 4.6). This demonstrated that expression of FnBPA and FnBPB in *L. lactis* was controlled by the availability of extracellular nisin. *L. lactis* expressing FnBPA adhered to the fibrinogen-coated wells with slightly higher affinity than *L. lactis* expressing FnBPB (Figures 4.5 and 4.6).

4.2.4 Expression of FnBPA truncates in *L. lactis*

The functional domains of FnBPA have been well defined. Fibronectin-binding activity has been mapped to 11 repeat domains within FnBPA, stretching from the C-terminus of the A domain right through to the D repeats of the protein (Schwarz-Linek *et al.*, 2003). Fibrinogen (and elastin) binding activity has been mapped to the N-terminal A domains of the protein (Wann *et al.*, 2000; Roche *et al.*, 2004). FnBPA truncates were expressed on the

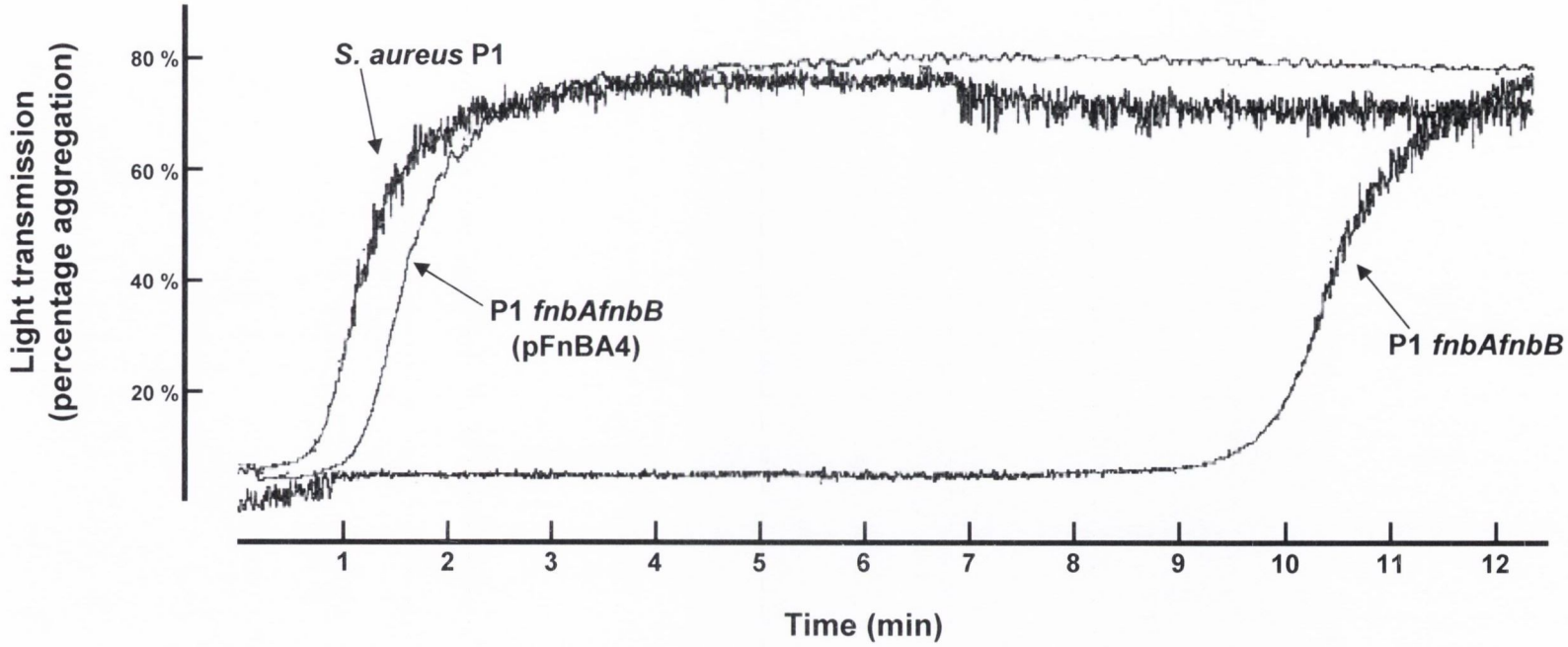


Figure 4.3. Aggregation of platelet-rich-plasma by *S. aureus* P1 derivative strains

S. aureus strains were grown to exponential phase. Washed cell suspensions (50 μl of OD_{600} 1.6) were added to human PRP samples (450 μl) in siliconized glass cuvettes with stirring at 37°C. Aggregation was monitored by light transmission in a PAP-4 platelet aggregometer. The aggregation trace shown is a representative of 3 different experiments.

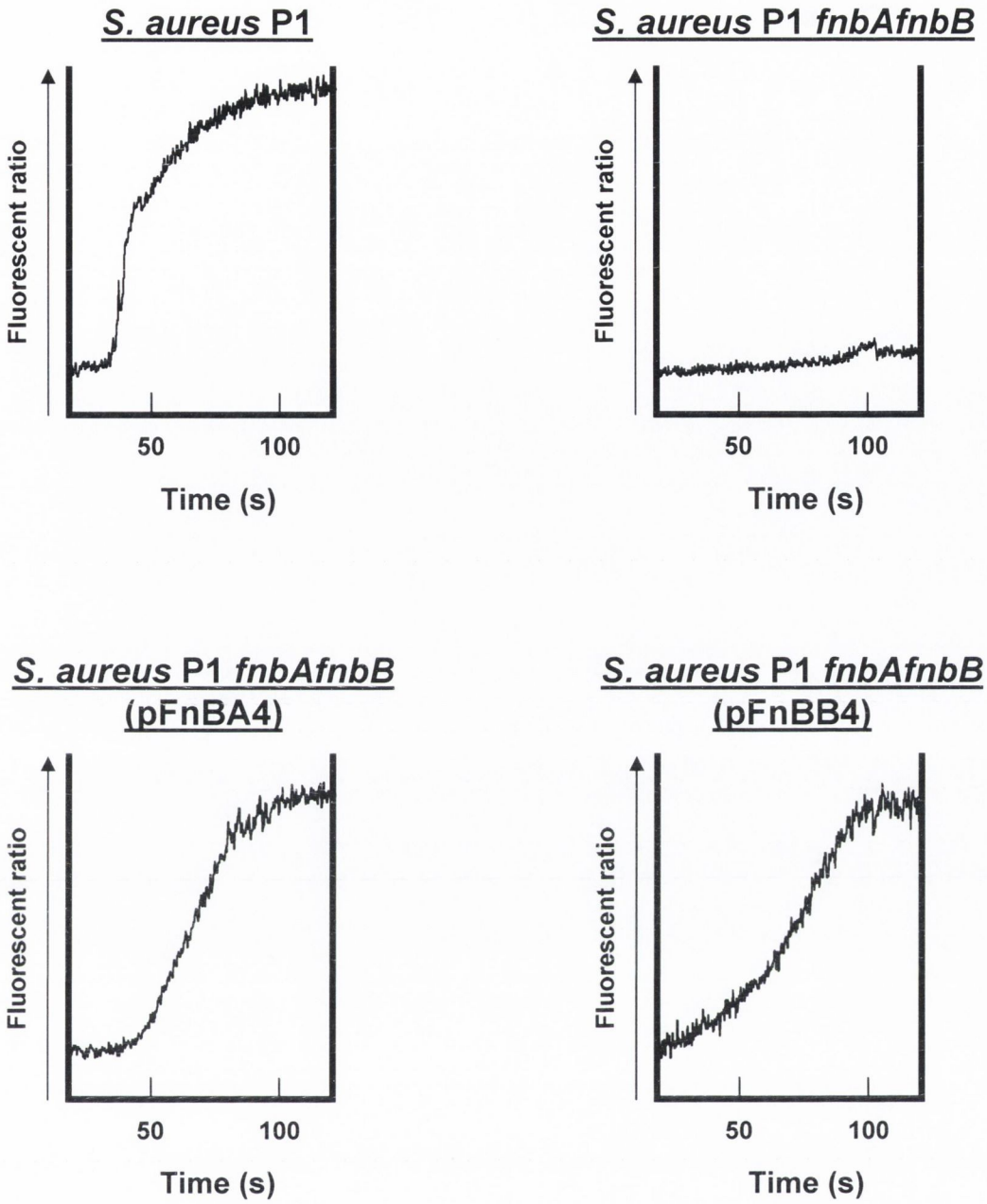


Figure 4.4. Direct measurement of FnBP-mediated platelet activation by calcium flux

S. aureus P1 derivative strains were grown to exponential phase and washed in PBS. Bacterial cell suspension (50 μl of OD_{600} 1.6) was added to FURA-2 labeled GFPs (8 x 10^7 platelets suspended in JNL with 50 % (v/v) plasma) in quartz cuvettes. Intracellular Ca^{2+} changes were monitored in a fluorimeter with stirring at 37°C. Increases in the fluorescent ratio indicate increasing cytosolic Ca^{2+}, indicative of activation. The traces shown are representative of 3 experiments

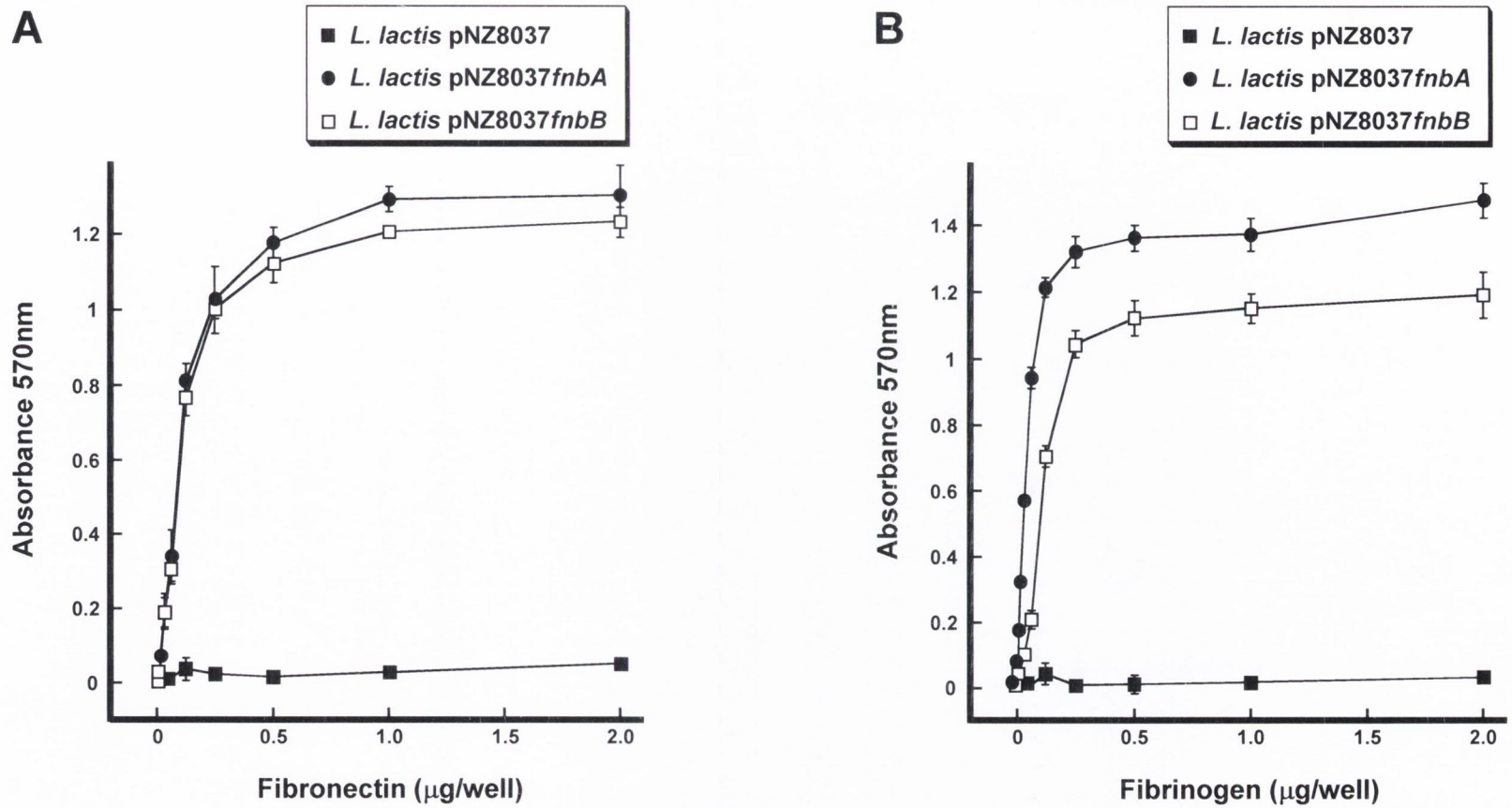


Figure 4.5. Adherence to immobilized fibrinogen and fibronectin of *L. lactis* strains expressing FnBPA and FnBPB

L. lactis (pNZ8037), *L. lactis* (pNZ8037*fnbA*) and *L. lactis* (pNZ8037*fnbB*) strains were grown for 16 h in the presence of 1.6 ng/ml nisin. Washed cell suspensions were added to microtitre wells coated with fibronectin (**A**: 4 – 2000 ng/well) or fibrinogen (**B**: 4 – 2000 ng/well) and allowed to adhere. Unbound cells were washed away and adherent cells were stained with crystal violet. Binding was quantified by measuring the absorbance of the wells at 570 nm in an ELISA plate reader.

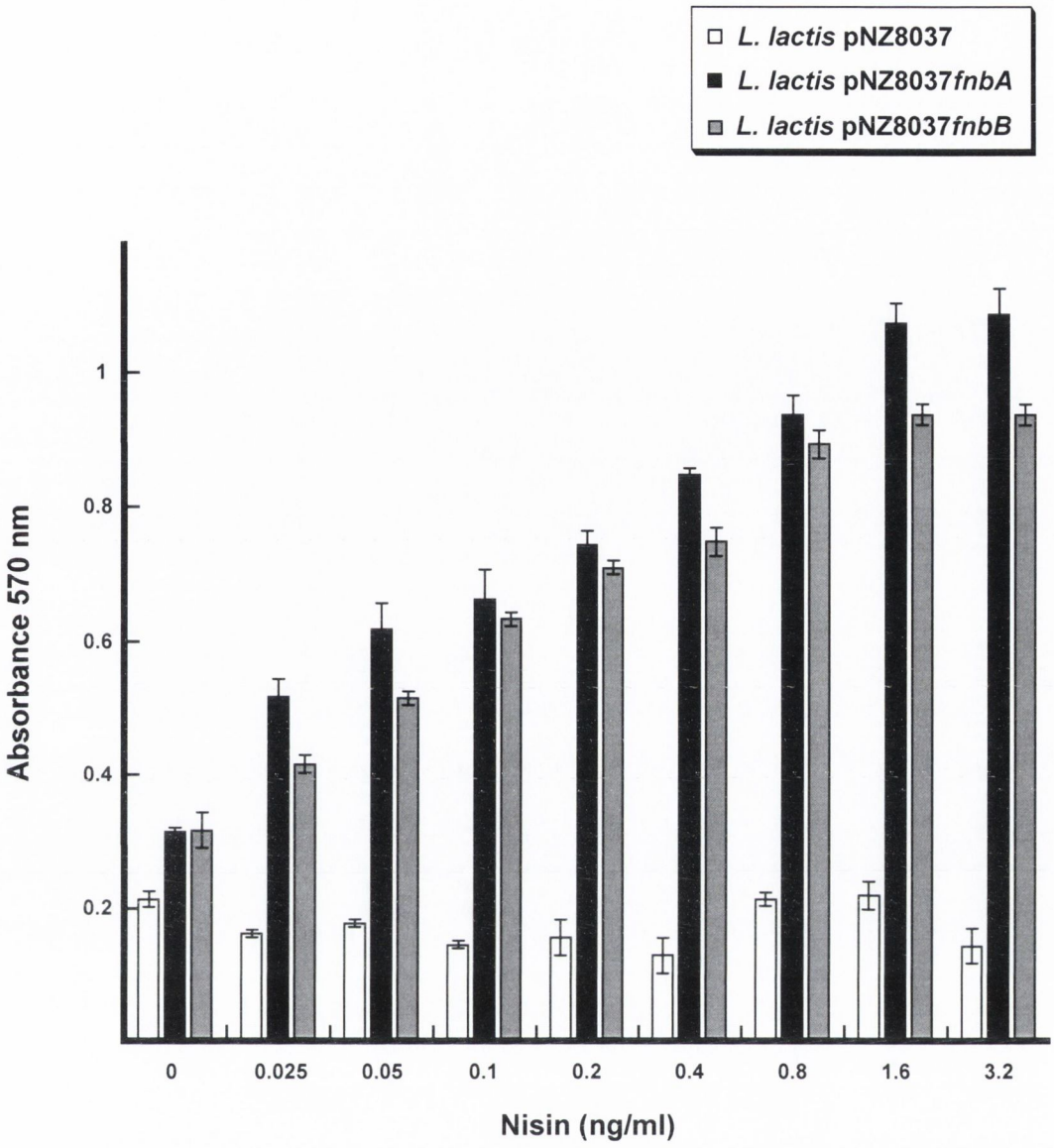


Figure 4.6. Controlled expression of FnBPA and FnBPB on the surface of *L. lactis* NZ9800

L. lactis (pNZ8037), *L. lactis* (pNZ8037*fnbA*) and *L. lactis* (pNZ8037*fnbB*) strains were grown for 16 h in the presence of nisin (0.025 – 3.2 ng/ml). Washed cell suspensions of each culture were added to microtitre wells coated with fibrinogen (500 ng/well) and allowed to adhere. Unbound cells were removed by washing and adherent cells were stained with crystal violet. Binding was quantified by measuring the absorbance of the wells at 570 nm in an ELISA plate reader.

surface of *L. lactis* NZ9800 in order to identify the functional domains that interact with platelets to induce activation. FnBPA proteins comprising either (1) the fibrinogen-binding A domain but lacking the fibronectin-binding BCD domains (FnBPA-A) or (2) the BCD domains but lacking the A domain (FnBPA-BCD) were expressed by *L. lactis*. Plasmids encoding these proteins were generated by an inverse PCR strategy as outlined in Figure 4.7.

L. lactis NZ9800 strains containing plasmids pNZ8037*fnbA*-A (expressing just the A domain) and pNZ8037*fnbA*-BCD (expressing just the BCD domains) were tested for adhesion to both fibrinogen and fibronectin. *L. lactis* expressing the A domain of FnBPA adhered strongly to immobilized fibrinogen, but the degree of adhesion was slightly lower than that seen for *L. lactis* expressing the full-length protein (Figure 4.8 A). *L. lactis* expressing the BCD domains did not adhere to fibrinogen-coated wells (Figure 4.8 A). In adhesion assays to immobilized fibronectin, *L. lactis* expressing full-length FnBPA and FnBPA-BCD adhered with high affinity (Figure 4.8 B). *L. lactis* expressing the FnBPA A domain could not bind to immobilized fibronectin (Figure 4.8 B).

L. lactis cells expressing the individual FnBPA domains were induced with nisin (0.025 – 3.2 ng/ml) and tested for adhesion to immobilized fibronectin and fibrinogen. Adherence of both *L. lactis* (pNZ8037*fnbA*) and *L. lactis* (pNZ8037*fnbA*-BCD) to fibronectin was promoted by increasing nisin concentrations in the growth medium (Figure 4.9 A). No adherence of *L. lactis* (pNZ8037*fnbA*-A) to fibronectin was observed at any nisin concentration tested (Figure 4.9 A). Similarly, *L. lactis* expressing the entire FnBPA protein or the A domain alone adhered to fibrinogen in a nisin concentration-dependent manner, whereas *L. lactis* expressing the BCD domains did not (Figure 4.9 B). These data indicate that the individual domains of FnBPA were expressed in a functional form on *L. lactis* and that the PCR strategy used to create the expression plasmids did not affect the integrity of the nisin-inducible promoter. The slightly impaired adhesion of cells expressing the A domain alone in comparison to the full-length protein may reflect the closer proximity of the A domain to the bacterial cell wall. It has been observed for ClfA that truncation of the SD repeats that project the A domain away from the cell surface impairs ligand-binding (Hartford *et al.*, 1997).

Whole cell immunoblotting experiments were performed to compare expression levels of FnBPA on the *L. lactis* surface with expression of FnBPA in *S. aureus* P1 *fnbAfnbB* (pFnBA4). The P1 derivative strain contained a mutation (*spa*::kan) to prevent non-immune reaction of antibodies with protein A. Cells were grown to exponential phase (*S. aureus*) or stationary phase with 1.6 ng/ml nisin (*L. lactis*). Washed cell suspensions were applied to

nitrocellulose membranes and probed with polyclonal antibodies against the A domain of FnBPA as described in section 2.7. Representative blots demonstrating FnBPA expression are shown in Figure 4.10. *S. aureus* P1 *fnbAfnbB* did not react with anti-FnBPA antibodies, whereas high level expression was observed in the same strain containing plasmid pFnBA4 (Figure 4.10). *L. lactis* (pNZ8037*fnbA*) and *L. lactis* (pNZ8037*fnbA*-A), when grown in the presence of 1.6 ng/ml nisin, expressed similar levels of immunoreactive protein to that observed in P1 *fnbAfnbB* (pFnBA4) (Figure 4.9). Neither *L. lactis* containing the empty pNZ8037 vector nor *L. lactis* (pNZ8037*fnbA*-BCD) reacted with anti-A domain antibodies (Figure 4.10).

4.2.5 Platelet activation and aggregation by FnBP-expressing *L. lactis*

L. lactis strains expressing full-length FnBPA and FnBPB were tested for the ability to stimulate aggregation of human platelets suspended in plasma. Cells were induced with 1.6 ng/ml nisin. This concentration was chosen as it was shown to induce FnBPA expression to levels comparable with *S. aureus* strains expressing FnBPA from plasmid pFnBA4 (Figure 4.10). *L. lactis* (pNZ8037) did not stimulate aggregation after incubation in PRP for 25 min (n = 3). *L. lactis* (pNZ8037*fnbB*) induced aggregation of PRP with extended lag times (2.5 ± 1 min; n = 3) compared to *L. lactis* expressing FnBPA (0.9 ± 0.2 min; n = 3). This suggests that perhaps expression of FnBPB by *L. lactis* is lower than that of FnBPA. Aggregation traces are shown in Figure 4.11. These data demonstrate that FnBPA and FnBPB can independently activate platelet aggregation in the absence of any other *S. aureus* co-factors.

Aggregation experiments were performed using *L. lactis* strains either expressing the FnBPA-A domain or FnBPA-BCD domains in order to identify the domain(s) of FnBPA responsible for activating platelet aggregation. Cells were grown in the presence of 1.6 ng/ml nisin, washed, and added to PRP samples. Rapid aggregation was promoted by both *L. lactis* expressing the FnBPA-A domain (1 ± 0.2 min; n = 3) and by *L. lactis* expressing the FnBPA-BCD domains (1.1 ± 0.1 min; n = 3). The lag times were comparable to that achieved by the full length protein expressed by *L. lactis* (Figure 4.11). These results support data obtained by Dr. J.R. Fitzgerald with *S. aureus* constitutively expressing similar truncates of FnBPA (personal communication).

To confirm that the aggregation response induced by *L. lactis* expressing FnBPA and the FnBPA truncates was the result of platelet activation, experiments were performed to

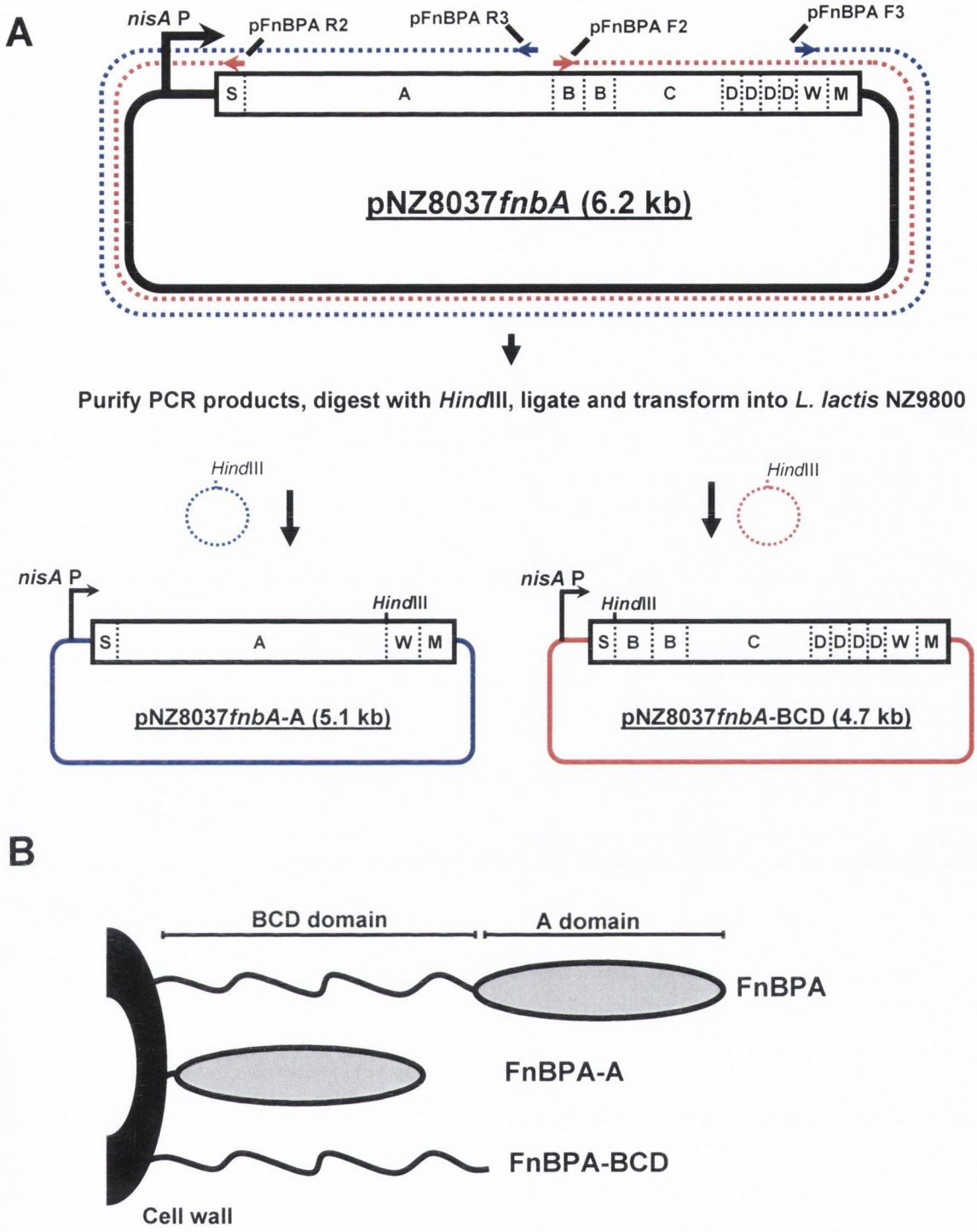


Figure 4.7. Construction of expression plasmids encoding truncated FnBPA proteins

A. Plasmid pNZ8037*fnbA* was used as a template in PCR reactions. Primers contained *Hind*III sites to facilitate self-ligation of the PCR products. The PCR product obtained with primers pFnBPA F3 and R3 was digested with *Hind*III, ligated and transformed into *L. lactis* NZ9800 to generate plasmid pNZ8037*fnbA*-A. PCR products generated with primers pFnBPA F2 and R2 were treated similarly, generated plasmid pNZ8037*fnbA*-BCD.

B. Schematic representation of the truncated FnBPA proteins used in this study

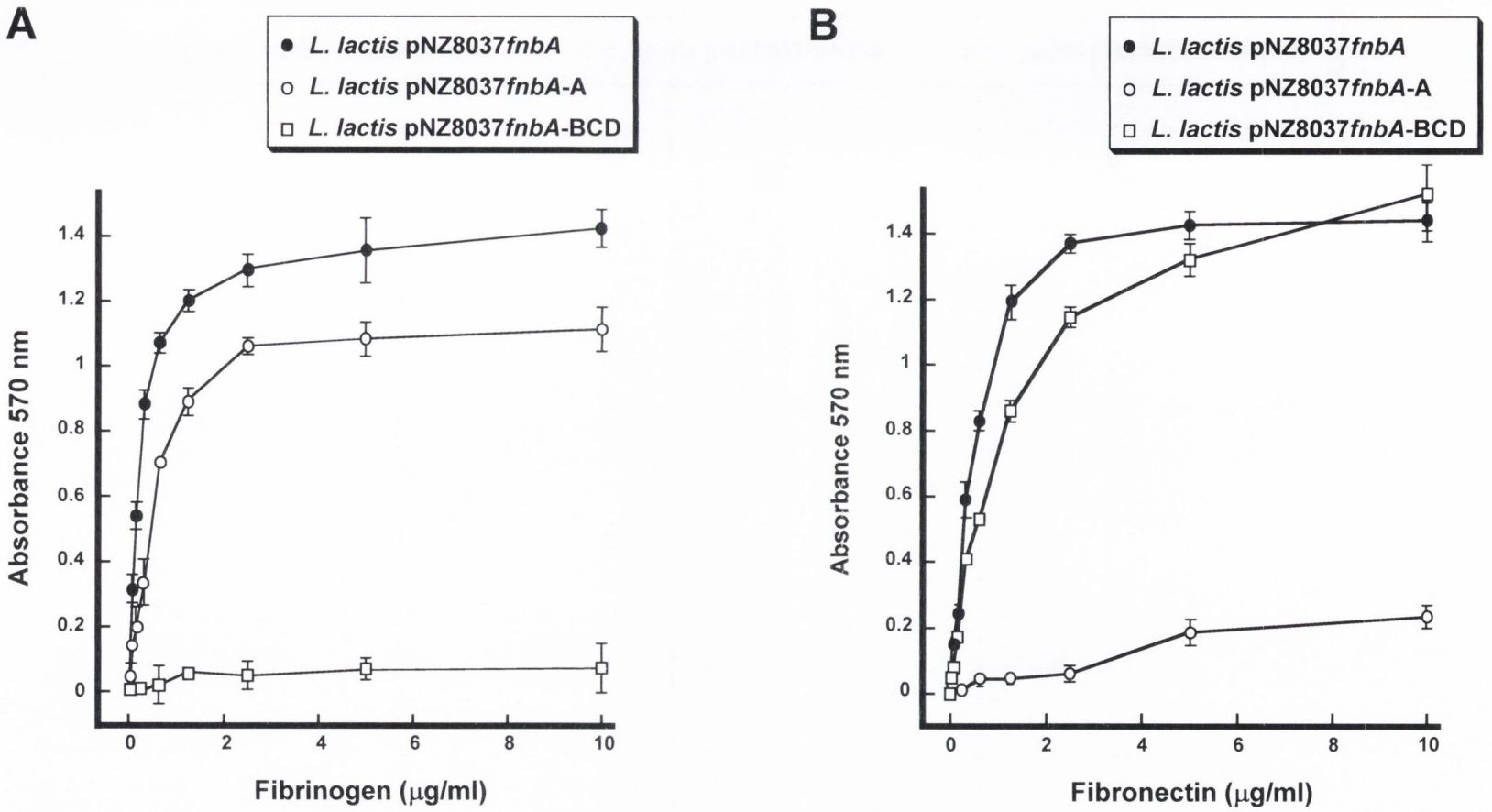


Figure 4.8. Adherence of *L. lactis* strains expressing FnBPA truncates to immobilized fibrinogen and fibronectin

L. lactis (pNZ8037*fnbA*), *L. lactis* (pNZ8037*fnbA*-A), and *L. lactis* (pNZ8037*fnbA*-BCD) strains were grown for 16 h in the presence of 1.6 ng/ml nisin. Washed cell suspensions were added to microtitre wells coated with fibrinogen (**A**: 4 – 2000 ng/well) or fibronectin (**B**: 4 – 2000 ng/well) and allowed to adhere. Unbound cells were washed away and adherent cells were stained with crystal violet. Binding was quantified by measuring the absorbance of the wells at 570 nm in an ELISA plate reader.

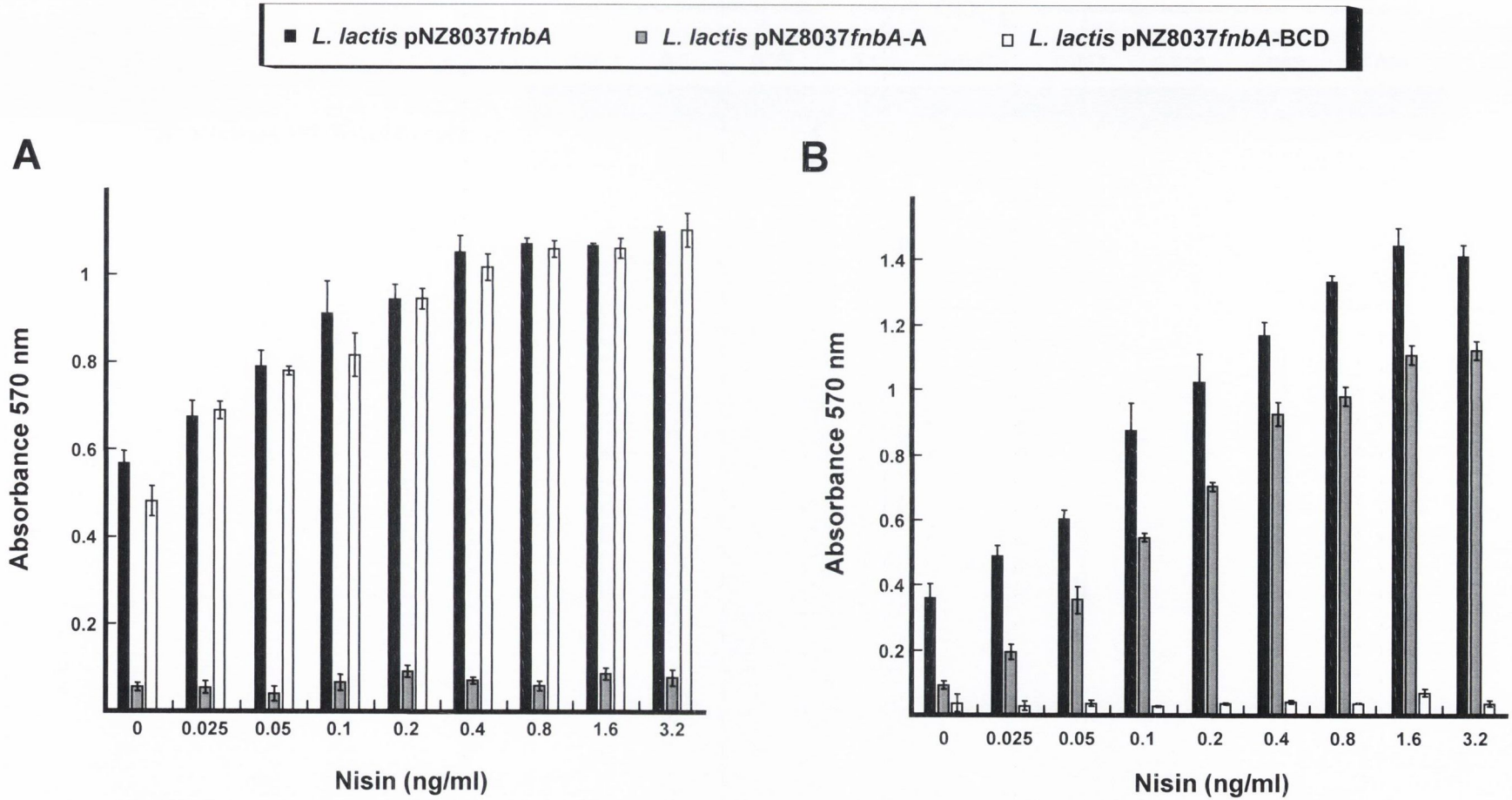


Figure 4.9. Controlled expression of FnBPA truncates on the surface of *L. lactis* NZ9800

L. lactis (pNZ8037*fnbA*), *L. lactis* (pNZ8037*fnbA*-A) and *L. lactis* (pNZ8037*fnbA*-BCD) strains were grown for 16 h in the presence of nisin (0.025 – 3.2 ng/ml). Washed cell suspensions of each culture were added to microtitre wells coated with fibronectin (**A:** 500 ng/well) or fibrinogen (**B:** 500 ng/well) and allowed to adhere. Unbound cells were removed by washing and adherent cells were stained with crystal violet. Binding was quantified by measuring the absorbance of the wells at 570 nm in an ELISA plate reader.

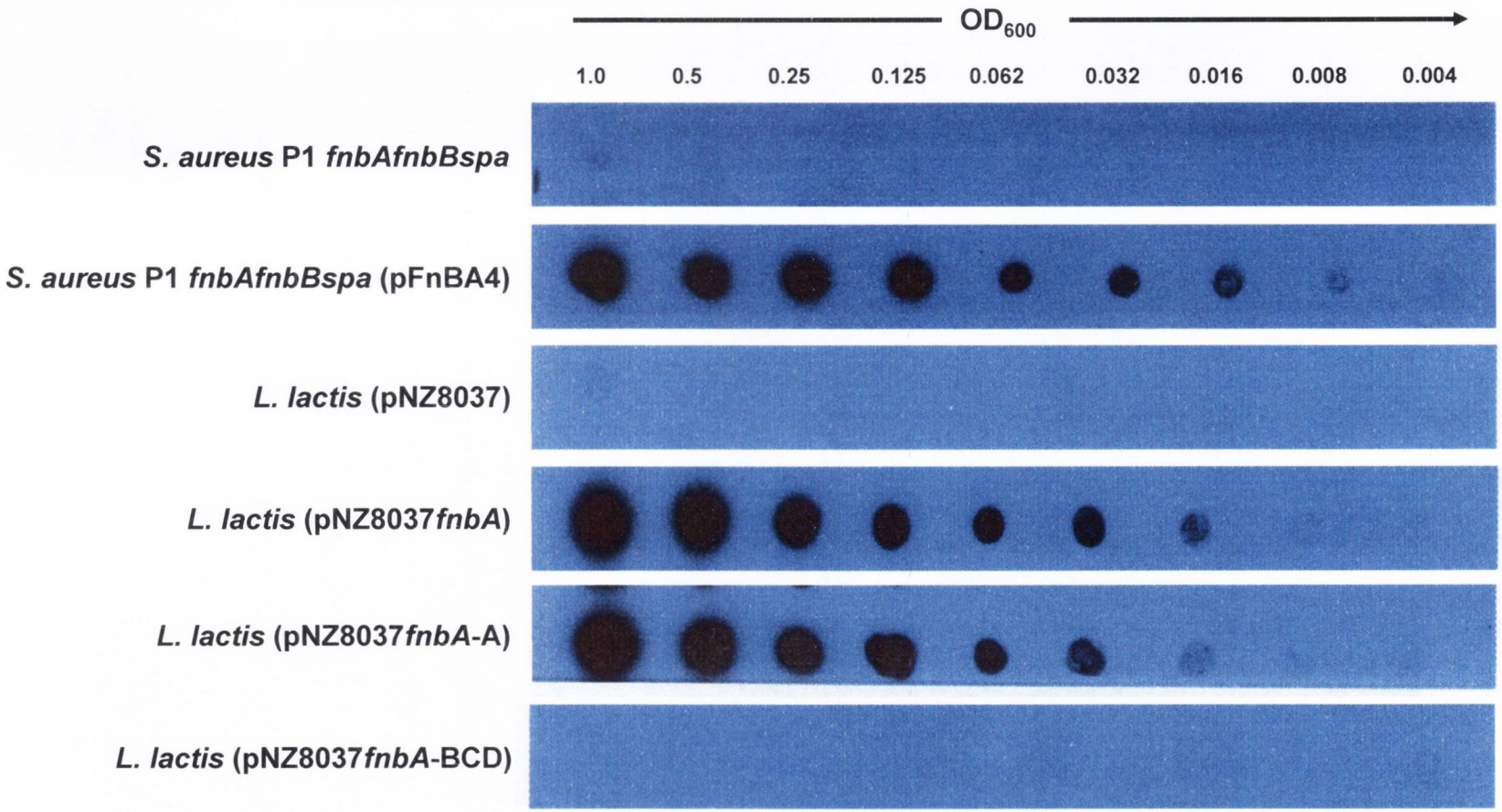


Figure 4.10. Whole cell dot-immunoblot analysis of FnBPA expression in *S. aureus* and *L. lactis*

S. aureus P1 *fnbAfnbB and S. aureus* P1 *fnbAfnbB* (pFnBA4) were grown to early exponential phase. *L. lactis* (pNZ8037), *L. lactis* (pNZ8037*fnbA*), *L. lactis* (pNZ8037*fnbA*-A), and *L. lactis* (pNZ8037*fnbA*-BCD) strains were grown for 16 h in the presence of 1.6 ng/ml nisin. Washed cell suspensions were serially diluted in PBS and 5 μl of each dilution was applied to nitrocellulose membranes. After blocking of the membranes in 10 % skimmed milk solution, anti-FnBPA A domain antibodies were added (1:1000 dilution) and incubated with the membranes for 1 h. Unbound antibody was removed by washing, and bound antibody was detected using goat-anti-rabbit antibodies conjugated to horseradish peroxidase. Membranes were developed by chemiluminescence.

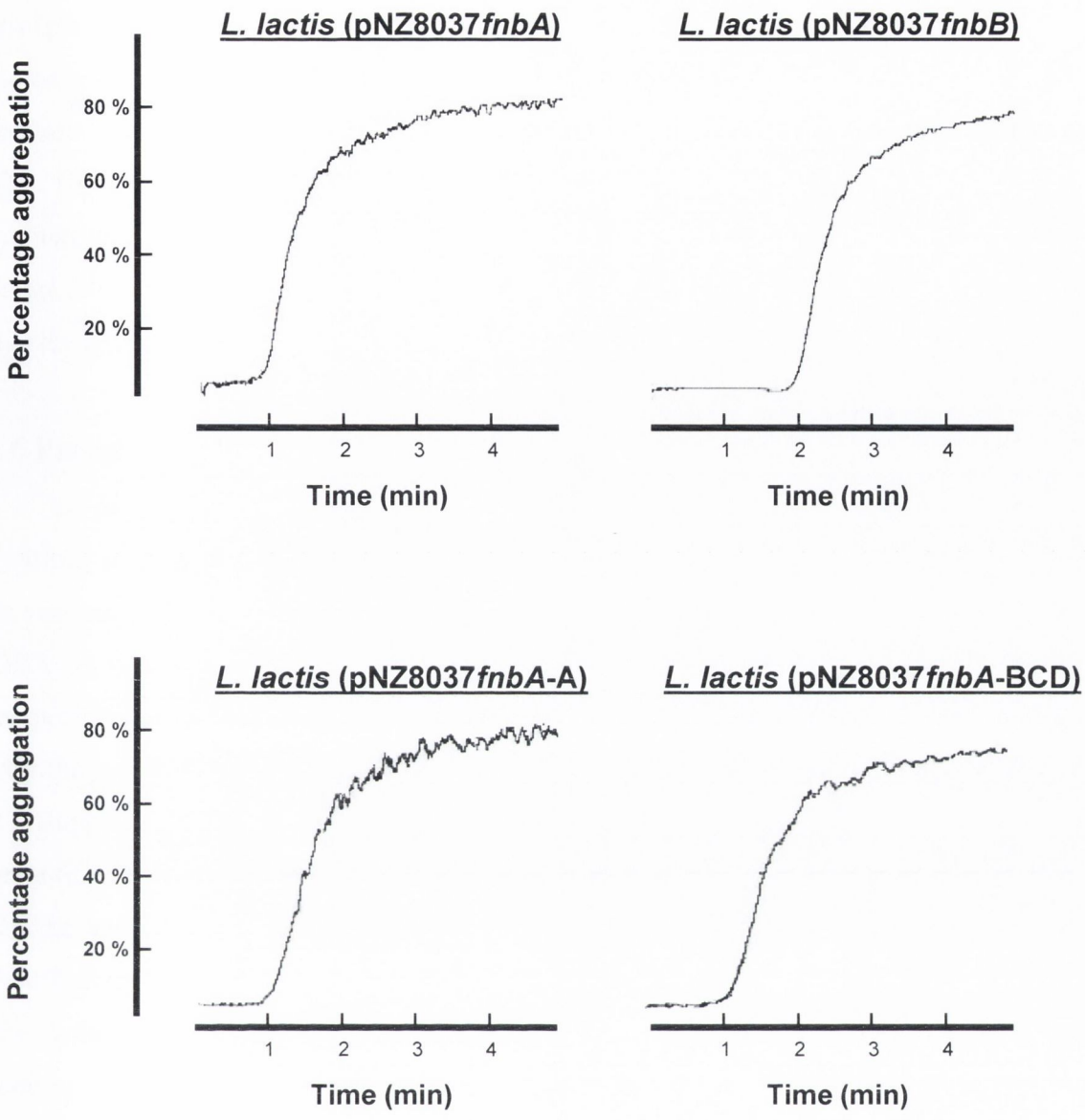


Figure 4.11. Aggregation of platelet-rich-plasma by *L. lactis* expressing FnBPA, FnBPB and FnBPA truncates

L. lactis strains were grown to stationary phase in the presence of 1.6 ng/ml nisin. Washed cell suspensions (50 μl of OD_{600} 1.6) were added to human PRP samples (450 μl) in siliconized glass cuvettes with stirrring at 37°C. Aggregation was monitored by light transmission in a platelet aggregometer. The aggregation traces are representative of 3 different experiments.

measure platelet intracellular calcium increases in response to *L. lactis* FnBPA+ strains. *L. lactis* (pNZ8037*fnbA*), *L. lactis* (pNZ8037*fnbA*-A) and *L. lactis* (pNZ8037*fnbA*-BCD) induced rapid calcium flux in FURA-2 labeled platelets suspended in 50 % plasma (Figure 4.12). No activation was observed in platelets that were incubated with *L. lactis* (pNZ8037) (Figure 4.12). Collectively, these data indicate that two independent mechanisms of activation are promoted by FnBPA, involving either the fibrinogen-binding A domain or the fibronectin-binding BCD domains. Activation occurs rapidly in the presence of plasma, and results in full, irreversible platelet aggregation.

4.2.6 Plasma factors are required for FnBPA-promoted platelet activation

In order to identify the plasma co-factors required for FnBPA-promoted platelet activation, aggregation experiments using plasma-depleted platelets (GFPs) supplemented with various purified plasma proteins were performed. *L. lactis* strains expressing full-length FnBPA, as well as its truncated derivatives, were tested in these assays. These experiments were performed in this laboratory by J.R. Fitzgerald and the results are described here to clarify the mechanism of activation by FnBPA (personal communication).

(1) Supplementation of GFPs with purified fibrinogen alone was not sufficient for aggregation by bacteria expressing FnBPA, FnBPA-A or FnBPA-BCD.

(2) The addition of both purified fibrinogen and pooled human IgG to GFPs was necessary and sufficient for aggregation stimulated by bacteria expressing either the full-length protein or the A domain of FnBPA. No aggregation was stimulated by *L. lactis* expressing the BCD domains.

(3) Fibronectin, in addition to IgG and fibrinogen, was required for aggregation stimulated by bacteria expressing the BCD domain protein.

The addition of IgG was crucial for aggregation by FnBPA and the FnBPA truncates. This is in agreement with the potent inhibition of FnBPA-promoted aggregation by the monoclonal anti-FcγRIIa antibody IV-3 (J. R. Fitzgerald, personal communication). This indicates that IgG binding to FcγRIIa on platelets is a crucial determinant in activation promoted by FnBPA, and indicates that ClfA and FnBPA share similarities in their mechanism of promoting activation. The requirement of fibronectin for activation triggered by the BCD domain protein indicates that fibronectin acts as a bridging molecule linking BCD with a

receptor on the platelet surface; this adhesion in conjunction with IgG-FcγRIIa interactions stimulate activation, and the presence of fibrinogen being required for cross-linking GPIIb/IIIa on adjacent platelets resulting in aggregate formation.

4.2.7 Fibrinogen and IgG are required for activation promoted by the A domain of FnBPA

As mentioned above, the presence of fibrinogen and IgG was sufficient for aggregation promoted by *L. lactis* expressing the A domain of FnBPA, either alone or as part of the full-length protein. The A domain of FnBPA shares 25 % identity with the A domain of ClfA, and both proteins bind to the C-terminus γ-chain of fibrinogen (Wann *et al.*, 2000). By using a non-fibrinogen binding mutant of ClfA, it was demonstrated that fibrinogen-binding by ClfA was necessary for rapid activation (see Chapter 3). In the absence of an equivalent mutation in the A domain of FnBPA, experiments directly measuring activation (calcium flux) were performed to determine if fibrinogen is required for activation promoted by the FnBPA A domain. Platelets were labeled with FURA-2 and separated from plasma as described in section 2.20. Labeled platelets were suspended in JNL buffer containing purified fibrinogen (1 mg/ml) and/or pooled human IgG (2 mg/ml). Washed cell suspensions of *L. lactis* (pNZ8037*fnbA*-A) were added to platelets and increases in cytosolic Ca^{2+} indicative of activation were measured. The presence of both fibrinogen and IgG was essential for activation promoted by the A domain of FnBPA (Figure 4.13). Neither fibrinogen nor IgG by themselves could support activation by *L. lactis* (pNZ8037*fnbA*-A) (Figure 4.13). This indicates that fibrinogen binding by the A domain of FnBPA is absolutely required for activation, in conjunction with IgG. This is supported by experiments demonstrating that a monoclonal antibody (7C5), which is specific for the A domain of FnBPA and inhibits fibrinogen binding, inhibited aggregation by bacteria expressing the A domain of FnBPA (J.R. Fitzgerald, personal communication).

4.2.8 FnBPA-specific IgG is required for activation by *S. aureus* and *L. lactis* expressing FnBPA

Platelet activation promoted by ClfA requires the presence of IgG specific for the adhesin, as discussed in Chapter 3. The requirement of IgG for FnBPA-promoted activation suggests that a similar mechanism exists for FnBPA. To test this hypothesis, pooled human

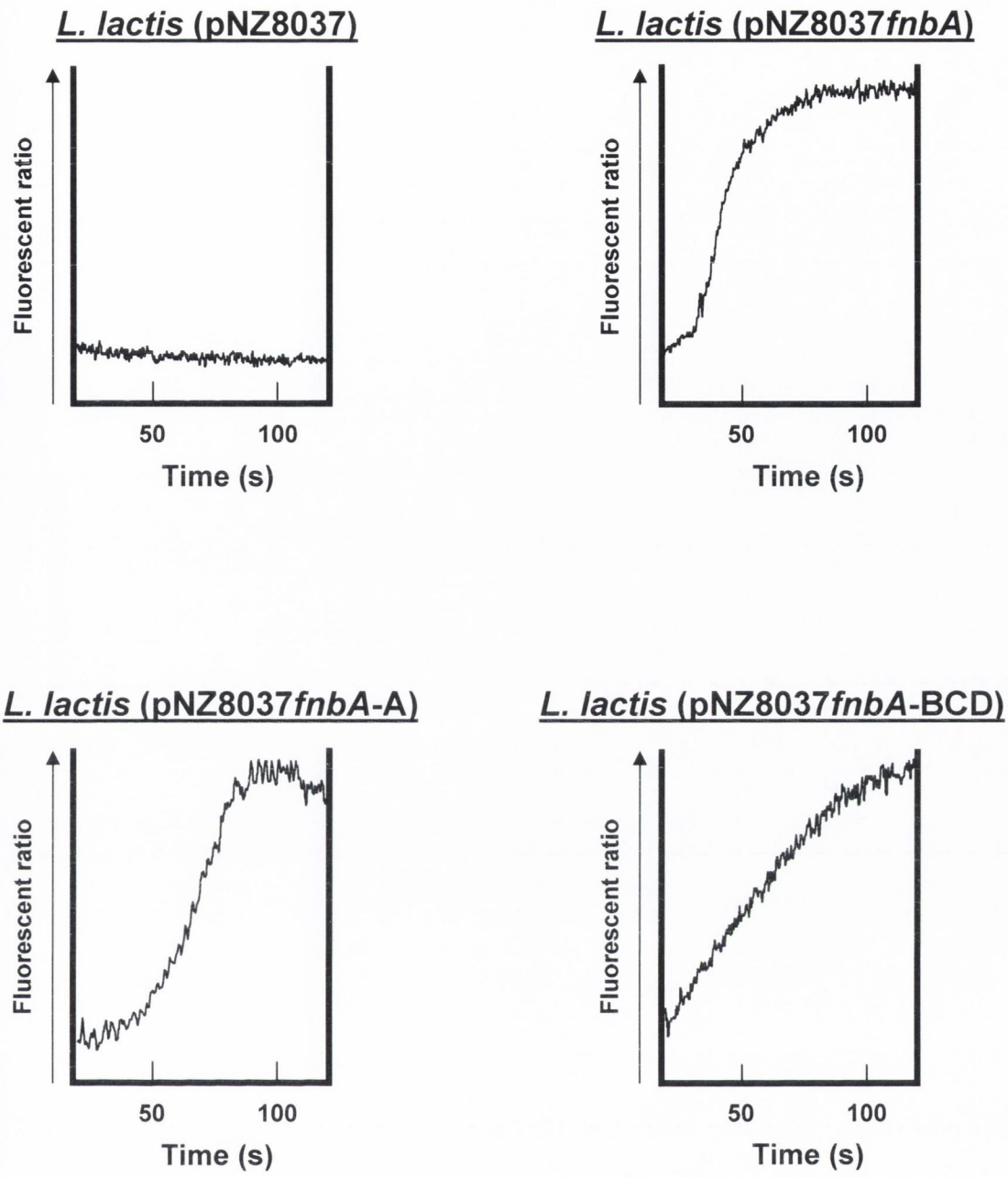


Figure 4.12. *L. lactis* expressing FnBPA and its truncated derivatives promote calcium flux in platelets

L. lactis strains were grown for 16 h in the presence of 1.6 ng/ml nisin and washed in PBS. Bacterial cell suspension (50 μl of OD_{600} 1.6) was added to FURA-2 labeled GFPs (8 x 10^7 platelets suspended in JNL with 50 % (v/v) plasma; final volume of 400 μl) in quartz cuvettes. Intracellular Ca^{2+} changes were monitored in a fluorimeter with stirring at 37°C. Increases in the fluorescent ratio indicate increasing cytosolic Ca^{2+}, indicative of activation. The traces shown are representative of 3 experiments

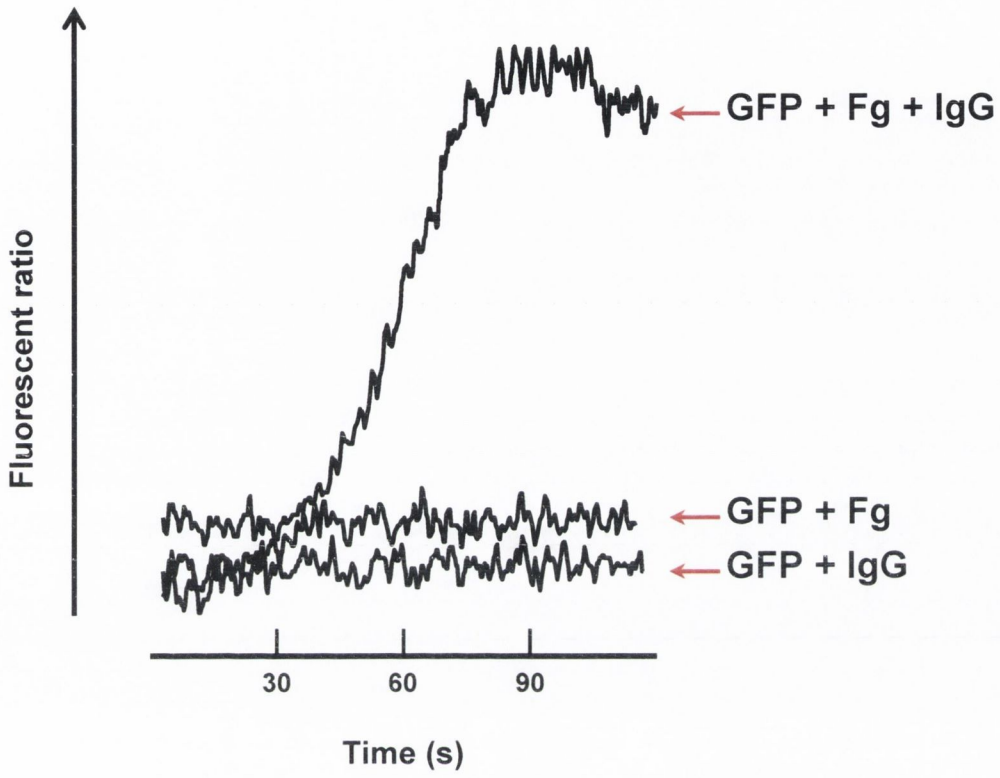


Figure 4.13. Fibrinogen and IgG are required for activation promoted by the A domain of FnBPA

L. lactis (pNZ8037*fnbA*-A) cell were induced with 1.6 ng/ml nisin and washed in PBS. Bacterial cell suspension (50 μl of OD_{600} 1.6) was added to FURA-2 labeled GFPs (8 x 10^7 platelets suspended in JNL) in quartz cuvettes. Platelets were supplemented with fibrinogen (1 mg/ml) and/or pooled human IgG (2 mg/ml) as indicated. Intracellular Ca^{2+} changes were monitored in a fluorimeter with stirring at 37°C. Increases in the fluorescent ratio indicate increasing cytosolic Ca^{2+}, indicative of activation.

IgG samples were depleted of antibodies recognizing the A domain and BCD domains of FnBPA. It is known that the immune response against the BCD domains is predominantly directed against neo-epitopes formed upon binding of fibronectin (Casolini *et al.*, 1998). Recombinant proteins encompassing either the A domain or the BCD domains were immobilized on affinity columns as described in section 2.15. The BCD protein was incubated with fibronectin prior to covalent attachment to the column, forming the complex to which the dominant part of the immune response is directed. Pooled human IgG samples were continuously passaged over the immobilized antigens to deplete specific antibodies as described in section 2.15.2. Unbound antibody samples were tested by ELISA to verify depletion of target IgG. Normal human pooled IgG contained significant levels of antibodies recognising FnBPA A domain or BCD domain bound to fibronectin (Figure 4.14). Passage of IgG samples over the immobilized FnBPA domains resulted in significant depletion of these antibodies (Figure 4.14).

Aggregation experiments using GFPs were performed to analyze the requirement of FnBPA-specific IgG in the response of platelets to FnBPA-expressing bacteria. GFPs supplemented with purified fibrinogen did not support full aggregation by either *S. aureus* P1 *fnbAfnbB* (pFnBA4) or *L. lactis* (pNZ8037*fnbA*) (24 ± 5 % aggregation and 15 ± 3 % aggregation respectively; n = 3). The addition of normal human IgG restored full aggregation by *S. aureus* P1 *fnbAfnbB* (pFnBA4) (73 ± 7 %; n = 3) and *L. lactis* (pNZ8037*fnbA*) (78 ± 10 %; n = 3). Human IgG that had been depleted of FnBPA-specific antibodies recognizing both the A domain and the BCD domain in complex with fibronectin supported greatly reduced aggregation by P1 *fnbAfnbB* (pFnBA4) (33 ± 5 %; n = 3). Aggregation of GFPs by *L. lactis* (pNZ8037*fnbA*) was also significantly reduced in the presence of anti-FnBPA depleted IgG (24 ± 2 %; n = 3). Representative aggregation traces are shown in Figure 4.15. This demonstrates that FnBPA-specific antibodies are required for platelet activation stimulated by either *S. aureus* or *L. lactis* expressing FnBPA. This suggests that antibodies to the surface protein engaging the resting platelet are required to stimulate activation, and that antibodies recognising other surface components could not support rapid activation.

4.2.9 Identification of plasma factors that promote adhesion of platelets to FnBPA expressing bacteria

The above findings suggest that bacteria expressing FnBPA interact with platelets either through fibrinogen-bridging (involving the A domain) or through fibronectin-bridging (involving the BCD domains) to a platelet receptor(s). Such interactions, in conjunction with FnBPA-bound IgG binding to FcγRIIa, trigger rapid activation and aggregation. To demonstrate that both fibrinogen and fibronectin promote adhesion of platelets to FnBPA-expressing cells, the ability of GFPs to adhere to FnBP-expressing *S. aureus* or *L. lactis* cells was tested. *S. aureus* P1 wild-type, its derivative *fnbAfnbB* strain, and the FnBPA complemented strain were grown, washed and immobilized in microtitre wells. GFPs alone, or supplemented either with plasma (10 % v/v), fibrinogen (1 mg/ml) or fibronectin (0.2 mg/ml) were added to the wells coated with bacteria. Low-level adhesion of platelets to the *S. aureus* strains was observed in the absence of plasma proteins (Figure 4.16 A). Adhesion of platelets to *S. aureus* P1 and P1 *fnbAfnbB* (pFnBA4) was enhanced three- to four-fold in the presence of plasma, fibrinogen, or fibronectin (Figure 4.16 A). Both plasma and fibrinogen promoted platelet adhesion to P1 *fnbAfnbB*, but not to the same extent as that observed for FnBP-expressing *S. aureus* (Figure 4.16 A). Fibrinogen-mediated adhesion may be due to expression of ClfA or ClfB by P1 *fnbAfnbB*. Fibronectin did not promote platelet adhesion to P1 *fnbAfnbB* (Figure 4.16 A). Fibronectin- and fibrinogen-dependent platelet adhesion mediated by FnBPA was blocked by the anti-GPIIb/IIIa monoclonal antibody abciximab (Figure 4.16 A). The low-level fibrinogen promoted adherence of platelets to P1 *fnbAfnbB* was also inhibited by abciximab (Figure 4.16).

No adhesion of platelets to *L. lactis* (pNZ8037) was observed in the presence or absence of plasma factors (Figure 4.16 B). Plasma, fibrinogen or fibronectin promoted platelet adhesion to immobilized *L. lactis* (pNZ8037*fnbA*) approximately three-fold compared to adhesion seen in the absence of plasma factors (Figure 4.16 B). This adhesion was GPIIb/IIIa-dependent, as it was completely inhibited by abciximab (Figure 4.16 B). These data indicate that either fibrinogen or fibronectin promote adhesion of platelets to bacteria expressing FnBPA and that GPIIb/IIIa on the platelet surface is the receptor involved in recognizing FnBPA-bound proteins.

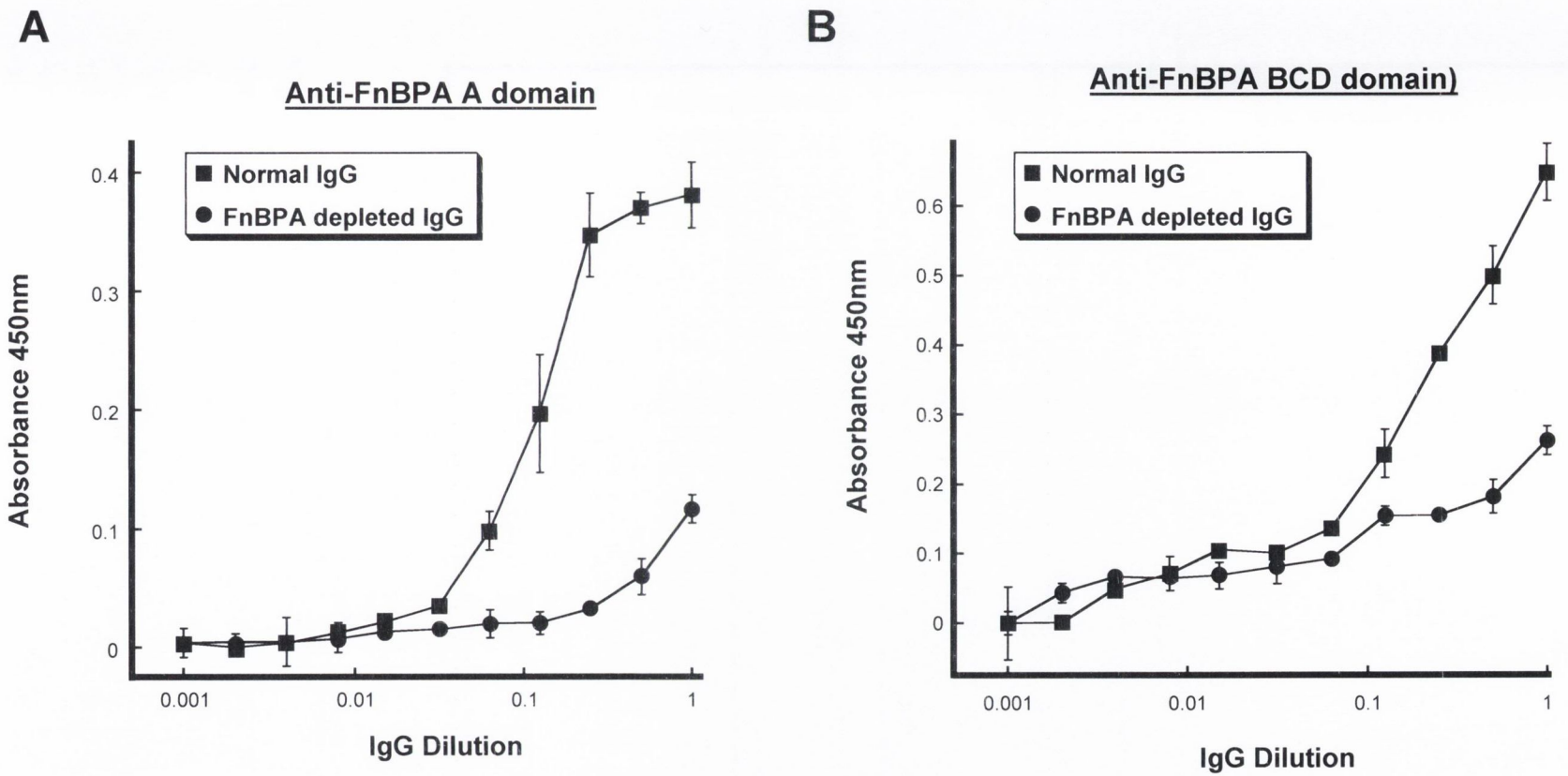


Figure 4.14. Anti-FnBPA specific antibody levels in pooled human IgG samples

Microtitre wells were coated with recombinant FnBPA A domain protein (**A**) or recombinant BCD protein bound to fibronectin (**B**). After blocking of the wells with BSA, IgG samples were serially diluted across a range of wells in PBS buffer and incubated for 1.5 h. Unbound antibody was removed by washing and bound IgG was detected using a goat-anti-human antibody conjugated to peroxidase.

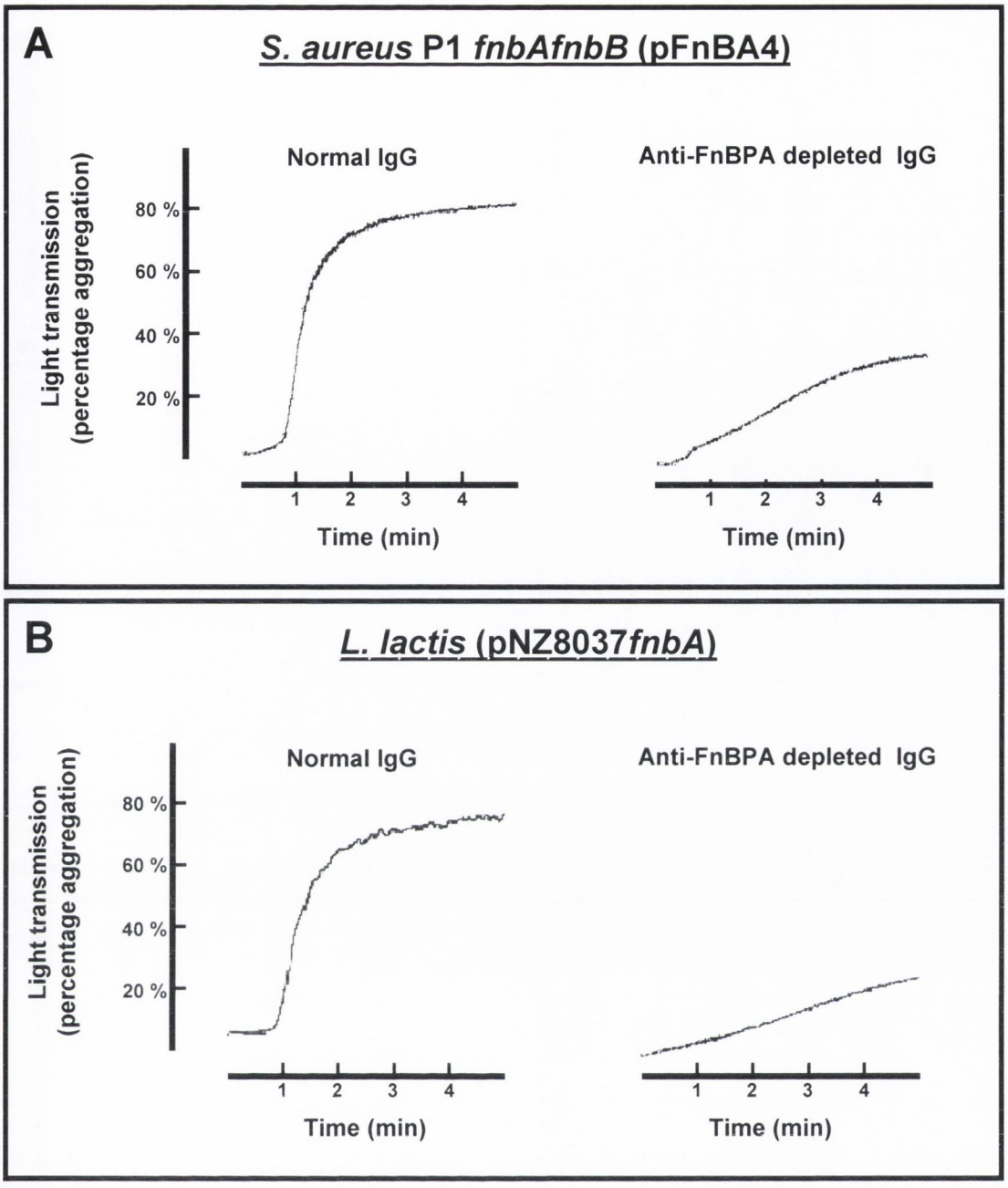


Figure 4.15. Antibodies specific for FnBPA are required for FnBPA-mediated platelet aggregation

S. aureus P1 *fnbAfnbB* (pFnBA4) cells were grown to exponential phase. *L. lactis* (pNZ8037*fnbA*) cells were induced with 1.6 ng/ml nisin for 16 h. Bacteria were washed in PBS and cell suspensions (50 μl of OD_{600} 1.6) were added to GFPs (8 x 10^7 platelets suspended in JNL with 1 mg/ml purified fibrinogen) in glass cuvettes. Normal pooled human IgG or IgG samples depleted of antibodies recognizing both the A domain and the BCD domain in complex with fibronectin were added (2 mg/ml) as indicated. Aggregation was monitored by light transmission at 37°C. The aggregation traces shown are representative of 3 different experiments.

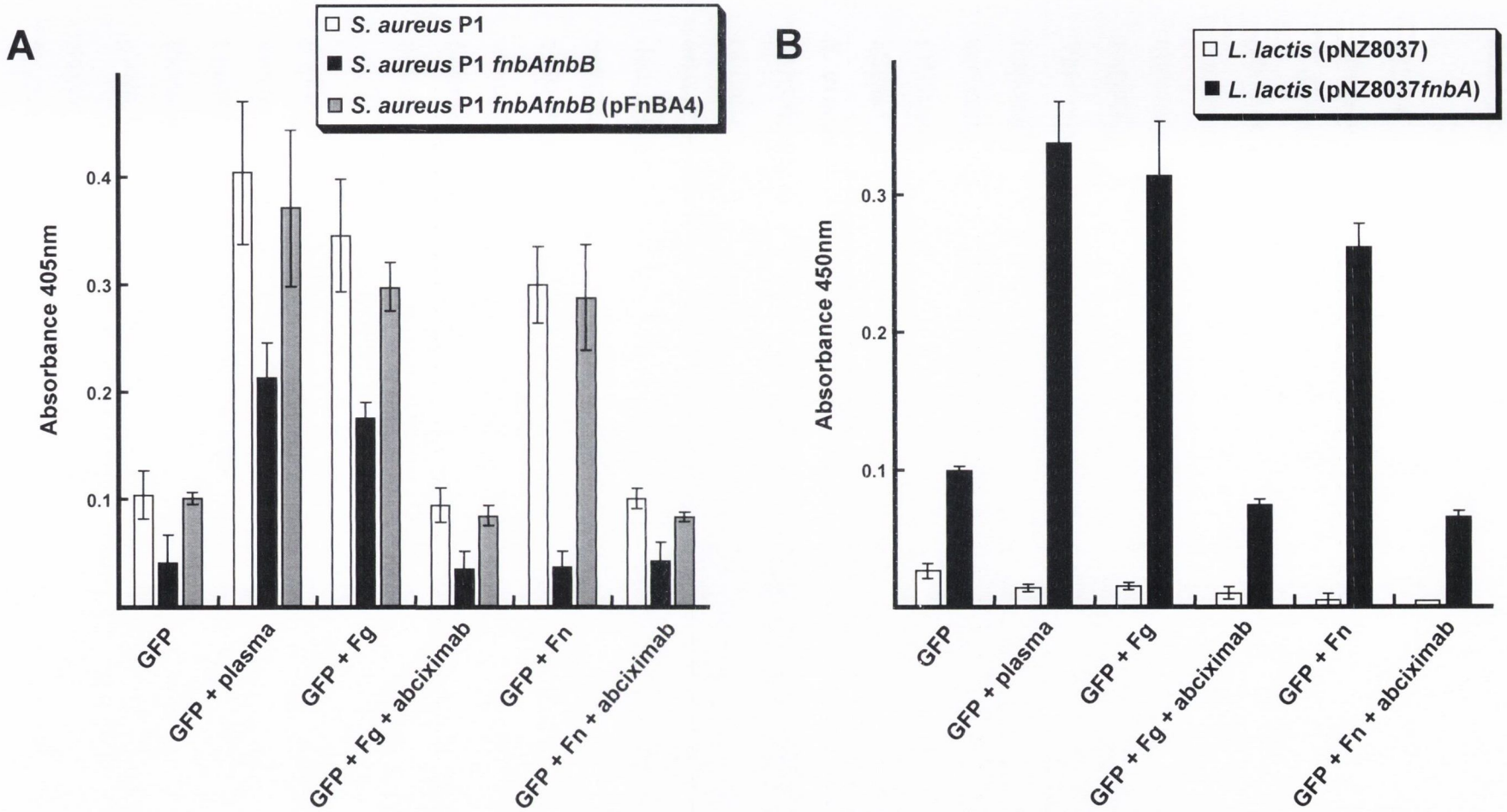


Figure 4.16. Adhesion of platelets to FnBPA-expressing bacteria

Microtitre wells were coated with *S. aureus* P1 strains (**A**) or *L. lactis* strains (**B**) as indicated. GFPs were prepared and supplemented with either plasma (10 % v/v), fibrinogen (Fg: 1 mg/ml) or fibronectin (Fn: 0.2 mg/ml). Platelets were pretreated with the anti-GPIIb/IIIa monoclonal antibody abciximab (10 μg/ml) for 20 min at 37°C where indicated. GFP suspensions were incubated with the bacteria-coated wells for 30 min. Non-adherent platelets were removed by gentle washing and bound platelets detected using a substrate for acid phosphatase.

4.2.10 Sequence diversity in the A domains of FnBPA and FnBPB

The fibronectin-binding repeat regions of FnBPA and FnBPB from *S. aureus* strain 8325-4 are highly homologous, sharing 94 % identical residues in the C and D domains (Jönsson *et al.*, 1991). FnBPB lacks the fibronectin-binding B repeats present in FnBPA. The A domains of FnBPA and FnBPB from strain 8325-4 share only 45 % amino acid identity (Jönsson *et al.*, 1991). Polyclonal antisera against the FnBPA A domain of strain 8325-4 did not significantly react with the FnBPB A domain of the same strain, and vice-versa. It was observed that antibodies raised against the recombinant A domains of $FnBPA_{8325\text{-}4}$ and $FnBPB_{8325\text{-}4}$ did not efficiently recognize the native FnBPs expressed by strain P1 (Figure 4.2). This raised the possibility that differences in amino acid sequences of the A domains was responsible for the difference in antibody reactivity. The regions of the *fnbA* and *fnbB* genes encoding the A domains of *S. aureus* P1 were amplified by PCR, cloned into plasmid pBluescript and sequenced. DNA sequences were translated into amino acid sequences and aligned with the A domain sequence of the 8325-4 FnBPs. The FnBPA A domain sequence of *S. aureus* P1 was found to be 73.5 % identical to the A domain of FnBPA from *S. aureus* 8325-4. For FnBPB, the A domains of P1 and 8325-4 were 80.6 % identical. The A domains of FnBPs are thought to be composed of 3 folded subdomains (N1, N2 and N3), similar to the organization of the A domains of ClfA and ClfB (Deivanayagam *et al.*, 2002). The N1 domains of FnBPA from 8325-4 and P1 were relatively similar (91.4 % identical) compared to domains N2 (78.3 % identical) and N3 (59.2 % identical). Similarly, the N1 domains of FnBPB from 8325-4 and P1 shared the highest identity, whereas the N2 and N3 domains were more divergent (Figure 4.17). This suggests that perhaps the low reactivity of the anti-$FnBP_{8325\text{-}4}$ antibodies against cell wall extracts of *S. aureus* P1 is the result of the presence of different epitopes on the P1 FnBPs.

The A domain sequences of FnBPA and FnBPB from the seven sequenced *S. aureus* genomes were compared to determine if diversity in these domains is common amongst *S. aureus* isolates. All of the sequenced strains contained genes encoding both FnBPA and FnBPB-like proteins, except strain MRSA252, which encodes a single FnBPA-like protein. Pairwise alignments of the FnBPA and FnBPB A domains from these strains revealed a similar diversity to that observed for the P1 FnBP proteins (Figure 4.18). The sequenced strains can be grouped according to their MLST profiles (see section 1.2). *S. aureus* strains 8325-4 and COL belong to different, but very similar sequence types (ST-8 and ST-250 respectively) (Lindsay *et al.*, 2004). The MRSA strains N315 and Mu50 both belong to ST-5

(Kuroda *et al.*, 2001), and strains MW2 and MSSA476 both belong to ST-1 (Baba *et al.*, 2002; Holden *et al.*, 2004). Strain MRSA252, belonging to ST-36 (Holden *et al.*, 2004) is the most divergent of the sequenced *S. aureus* strains (Lindsay *et al.*, 2004). MRSA252 contains a single *fnb* gene whose product is more homologous to FnBPA than FnBPB, and is classified as FnBPA in the analysis here. Strains from the same sequence type shared nearly identical A domains for both FnBPA and FnBPB. For example, the A domains of FnBPA from strains 8325-4 and COL are 99.8 % identical, and the A domains of FnBPB from these strains share 100 % identity (Figure 4.18). ST-1 strains (MW2 and MSSA476) share identical A domains for both FnBPs, and ST-5 strains (N315 and Mu50) also share identical FnBP A domains (Figure 4.18).

However, comparison of A domain sequences of strains from different sequence types revealed that significant diversity exists. As an example, the FnBPA A domain of strain 8325-4 (ST-8) shares only 76.7 % identity with the corresponding domain of strain MW2 (ST-1) and 75.3 % identity with the FnBPA A domain of strain N315 (ST-5; Figure 4.18). Strain MRSA252, which does not cluster in the same sequence type as any of the other sequenced strains, shared between 73 – 79 % identity in the A domains of FnBPA with all the other sequenced strains (Figure 4.18). Similar diversity in the A domains of FnBPB amongst the sequenced *S. aureus* strains was observed (Figure 4.18). The FnBPA and FnBPB A domains of *S. aureus* P1 were between 73 and 84 % identical to the corresponding domains of other *S. aureus* isolates (Figure 4.18). These data indicate that the observed diversity between the A domains of FnBPs from *S. aureus* 8325-4 and P1 is not unique, and may be common in different clonal lineages of *S. aureus*.

Comparison of the individual sub-domains (N1, N2 and N3) revealed that domain N1 is relatively conserved, with domains N2 and N3 being more divergent. The 8325-4 FnBPA N1 domain is 91 – 93 % identical to the N1 domains of the other sequenced strains. The FnBPA N2 domain of 8325-4 is 70 – 79 % identical to other FnBPA N2 domains. Domain N3 appears to be the most divergent, with the 8325-4 FnBPA N3 domain sharing only 59 – 64 % identity with FnBPA N3 domains of the other sequenced strains. Alignments of FnBPA N2 and N3 domains are shown in Figure 4.19. A similar pattern was observed for FnBPB. The residues N304 and F306 in the FnBPA A domain (analogous to P336 and Y338 in ClfA) have recently been demonstrated to be crucial for fibrinogen-binding by the recombinant form of the 8325-4 protein (F. Keane, personal communication). These residues are conserved

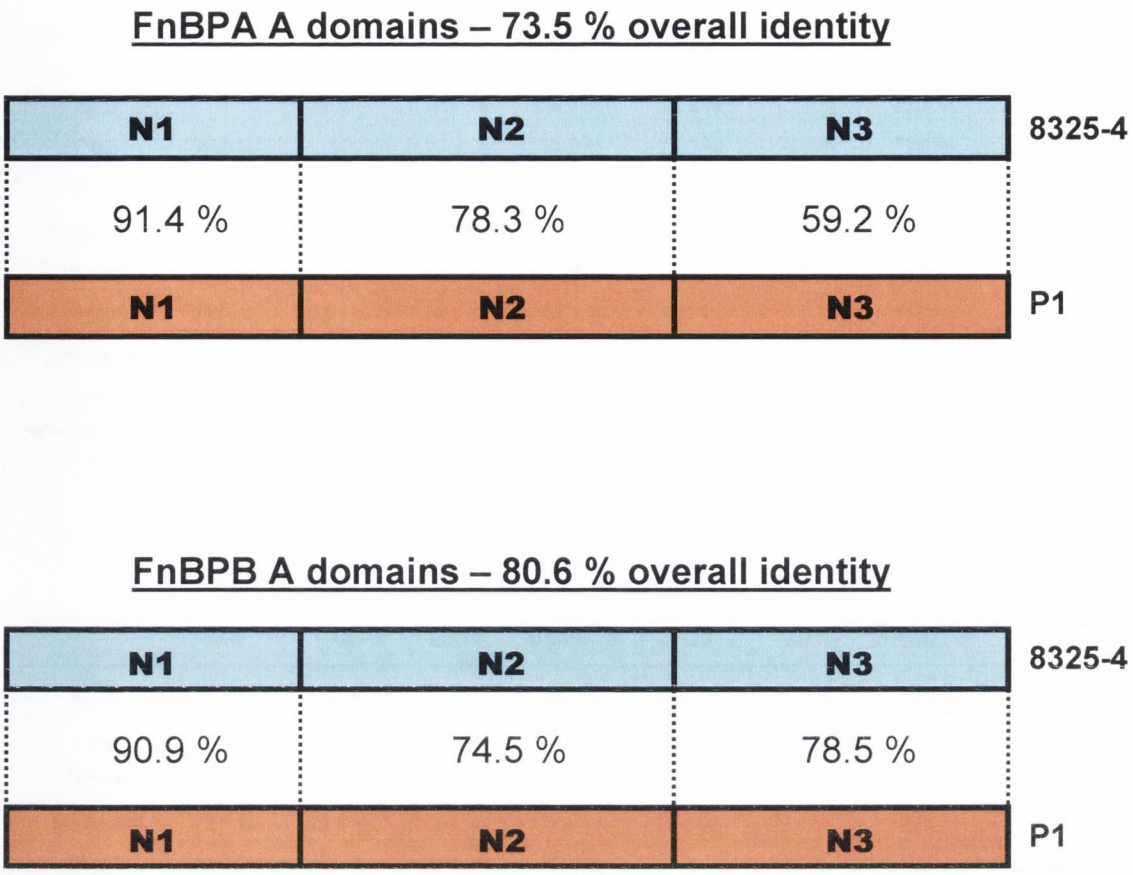


Figure 4.17. Diversity in the A domain amino acid sequences of FnBPA and FnBPB from *S. aureus* strains 8325-4 and P1

Genomic DNA of *S. aureus* P1 was used to clone the DNA sequences encoding the FnBPA and FnBPB A domains. Sequenced DNA was translated into amino acid sequences and aligned with the A domain sequences of the 8325-4 FnBPs. The percentage amino acid identities between the subdomains (N1, N2 and N3) are shown.

FnBPA A domain

	8325	COL	MW2	MSSA476	Mu50	N315	MRSA252	P1
8325	-	99.8 %	76.7 %	76.7 %	75.3 %	75.3 %	73.8 %	73.5 %
COL	99.8 %	-	76.9 %	76.9 %	75.5 %	75.5 %	73.0 %	73.7 %
MW2	76.7 %	76.9 %	-	100 %	94.7 %	94.7 %	79.3 %	77.8 %
MSSA476	76.7 %	76.9 %	100 %	-	94.7 %	94.7 %	79.2 %	77.8 %
Mu50	75.3 %	75.5 %	94.7 %	94.7 %	-	100 %	75.9 %	76.0 %
N315	75.3 %	75.5 %	94.7 %	94.7 %	100 %	-	75.9 %	76.0 %
MRSA252	73.8 %	73.0 %	79.3 %	79.2 %	75.9 %	75.9 %	-	75.9 %
P1	73.5 %	73.7 %	77.8 %	77.8 %	76.0 %	76.0 %	75.9 %	-

FnBPB A domain

	8325	COL	MW2	MSSA476	Mu50	N315	MRSA252	P1
8325	-	100 %	72.7 %	72.7 %	78.9 %	78.9 %	N/A	80.6 %
COL	100 %	-	72.7 %	72.7 %	78.9 %	78.9 %	N/A	80.6 %
MW2	72.7 %	72.7 %	-	100 %	75.1 %	75.1 %	N/A	72.9 %
MSSA476	72.7 %	72.7 %	100 %	-	75.1 %	75.1 %	N/A	72.9 %
Mu50	78.9 %	78.9 %	75.1 %	75.1 %	-	100 %	N/A	83.9 %
N315	78.9 %	78.9 %	75.1 %	75.1 %	100 %	-	N/A	83.9 %
MRSA252	N/A	N/A	N/A	N/A	N/A	N/A	-	N/A
P1	80.6 %	80.6 %	72.9 %	72.9 %	83.9 %	83.9 %	N/A	-

Figure 4.18. Divergence in FnBP A domain amino acid sequences of sequenced *S. aureus* strains

Genomic sequences from *S. aureus* strains were searched for proteins showing homology to FnBPA and FnBPB from *S. aureus* 8325-4. A domain amino acid sequences for each protein were aligned with corresponding domains from other strains in a pairwise fashion. The overall percentage of identical residues between the aligned domains is shown. The deduced sequences of FnBPA and FnBPB from *S. aureus* P1 were included in the alignments and are shown in red. Similarities of FnBPA A domains between strains is shown in the upper table. The lower table shows similarities of FnBPB A domains between strains. MRSA252 contains a single *fnb* gene whose product is homologous to FnBPA.

Figure 4.19. Alignments of FnBPA A domain sequences from sequenced *S. aureus* genomes.

FnBPA A domain amino acid sequences were divided into subdomains N1, N2 and N3 based on homology to the A domains of ClfA and ClfB (Deivanayagam *et al.*, 2002). Multiple alignments of the N2 and N3 subdomains of FnBPA are shown. Conserved residues within the domains are hightlighted with an asterisk.

amongst all FnBPA proteins from the sequenced strains, and are also conserved in FnBPA from *S. aureus* P1.

4.3 Discussion

The mechanism(s) by which exponentially-growing *S. aureus* cells stimulate platelet aggregation has not been previously addressed. Investigations in this laboratory demonstrated that platelet aggregation by exponential phase *S. aureus* strain P1 is primarily mediated by surface-expressed fibronectin-binding proteins. The experiments described in this chapter were performed to investigate the molecular mechanisms by which *S. aureus* FnBPs interact with resting platelets to stimulate platelet activation, a factor that may be important in the pathogenesis of vascular infections.

S. aureus P1 cells, either expressing its native FnBPs, or expressing FnBPA or FnBPB from multicopy plasmids, stimulated rapid aggregation of platelets suspended in plasma. Lag times to aggregation, typically 1 to 1.5 min for the wild-type strain, increased approximately 10-fold upon removal of FnBP(s) from the cell surface. Aggregation was accompanied by rapid intracellular calcium mobilization (flux) in platelets exposed to FnBP-expressing *S. aureus*. No Ca^{2+} increases were detected in platelets exposed to an FnBP-deficient mutant. This demonstrated that the fast aggregation response was the result of platelet activation. FnBPA and FnBPB are therefore major mediators of platelet activation when expressed on the surface of *S. aureus*.

The functional expression of either FnBPA or FnBPB in *L. lactis* demonstrated that each protein could independently trigger aggregation. *L. lactis* expressing FnBPA aggregated platelets with the same short lag times as observed for *S. aureus* P1. FnBPB expressed in *L. lactis* was not as potent in aggregating platelets, with lag times of between 2.5 and 3.5 minutes. This may be due to lower-level expression of FnBPB in *L. lactis* compared to FnBPA. The expression of truncated FnBPA proteins on the *L. lactis* surface allowed identification of the domains involved in triggering aggregation. Proteins comprising either the fibrinogen-binding A domain or the fibronectin-binding BCD domains were equally potent in triggering aggregation. Lag times were indistinguishable to those seen for the full-length FnBPA expressed in *L. lactis*. Rapid Ca^{2+} flux occurred in platelets when exposed to *L. lactis*

expressing either full-length FnBPA or the FnBPA truncates. No increases in cytosolic Ca^{2+} were observed when platelets were mixed with the *L. lactis* host strain. This shows that FnBPA can activate platelets, resulting in irreversible aggregation, by 2 independent mechanisms.

The A domains of FnBPA and ClfA are 25 % identical. Although the crystal structure of the FnBPA A domain has not been determined, it is predicted to form a folded structure similar to that for ClfA (Deivanayagam *et al.*, 2002). Both proteins specifically recognize the extreme C-terminus of the γ-chain of fibrinogen (McDevitt *et al.*, 1997; Wann *et al.*, 2000), so it is reasonable to predict that both domains stimulate platelet activation in an analogous manner. Rapid activation promoted by *L. lactis* expressing ClfA is critically dependent on the binding of fibrinogen to the A domain, which links the bacterium to GPIIb/IIIa. Similarly, it was found that bacteria expressing the A domain of FnBPA required fibrinogen (and IgG) to cause calcium flux in washed platelets. No activation was observed in the presence of IgG alone. This suggests that fibrinogen-binding by the A domain is important in promoting activation. Fibrinogen was capable of supporting platelet adhesion to bacteria expressing FnBPA, and adhesion was dependent on platelet GPIIb/IIIa. Collectively, these data indicate that a high-affinity binding mechanism, involving fibrinogen-bridging between bacteria expressing the FnBPA A domain and GPIIb/IIIa on platelets is required for activation.

The unfolded BCD domain of FnBPA mediates binding to fibronectin (Schwarz-Linek *et al.*, 2003). *L. lactis* expressing the BCD domain could only cause aggregation of washed platelets when fibronectin was present (J.R. Fitzgerald, personal communication) indicating a requirement for fibronectin in the activation process. Fibronectin was capable of supporting platelet adhesion to *L. lactis* or *S. aureus* cells expressing FnBPA. Adhesion was blocked by abciximab, indicating that GPIIb/IIIa is the platelet receptor recognizing fibronectin bound to FnBPA. GPIIb/IIIa is known to bind fibronectin at multiple sites, including the RGD motif in module 10 (Bowditch *et al.*, 1991).

The presence of 2 independent mechanisms of activation by FnBPA stems from the ability of the individual domains of this protein to interact with the platelet adhesive ligands fibrinogen and fibronectin. These plasma proteins mediate bacterial-platelet adhesion, interactions that are required for activation. However, a crucial requirement for IgG was also demonstrated, with the addition of pooled human IgG to washed platelets suspended in fibrinogen being required for full aggregation by *S. aureus* or *L. lactis* expressing FnBPA. The aggregation of PRP samples by *S. aureus* or *L. lactis* expressing FnBPA and its truncated

derivatives was completely blocked by the function-blocking anti-FcγRIIa monoclonal antibody IV-3 (J.R. Fitzgerald, personal communication). This demonstrates that the presence of functional FcγRIIa on the platelet is required for activation by both mechanisms. The most likely scenario is FcγRIIa recognizes the Fc region of specific antibodies bound to the FnBPA domains engaging the resting platelet through plasma protein bridging. ELISA experiments demonstrated the presence of antibodies recognizing both of the FnBPA-platelet interactive domains (A domain and BCD domains) in human pooled IgG. Antibodies directed against the BCD domain only recognized the recombinant antigen in the presence of fibronectin (data not shown). This is probably the result of antibodies binding to neo-epitopes created upon binding of the BCD domain to fibronectin (Casolini *et al.*, 1998). Human IgG samples specifically depleted of antibodies recognizing both FnBPA-A and FnBPA-BCD in complex with fibronectin did not support full platelet aggregation by bacteria (*S. aureus* or *L. lactis*) expressing FnBPA. This demonstrated that antibodies specific for FnBPA are required for platelet activation by FnBPA-expressing bacteria.

Taken together, these data allow a model to be proposed for platelet activation by bacteria expressing FnBPA. Plasma proteins (fibrinogen or fibronectin) recognize and bind to domains within FnBPA, and are used as bridging molecules mediating bacterial adhesion to GPIIb/IIIa on platelets. This allows the interaction of antibodies bound to FnBPA with the low-affinity FcγRIIa receptor on platelets. This is depicted schematically in Figure 4.20. It is likely that recognition of FnBPA-bound IgG by FcγRIIa causes signaling resulting in platelet activation. GPIIb/IIIa activation enhances platelet binding to soluble fibrinogen and the cross-linking of adjacent platelets into large aggregates. While not demonstrated here, it is proposed that a similar mechanism exists for FnBPB.

Bacteria expressing FnBPA utilize host plasma proteins and IgG specific for the adhesin to induce platelet activation. Pooled human IgG samples used in this study contained antibodies recognizing both the A domain and neo-epitopes on the BCD domain created upon binding to fibronectin. This suggests that antibodies against FnBPA are present in sera from normal healthy individuals. The presence of low level antibodies against FnBPA in sera from healthy individuals colonized by *S. aureus* (nasal carrier) and non-carriers has been demonstrated (Dryla *et al.*, 2005). Sera obtained from patients with documented *S. aureus* infections showed increased anti-FnBPA antibodies, suggesting that expression of FnBPA occurs during infection (Dryla *et al.*, 2005). FnBPA-expressing bacterial cells utilize low-

level specific antibodies present in donors' plasma to induce platelet activation. *In vivo*, FnBPA-mediated platelet activation may occur through similar mechanisms, which may contribute to thrombus formation and vegetation development on heart valves.

The model proposed above for platelet activation by bacteria expressing FnBPA is strikingly similar to that for ClfA expressing cells, discussed in chapter 3. In both instances, plasma proteins act as bridging molecules linking the bacterial adhesin to GPIIb/IIIa on the platelet. Antibodies that are bound to the adhesin are recognized by platelet FcγRIIa. Activation is triggered resulting in platelet aggregation characterized by short lag times (1 – 1.5 min). Platelet aggregation by a series of *S. aureus* isolates from confirmed endocarditis cases occurred with similar short lag times of 1 to 2 minutes (J.R. Fitzgerald, personal communication). Both exponential and stationary phase cells triggered fast aggregation which was completely inhibited by blockade of FcγRIIa. A general mechanism is proposed for platelet activation by *S. aureus* in both exponential and stationary growth phases. The expression of surface proteins that bind to platelet-reactive plasma factors mediate high-affinity binding of *S. aureus* to the platelet. Antibodies specific for the surface proteins interacting with the platelet are recognized by FcγRIIa, resulting in activation and aggregation. *S. aureus* cells lacking the major pro-aggregatory surface proteins (FnBPA/FnBPB in exponential phase and ClfA in stationary phase) still caused aggregation, but the lag times were significantly prolonged. This may be due to expression of other surface proteins such as SdrE, shown to cause platelet aggregation with lag times greater than 10 minutes when expressed by *S. aureus* or *L. lactis* (O'Brien *et al.*, 2002). The anti-FcγRIIa antibody IV-3 completely inhibited platelet aggregation by *S. aureus* cells, and by *L. lactis* cells expressing SdrE (M. Brennan, personal communication), indicating that slow aggregation by *S. aureus* is also dependent on FcγRIIa. The interactions of other pathogens with platelets, including *Strep. sanguis*, *Strep. pyogenes* and *H. pylori*, can also be prevented by the use of IV-3 (Ford *et al.*, 1997; Kerrigan *et al.*, 2002; Byrne *et al.*, 2003) suggesting that the mechanism described here may represent a common strategy employed by bacterial pathogens of the vascular system.

It was demonstrated here that *S. aureus* P1 expresses a high-molecular weight FnBP, responsible for the strong adhesion of this strain to fibronectin. Elimination of FnBP-expression correlated with impaired fibronectin-binding and delayed platelet aggregation. Polyclonal antiserum, raised against FnBPA-$A_{8325\text{-}4}$ and FnBPB-$A_{8325\text{-}4}$, did not efficiently

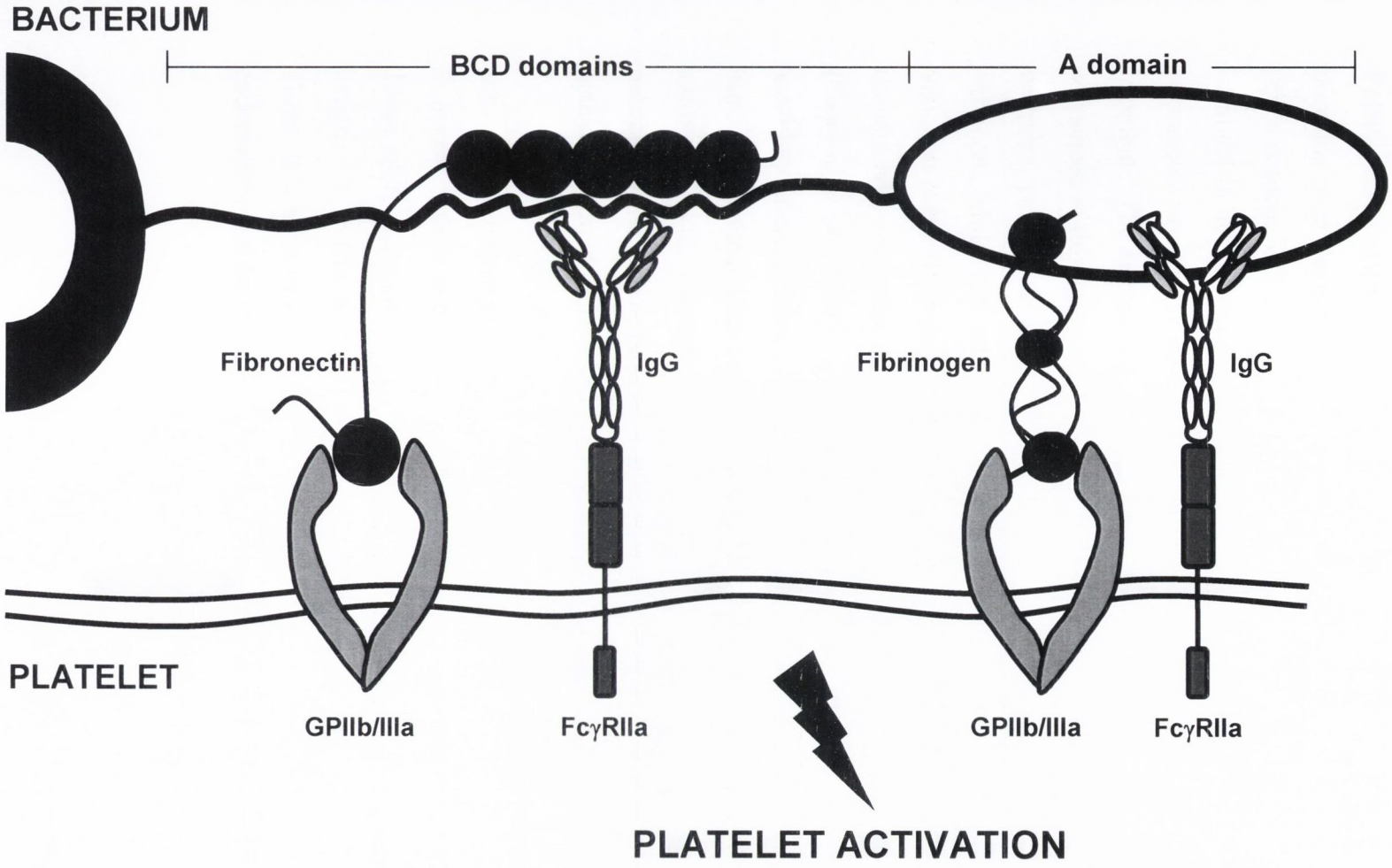


Figure 4.20. Model for platelet activation by FnBPA

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

recognize the FnBP expressed by *S. aureus* P1. The A domains of FnBPA and FnBPB from strain P1 were found to be 73.5 % and 80.6 % identical to the A domains of *S. aureus* 8325-4 FnBPA and FnBPB, respectively. Similar levels of diversity were found in the FnBP A domains from the genome sequences of *S. aureus* strains. The A domains of ClfA from the seven sequenced *S. aureus* genomes did not display such diversity, and were greater than 90 % identical in any pairwise alignment. The fibronectin-binding domains (BCD domains) in the sequenced strains did not display any significant differences, and were greater than 95 % identical. The fibronectin-binding domains of FnBPA and FnBPB from strain P1 were not sequenced in this study. This suggests a selective advantage in variation in FnBPA/FnBPB A domains, perhaps as a mechanism of evasion of host immune responses through antigenic variation, while still retaining ligand-binding activity. A similar mechanism of antigenic variation in the BCD domains would not confer a similar advantage, as antibodies against this domain only recognize neo-epitopes formed upon binding of fibronectin to the BCD domain (Casolini *et al.*, 1998). A reduction in the host immune response against the BCD domain would therefore involve a reduction in binding of this domain to fibronectin. This may affect aspects of pathogenesis such as cell invasion and would be counter-productive. Reduction in antibody binding through antigenic variation in the A domains of FnBPA and FnBPB would not compromise the interaction of FnBPs with platelet FcγRIIa, platelet activation, as antibodies bound to the BCD domain could still participate.

In summary, the fibronectin-binding proteins have been shown to be the major platelet activation factors expressed by *S. aureus* during exponential growth. The interactions between *S. aureus* FnBPs and human platelets that lead to platelet activation have been determined. These interactions are mediated either through fibronectin or fibrinogen bridges to the platelet integrin GPIIb/IIIa, and require specific IgG to trigger activation through the FcγRIIa receptor. These data have broad implications for our understanding of the pathogenesis of infective endocarditis and for the development of novel therapeutics against vascular infections.

Chapter 5

Role of complement proteins in platelet aggregation by *S. aureus*

5.1 Introduction

The expression of high levels of a non-fibrinogen binding ClfA mutant protein (ClfA PY) on the surface of *L. lactis* was shown to stimulate the aggregation of platelets suspended in plasma. This was characterized by significantly longer lag times to aggregation than the rapid aggregation stimulated by *L. lactis* expressing the wild-type ClfA protein. *L. lactis* expressing ClfA or ClfA PY could stimulate calcium flux in plasma-depleted platelets when bacterial cells were pre-incubated in plasma and then washed. This indicates that the plasma components required for the interaction of ClfA or ClfA PY with platelets are bound to the bacterial surface, where they are recognized by specific platelet receptors, leading to platelet activation. Rapid activation and aggregation was the result of a high-affinity fibrinogen-dependent interaction of ClfA with GPIIb/IIIa on platelets. The fibrinogen-bridge, in conjunction with ClfA-bound IgG interacting with FcγRIIa, was both necessary and sufficient to cause the full, irreversible aggregation of resting platelets in *in vitro* thrombi.

High-level aggregation of platelets by *L. lactis* ClfA PY+ also required the presence of IgG specific for ClfA interacting with FcγRIIa as was observed for platelet activation mediated by cells expressing wild-type ClfA. ClfA PY-expressing bacteria cannot interact with platelets through a fibrinogen bridge, and were incapable of aggregating plasma-depleted platelets in the presence of fibrinogen and IgG (unlike bacteria expressing the wild-type protein). Therefore, another plasma component is required for platelet activation stimulated by cells expressing ClfA PY (in addition to ClfA-specific IgG), compared to cells expressing wild-type ClfA. Identification of the plasma component(s) required for ClfA PY-mediated platelet aggregation is the subject of the research described in this chapter.

Plasma factors that have been shown to mediate the interaction of various bacterial species with platelets, leading to platelet aggregation, include fibrinogen, fibronectin, IgG, von Willebrand factor and complement proteins (Sullam *et al.*, 1988; Ford *et al.*, 1996, 1997; O'Brien *et al.*, 2002; Byrne *et al.*, 2003; Pawar *et al.*, 2004; Fitzgerald *et al.*, 2005). Fibrinogen, fibronectin or von Willebrand factor acted as bridging molecules linking specific bacterial adhesins to receptors on the platelet surface. Platelet aggregation caused by *Strep. sanguis* NCTC 7863 and by some strains of enterococci was shown to proceed through complement-dependent mechanisms (Usui *et al.*, 1991a; Ford *et al.*, 1996). Complement deposition on the *Strep. sanguis* NCTC 7863 cell surface along with bacterial-bound IgG was required to stimulate the aggregation of resting platelets (Ford *et al.*, 1996, 1997). Activation

of the complement cascade is triggered by many bacteria, raising the possibility that *L. lactis* cells expressing ClfA PY interact with platelets in a manner similar to *Strep. sanguis* NCTC 7863.

The complement system is a chief component of the innate immune system, and is also involved in initiation of the adaptive immune response (Song *et al.*, 2000; Carroll, 2004). More than 30 distinct proteins, including regulatory factors and specific cellular receptors, together comprise the complement system (Cooper, 1999). Complement activation results in a controlled series of reactions; sequential activation of complement components through enzymatic cleavage or protein-protein interactions results in deposition of complement proteins on the bacterial surface and the formation of the terminal membrane-attack-complex (MAC). The insertion of MAC-complexes into the cell membranes of certain pathogens creates trans-membrane pores resulting in cell death through osmotic lysis (Cooper, 1999).

The complement system acts via three separate pathways that differ in their mode of recognition. The classical complement pathway (CCP) is triggered by recognition of antigen-bound IgG or IgM molecules by the C1q component of the C1 complex. C1 is present in plasma as a non-covalent complex containing one C1q molecule, two C1r molecules and two C1s molecules. C1q binding to antibody-antigen complexes activates C1r through a process involving cleavage, which in turn activates C1s. Activated C1s in the activator-bound macromolecular C1 complex cleaves component C4, the next reacting component, thereby yielding small (C4a) and large (C4b) fragments. C4a, an anaphylatoxin that is a weak activator of inflammation, diffuses away from the cell surface. C4b covalently binds to the activator surface, forming clusters around the bound, activated C1 molecules. The next reacting component, C2, binds to immobilized C4b in a Mg^{2+}-dependent manner. Activated C1s in the nearby C1 molecules cleave C2 into large (C2a) and small (C2b) fragments. C2b diffuses away, and C2a remains bound to C4b, where it acquires serine-protease activity. This bound bimolecular C4b2a complex is the C3 convertase of the CCP.

The mannose-binding lectin (MBL) pathway of complement activation is extremely similar to the CCP. However, the stimulus activating the MBL pathway is different. The recognition molecule of this pathway, mannose-binding lectin (MBL), recognizes conserved sugar patterns on microbes. MBL and two other proteins (MASP-1 and MASP-2) form a complex that is homologous to the C1 complex of the CCP. This complex utilizes C4 and C2 in an identical manner to the CCP to generate the C4b2a C3 convertase.

The final pathway of complement activation, the alternative pathway (ACP), is distinct from the CCP and MBL pathways in that it lacks a specific recognition molecule. Rather, low-level spontaneous cleavage of component C3 in plasma generates C3b which can bind covalently to the pathogen surface. Binding of the ACP specific component, factor B, to surface-bound C3b occurs in a Mg^{2+}-dependent manner. Factor B in the C3bB complex is cleaved by the circulating serine-protease (Factor D), yielding a larger fragment (Bb) that remains bound to C3b and a smaller (Ba) fragment that diffuses away. Bound C3bBb complexes form the C3 convertase of the ACP.

The generation of C3 convertases (C4b2a for CCP/MBL and C3bBb for the ACP) marks the point at which the 3 pathways of complement activation converge. The remaining steps of the complement cascade are identical, regardless of the mode of initiation/activation. C3 convertase complexes serve to cleave massive amounts of the central complement component C3, which is the most abundant (1.2 mg/ml) complement component in plasma. C3 cleavage results in the liberation of small (C3a) and large (C3b) fragments. The anaphylatoxin, C3a, diffuses away from the cell and is involved in stimulating inflammation. Generated C3b molecules become covalently attached to the activator surface (a process called opsonization) and can fulfill a number of functions. (1) Complement receptors expressed by professional phagocytes (neutrophils and macrophages) recognize C3b and its degradation products (iC3b and C3dg) on opsonized cells; such interactions help to promote phagocytosis. (2) A single C3b molecule can covalently bind to a C3 convertase complex (C4b2a or C3bBb) forming a C5 convertase complex (C4b2a3b or $C3b_2Bb$). C5 convertases initiate the terminal events in the complement cascade. (3) Binding of C3b to the activator surface provides an efficient substrate for the generation of nascent C3 convertases by the ACP. This C3b amplification feedback loop serves to accelerate complement fixation by increasing the rate of C3b deposition. This increases the efficiency of phagocytosis and aids in the generation of C5 convertase complexes.

C5 convertases catalyze the cleavage of complement component C5 into large (C5b) and small (C5a) fragments. C5a is an anaphylatoxin, released from the cell surface, which induces potent pro-inflammatory responses, and mediates other effects such as triggering of chemotaxis and augmentation of receptor expression on cells. C5b is the first component involved in a series of protein-protein interactions that culminate in the formation of the terminal C5b-9 membrane-attack-complex (MAC). Initially, C5b binds to component C6, followed by binding of C7 to C5b6. This complex (C5b-7) inserts into target membranes

without causing pore formation. Each membrane-inserted C5b-7 complex then binds one C8 molecule, resulting in deeper insertion of the complex into the membrane (Hu *et al.*, 1981). Polymerization of component C9 upon the membrane-inserted C5b-8 complex generates the mature MAC, which can contain up to 16 C9 molecules generating a pore size of about 10 nm in diameter (Hu *et al.*, 1981; Cooper, 1999). This results in osmotic lysis of susceptible cells (such as gram-negative bacteria expressing rough-type LPS molecules). Gram-positive bacteria are resistant to MAC-mediated cell lysis, presumably because the thick peptidoglycan layer restricts access of the complex to the cytoplasmic membrane (Cooper, 1999). One of the main purposes of complement fixation on Gram-positive bacteria is opsonization, whereby complement components that become deposited on the bacterial surface mark the cell for destruction by host phagocytic cells (Rooijakkers *et al.*, 2005).

The complement system composes an orderly set of precisely regulated interactions of complement components with each other, with the activator, and with the cell membrane. The CCP is depicted schematically in Figure 5.1. *Strep. sanguis* NCTC 7863 required a functional complement system to stimulate the aggregation of platelets suspended in plasma, characterized by lag times of greater than 7 minutes before the onset of aggregation. Progressive formation of C5b-9 could be detected on the bacterial surface over time when incubated in plasma, the rate of which correlated with the lag times to aggregation (Ford *et al.*, 1996). Platelet aggregation by this strain also required recognition of specific antibodies bound to the bacterial surface by FcγRIIa (Ford *et al.*, 1997). This indicated that platelet aggregation by some *Strep. sanguis* strains required multiple stimuli, including IgG-Fc receptor interactions and the interaction of complement components and the platelet. The identity of the specific complement components mediating the interaction with platelets, or the identity of the platelet receptors that recognize bacterial-bound complement proteins were not elucidated, although a role for C5b-9 was suggested (Ford *et al.*, 1996). It is reasonable to presume that exposure of *L. lactis* cells expressing ClfA PY to plasma could activate the complement cascade, resulting in the deposition of complement proteins on the bacterial surface. The binding of specific IgG in plasma to ClfA PY expressed on the cell surface certainly should be sufficient to trigger complement activation through the classical pathway. This could lead to platelet aggregation through a similar mechanism as that described for *Strep. sanguis* NCTC7863 (Ford *et al.*, 1996). Experiments were designed to investigate

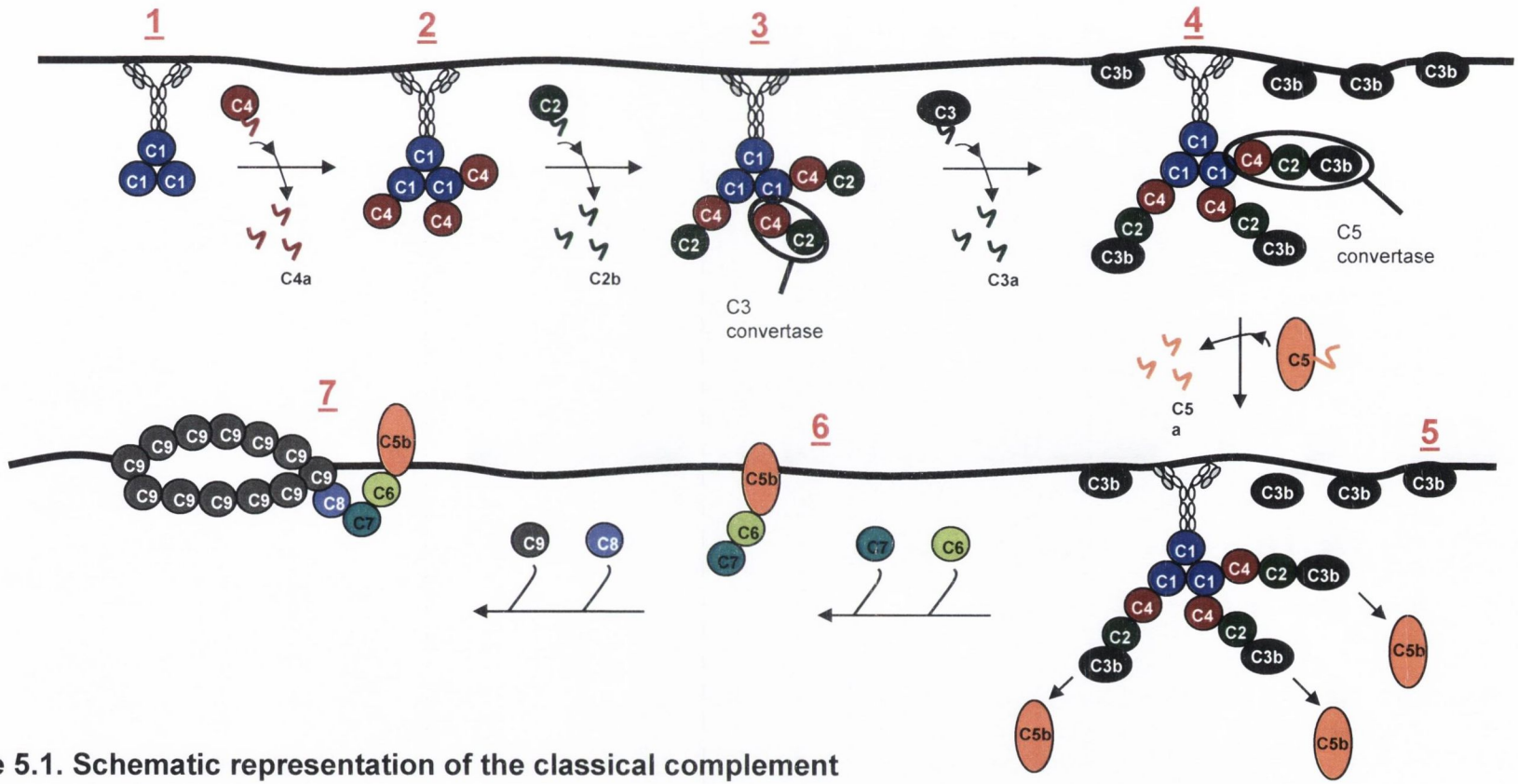


Figure 5.1. Schematic representation of the classical complement activation pathway

1. C1q of the C1 complex binds to antibody-antigen complexes.
2. C4 cleavage by C1s liberates C4a peptide and C4b binds to C1.
3. C2 binds to C4b, allowing C2 cleavage by C1s. The C2b peptide is liberated and C2a binding to C4b generates the C3 convertase
4. C3 cleavage by C4b2a releases C3a. C3b covalently binds to surfaces (opsonization) or to C4b2a complexes (generating C5 convertase)
5. C5 cleavage by C4b2a3b generates C5a and C5b.
6. Binding of C6 and C7 to C5b generates the C5b-7 complex that inserts into the target membrane.
7. Binding of C8 to C5b-7 is followed by polymerisation of C9 molecules to form the terminal C5b-9 membrane-attack-complex.

whether complement formation participates in the slow, IgG-FcγRIIa-dependent aggregation of platelets triggered by *L. lactis* expressing ClfA PY.

5.2 Results

5.2.1 A functional complement system is required for platelet aggregation by *L. lactis* expressing ClfA PY

L. lactis expressing a non-fibrinogen binding ClfA mutant (ClfA PY) aggregated platelets suspended in plasma with longer and more variable lag times than cells expressing wild-type ClfA. This fibrinogen-independent mechanism of activation required ClfA-specific IgG to interact with FcγRIIa and an unidentified plasma factor that was not required for activation by the wild-type protein. Experiments were performed to assess if a functional complement system is required for slow platelet aggregation by *L. lactis* expressing ClfA PY. The ability of *L. lactis* ClfA PY+ to aggregate GFPs required fibrinogen and serum (Figure 3.21). Human serum samples were depleted of complement proteins and tested for their ability to support platelet aggregation by *L. lactis* ClfA PY+. Complement was depleted from serum either by heat inactivation (56°C for 10 min) or by adsorption with a concentrated suspension (100 mg/ml for 30 min) of zymosan isolated from *Saccharomyces cerevisiae*. Zymosan is a yeast-derived polysaccharide which is known to activate complement by the alternative pathway (Göetze and Müller-Eberhard, 1976), and which can cause platelet aggregation in a complement-dependent manner (Zucker *et al.*, 1974). Accumulation of complement components on the polysaccharide surface results in a depletion of complement proteins from serum samples, which cannot support zymosan-induced platelet aggregation (Ford *et al.*, 1996).

L. lactis ClfA PY+ caused full aggregation of GFPs suspended in fibrinogen and normal human serum (66 ± 5 % aggregation; n = 3; Figure 5.2). The lag times to aggregation in GFPs supplemented with fibrinogen and serum were similar (6 to 9 min) to that seen in PRP preparations. GFPs suspended in fibrinogen and heat-treated serum did not aggregate in response to *L. lactis* ClfA PY+ (6 ± 4 % aggregation; n = 3; $p < 0.05$; Figure 5.2). Similarly, zymosan-adsorbed serum did not support the aggregation of GFP / fibrinogen mixtures by *L. lactis* ClfA PY+ (4 ± 2 % aggregation; n = 3; $p < 0.05$; Figure 5.2). Complement-depleted

serum supported full aggregation of GFP / fibrinogen by *L. lactis* expressing the wild-type ClfA protein (Figure 5.2). These data demonstrate that an intact complement system is crucial for platelet activation and aggregation by bacteria expressing ClfA PY.

The rate of complement fixation on *L. lactis* ClfA PY may explain the long lag times to aggregation in PRP. It has been demonstrated that complement-mediated platelet aggregation by *Strep. sanguis* NCTC 7863 occurs with long, variable lag times, which correlate with the rate of complement fixation on the bacterial cell (Ford *et al.*, 1996). *L. lactis* ClfA PY+ cells, which had been incubated in human plasma samples and then washed in PBS, supported full aggregation of GFP / fibrinogen mixtures with significantly faster lag times (2.1 ± 0.3 min; $n = 3$; $p < 0.05$). This shows that all the plasma factors required for the interaction of ClfA PY-expressing bacteria with platelets become bound to the bacterial surface and are presumably then recognized by specific platelet receptors. The shorter lag time to the onset of aggregation upon pre-incubation of bacteria in plasma suggests that the normal lag phase is due to the progressive binding of plasma factors (IgG and complement proteins) to the bacterial surface before platelet activation can be triggered.

5.2.2 Inhibition of platelet aggregation by antibodies to complement components

Polyclonal antibodies recognizing individual components of the complement system were tested for their ability to inhibit *L. lactis* ClfA PY+-induced aggregation of platelets suspended in plasma. C3 is a central component of the complement cascade, and all three complement activation pathways converge upon the formation of a C3 convertase complex that cleaves C3 molecules in plasma. Covalent attachment of the C3 cleavage product C3b to the microbial surface results in opsonization through recognition of C3b and its cleavage products by phagocytic cells. C3b also forms part of the C5 convertase, required for generation of the terminal C5b-9 complex. Polyclonal antibodies against C3 were diluted 1:100 into PRP samples and incubated for 20 min. *L. lactis* ClfA+ or *L. lactis* ClfA PY+ cells were added to samples and aggregation was monitored. Platelet aggregation by *L. lactis* ClfA PY+ was completely inhibited by treatment of PRP with anti-C3 antibodies (1 ± 1 % aggregation; $n = 3$; $p < 0.05$; Figure 5.3). *L. lactis* ClfA+ stimulated full aggregation with rapid (1.5 min) lag times in PRP treated with anti-C3 (70 ± 6 % aggregation; $n = 3$; Figure 5.3). This indicates that the interaction of *L. lactis* ClfA PY+ with platelets requires complement activation on the bacterial surface to a point involving C3 convertase-mediated cleavage of C3.

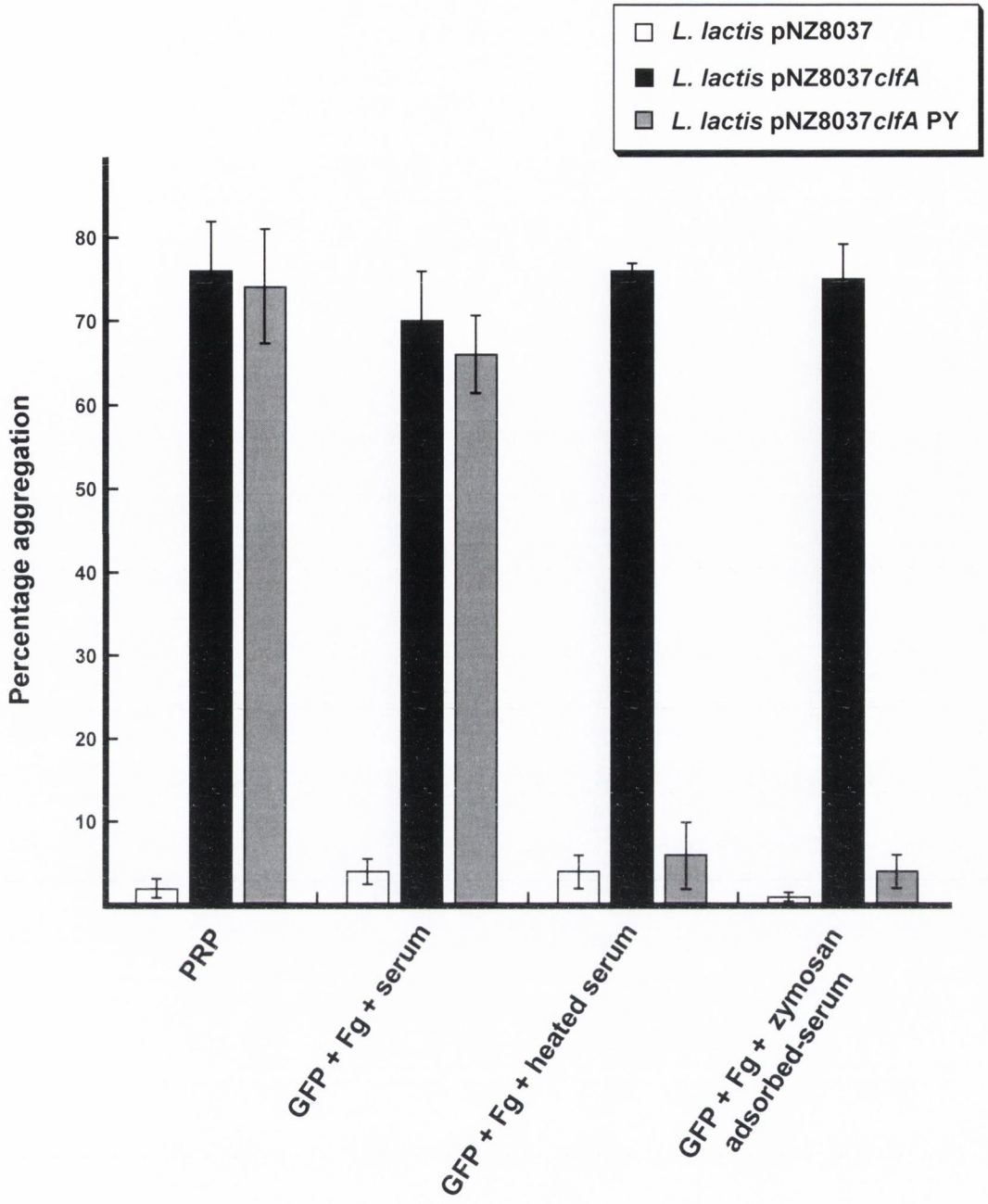


Figure 5.2. Complement proteins are required for platelet aggregation by *L. lactis* ClfA PY

L. lactis (pNZ8037), *L. lactis* (pNZ8037*clfA*) and *L. lactis* (pNZ8037*clfA* PY) cells were grown for 16 h in the presence of 1.6 ng/ml nisin. Twenty-five μl of washed cell suspensions (OD_{600} 1.6) were added to GFP suspensions, containing 5 x 10^7 platelets in 225 μl buffer. GFP samples were supplemented with purified fibrinogen (Fg: 1 mg/ml) and human serum samples (20 % v/v) as indicated. Data is presented as mean percentage aggregation ± SD after 20 min incubation of bacteria with platelets. Percentage aggregation observed in control PRP samples is shown. This experiment was performed 3 times using platelets from 3 different donors.

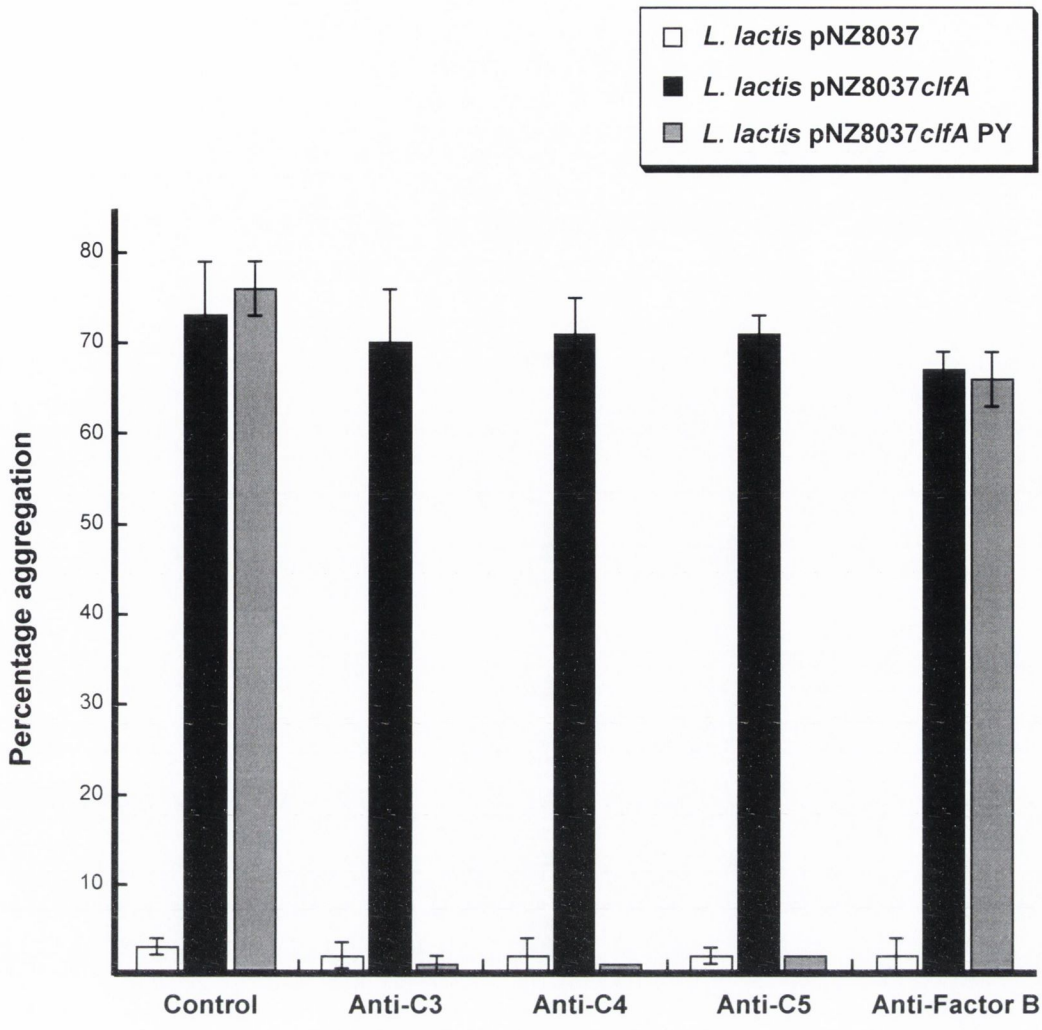


Figure 5.3. Inhibition of ClfA PY-mediated aggregation by antibodies to complement proteins

L. lactis (pNZ8037), *L. lactis* (pNZ8037*clfA*) and *L. lactis* (pNZ8037*clfA* PY) cells were grown for 16 h in the presence of 1.6 ng/ml nisin. Fifty μl of washed cell suspensions (OD_{600} 1.6) were added to PRP suspensions that had previously been incubated with polyclonal antibodies to the complement proteins C3, C4, C5 or factor B as indicated. Anti-complement antisera were diluted 1:100 into PRP suspensions and incubated for 20 min prior to the addition of bacterial cells. Data is presented as mean percentage aggregation ± SD after 20 min incubation of bacteria with platelets. This experiment was performed 3 times using platelets from 3 different donors.

Complement component C4 is cleaved by C1s as part of the classical pathway of complement activation. The C4 cleavage product C4b forms part of the classical pathway C3 convertase. Antibodies against the complement C4 component were incubated with PRP samples (1:100 dilution) and *L. lactis* cells expressing ClfA or ClfA PY were added. *L. lactis* expressing ClfA caused full aggregation of PRP treated with anti-C4 (71 ± 1 % aggregation; n = 3; Figure 5.3). Aggregation by *L. lactis* ClfA PY+ was completely inhibited by anti-C4 antibodies (1 ± 0 % aggregation; n = 3; $p < 0.05$; Figure 5.3), indicating a requirement for C4 in the complement cascade leading to platelet activation by ClfA PY.

Component C5 is cleaved by C5 convertases. Liberated C5b molecules then sequentially bind to components C6, C7, C8 and C9 forming the terminal attack complex that binds to the bacterial cell surface. Polyclonal antibodies to C5 were used to determine if this component is required for the interaction of *L. lactis* ClfA PY+ with platelets leading to activation and aggregation. Incubation of PRP samples with anti-C5 antibodies completely inhibited aggregation by *L. lactis* ClfA PY+ (1 ± 0 % aggregation; n = 3; $p < 0.05$; Figure 5.3) but had no effect on aggregation by *L. lactis* expressing wild-type ClfA (71 ± 2 % aggregation; n = 3; Figure 5.3). This indicates that C5 or its cleavage product C5b are involved in the interaction of *L. lactis* ClfA PY+ with platelets leading to aggregation.

Factor B is specific to the alternative pathway of complement activation. Polyclonal antibodies to factor B were incubated with PRP samples to determine what role the alternative complement pathway plays in the interaction of *L. lactis* ClfA PY+ with platelets. PRP incubated with anti-factor B antibodies (1:100 dilution) aggregated rapidly (1.5 min lag time) in response to *L. lactis* expressing wild-type ClfA (67 ± 2 % aggregation; n = 3; Figure 5.3). PRP treated with anti-factor B antibodies aggregated fully in response to *L. lactis* ClfA PY+ (66 ± 3 % aggregation; n = 3; Figure 5.3). However, the lag time to the onset of aggregation was doubled for *L. lactis* expressing ClfA PY in PRP treated with anti-factor B (11.5 ± 1.5 min lag time; n = 3; $p < 0.05$) compared to control untreated PRP samples (6.5 ± 0.5 min lag time; n = 3). This indicates that the alternative pathway of complement activation is not crucial for the interaction of cells expressing ClfA PY with platelets. However, it is possible that the concentration of antibodies used here were not sufficient to deplete factor B from PRP preparations. Anti-Factor B antibodies used at lower concentrations (diluted 1: 500 into PRP) did not effect the lag time or extent of aggregation caused by *L. lactis* ClfA PY+ (data not shown).

5.2.3 An antibody to complement receptor type 4 (CR4) inhibits aggregation by *L. lactis* expressing ClfA PY

The requirement of an intact complement system for platelet aggregation by *L. lactis* expressing ClfA PY suggests that a platelet complement receptor recognizes some component(s) of the complement system bound to the bacterial surface as part of the activation process. Many types of complement receptors have been characterized and are generally involved in recognition and clearance of immune complexes from the circulation. Complement receptor 1 (CR1) binds either to C3b or C4b molecules that become attached to the complement-activating surface. CR2, CR3 and CR4 bind to inactivated, degradation products of C3b (iC3b and/or C3dg) which remain attached to the pathogen surface (Cooper, 1999). Platelets do not express CR1, CR2 or CR3, but expression of CR4 has been demonstrated on activated and resting platelets (Vik and Fearon, 1987). CR4 is a β2 integrin that preferentially binds to C3 degradation product iC3b (Cooper, 1999), but has also been reported to bind C3dg (Vik and Fearon, 1987). Platelets also express the C1q receptor gC1qR/p33, but surface expression of this receptor can only be detected on activated platelets (Peerschke *et al.*, 2003). An antibody to CR4 (also known as CD11c) was incubated with PRP preparations and the ability of *L. lactis* cells expressing ClfA or ClfA PY to stimulate aggregation was tested. Incubation of PRP with anti-CD11c antibodies did not affect the ability of ClfA to cause rapid aggregation (lag time 1.2 ± 0.2 min; n = 3). However, the level of aggregation caused by *L. lactis* expressing wild-type ClfA was somewhat reduced by treatment of platelets with anti-CD11c antibodies (65 ± 2 % aggregation in PRP treated with anti-CD11c; n = 3; $p < 0.05$; Figure 5.4) compared to control untreated PRP (83 ± 6 % aggregation; n = 3; Figure 5.4). Platelet aggregation by *L. lactis* ClfA PY+ was completely blocked by pre-incubation of PRP with anti-CD11c antibodies (65 ± 2 % aggregation in PRP treated with anti-CD11c; n = 3; $p < 0.05$; Figure 5.4). These data suggest that CD11c (CR4) is involved in the complement-dependent response of platelets to *L. lactis* expressing ClfA PY, perhaps through recognition of iC3b/C3dg bound to the bacterial cell surface.

5.2.4 Identification of the complement activation pathway required for platelet aggregation stimulated by *L. lactis* expressing ClfA PY

Experiments were performed to clarify which complement activation mechanism is required for platelet aggregation by *L. lactis* expressing ClfA PY. Human serum samples,

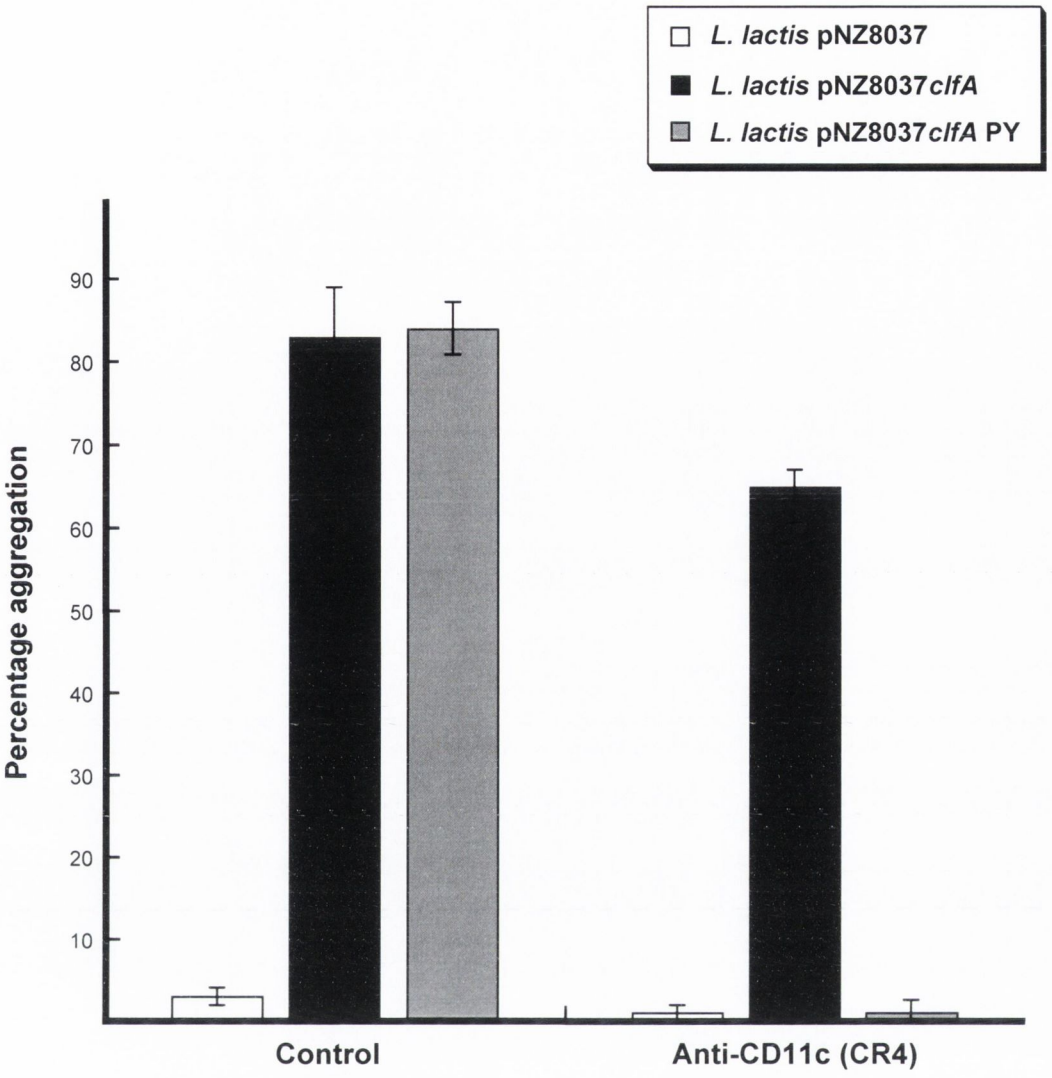


Figure 5.4. Inhibition of ClfA PY-mediated platelet aggregation by an antibody to complement receptor (CR) 4

L. lactis (pNZ8037), *L. lactis* (pNZ8037*clfA*) and *L. lactis* (pNZ8037*clfA* PY) cells were grown for 16 h in the presence of 1.6 ng/ml nisin. Mouse-anti-CD11c (CR4) antibodies were diluted 1:50 into PRP suspensions and incubated for 20 min prior to the addition of bacterial cells. Fifty μl of washed cell suspensions (OD_{600} 1.6) were added to PRP suspensions as indicated. Data is presented as mean percentage aggregation ± SD after 20 min incubation of bacteria with platelets.

specifically depleted of individual complement proteins, were used to supplement GFPs suspended in purified fibrinogen in platelet aggregation assays. Serum depleted of C1q (part of the C1 component that initiates the classical pathway through recognition of antibody-antigen complexes) was used to determine the role of the classical complement pathway in platelet aggregation by *L. lactis* ClfA PY+. Factor B-depleted serum was used to demonstrate the contribution of the alternative complement pathway. Serum depleted of C9, the final component of the terminal attack complex, was used to analyze the requirement for C5b-9 formation in platelet aggregation by bacteria expressing ClfA PY.

As previously observed, the addition of fibrinogen and normal human serum to GFPs was sufficient for full platelet aggregation by *L. lactis* ClfA PY+ (61 ± 3 % aggregation; n = 3; Figure 5.5). Human serum, incubated at 56°C for 10 min, served as a control to demonstrate the requirement for complement. GFPs suspended in fibrinogen and heated serum did not aggregate in response to *L. lactis* ClfA PY+ (5 ± 3 % aggregation; n = 3; $p < 0.05$; Figure 5.5). C1q-depleted human serum did not support aggregation of GFP / fibrinogen by *L. lactis* ClfA PY+ (7 ± 2 % aggregation after 25 min incubation; n = 4; $p < 0.05$; Figure 5.5) indicating a crucial role for the classical pathway of complement activation in the aggregation of platelets by bacteria expressing ClfA PY. Serum-depleted of either C9 or Factor B supported aggregation of GFPs suspended in fibrinogen by *L. lactis* ClfA PY+ (Figure 5.5). The lag time to aggregation of GFPs suspended in fibrinogen and C9-depleted serum was extended (11.5 ± 2.5 min; n = 3) compared to that observed in GFP / fibrinogen / normal serum (5.8 ± 1.3 min; n = 3). Similarly, longer lag times to aggregation were observed for GFPs suspended in fibrinogen and factor B-depleted serum (9.1 ± 1.8 min; n = 3), although this was not as pronounced as that seen with C9-depleted serum. This indicates that the complement activation by the alternative pathway is not crucial for platelet aggregation by *L. lactis* ClfA PY+, but the presence of this pathway enhances the interaction with platelets resulting in shortened lag times to aggregation. Similarly, formation of C5b-9 on the surface of *L. lactis* ClfA PY+ was not essential for aggregation, although shorter lag times to aggregation were associated with the presence of C9 in serum samples. The formation of C5b-9 on the bacterial cell may enhance platelet activation, perhaps through binding to low-levels of activated GPIIb/IIIa receptors (Røger *et al.*, 1995).

5.2.5 Binding of plasma components to the surface of *L. lactis* and *L. lactis* ClfA PY+

The interactions between *L. lactis* cells expressing ClfA PY and platelets apparently involve adhesin-bound IgG recognition by FcγRIIa and complement fixation through the classical pathway. Surface-bound complement components may recognize the platelet through one or more interactions (e.g bacterial-bound iC3b binding to CR4 or C5b-9 binding to GPIIb/IIIa). This suggests that the inability of the *L. lactis* host strain to stimulate platelet aggregation is either the result of low-levels of complement deposition on the bacterium or low levels of IgG in plasma that recognize the lactococcal surface. Either or both of these scenarios would impair the interaction of bacteria and platelets and would explain the failure of *L. lactis* to stimulate platelet aggregation. Experiments were performed to compare the rate and extent of C5b-9 formation on the surface of *L. lactis* and *L. lactis* ClfA PY+. Washed bacterial cells were incubated in plasma samples with stirring at 37°C to mimic the conditions found in platelet aggregation assays. Plasma samples which had been heated at 56°C to deplete complement were used as controls. At selected time points, aliquots of cells were removed into a solution of EDTA to terminate the complement reaction. Cells were extensively washed with PBS to remove non-specifically bound plasma components and the cells were then coated onto microtitre wells. A polyclonal anti-C5b-9 antibody was used to probe the wells to detect the C5b-9 complex formed on the bacterial cell during incubation in plasma. As expected, only background level anti-C5b-9 binding was observed to cells that were incubated in complement-depleted (heated) plasma (Figure 5.6). *L. lactis* (pNZ8037) cells that were incubated in plasma formed C5b-9 complexes on their surface (Figure 5.6). C5b-9 formation was rapid and readily detectable on cells after 1 min incubation in plasma. Maximal C5b-9 formation was observed between 5 to 10 minutes exposure to plasma, and did not markedly increase after 20 min incubation (Figure 5.6). Very similar patterns of C5b-9 formation were observed on *L. lactis* ClfA PY+ cells incubated in normal plasma (Figure 5.6), with C5b-9 levels reaching saturation between 5 and 10 minutes incubation. Of note, PRP samples from the plasma donors used for this experiment aggregated with lag times of 6.3 ± 1.1 min (n = 3) in response to *L. lactis* ClfA PY cells. These data indicate that expression of ClfA PY by *L. lactis* does not increase the rate or extent of complement activation on the bacterial surface. Therefore it does not seem likely that the failure of *L. lactis* (pNZ8037) to

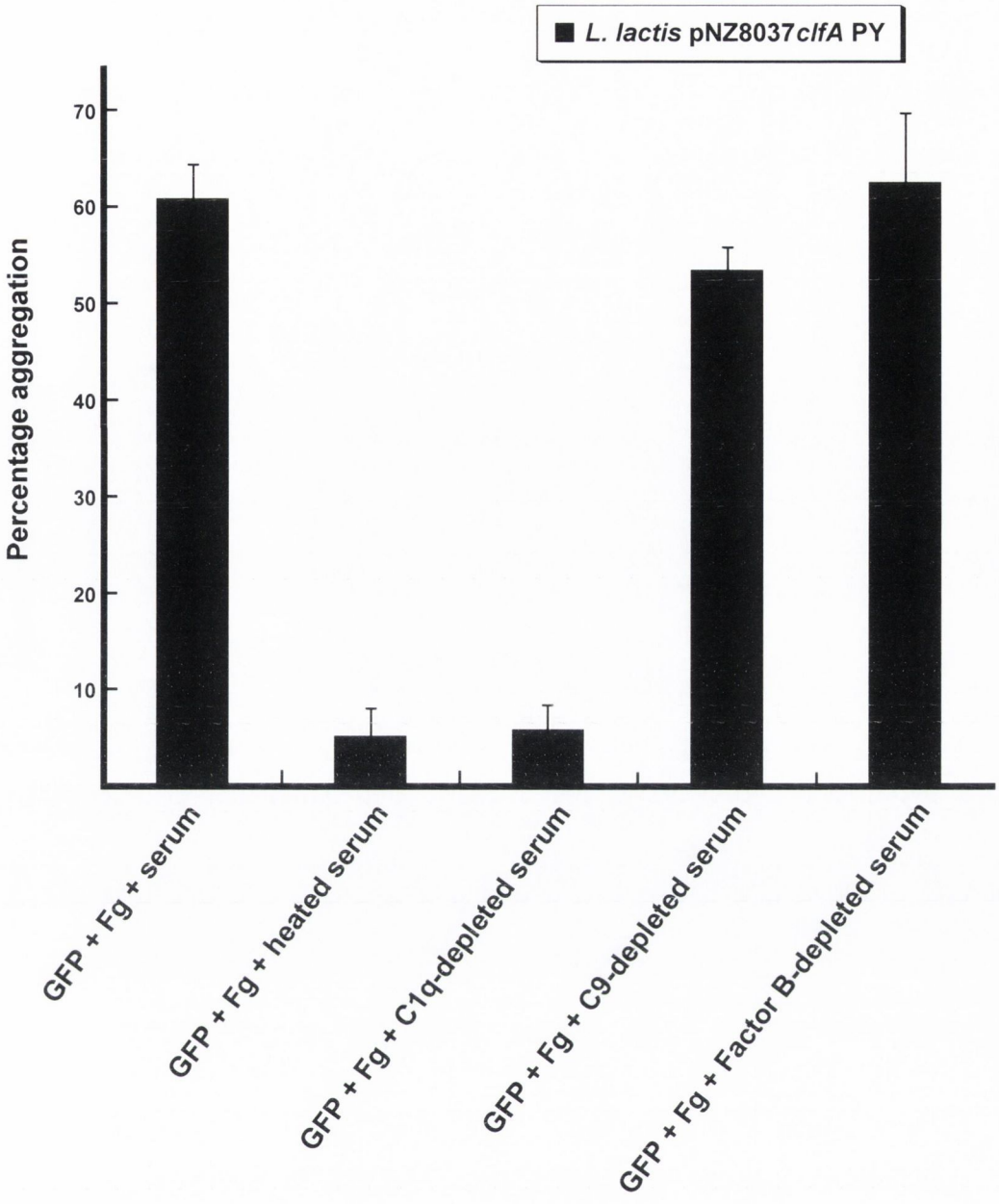


Figure 5.5. ClfA PY-mediated platelet aggregation is dependent on the classical complement pathway

L. lactis (pNZ8037*clfA* PY) cells were grown for 16 h in the presence of 1.6 ng/ml nisin. Twenty-five μl of washed cell suspensions (OD_{600} 1.6) were added to GFP suspensions, containing 5 x 10^7 platelets in 225 μl buffer. GFP samples were supplemented with purified fibrinogen (Fg: 1 mg/ml) and human serum samples depleted of specific complement proteins (20 % v/v) as indicated. Data is presented as mean percentage aggregation ± SD after 20 min incubation of bacteria with platelets. This experiment was performed 3 times using platelets from 3 different donors.

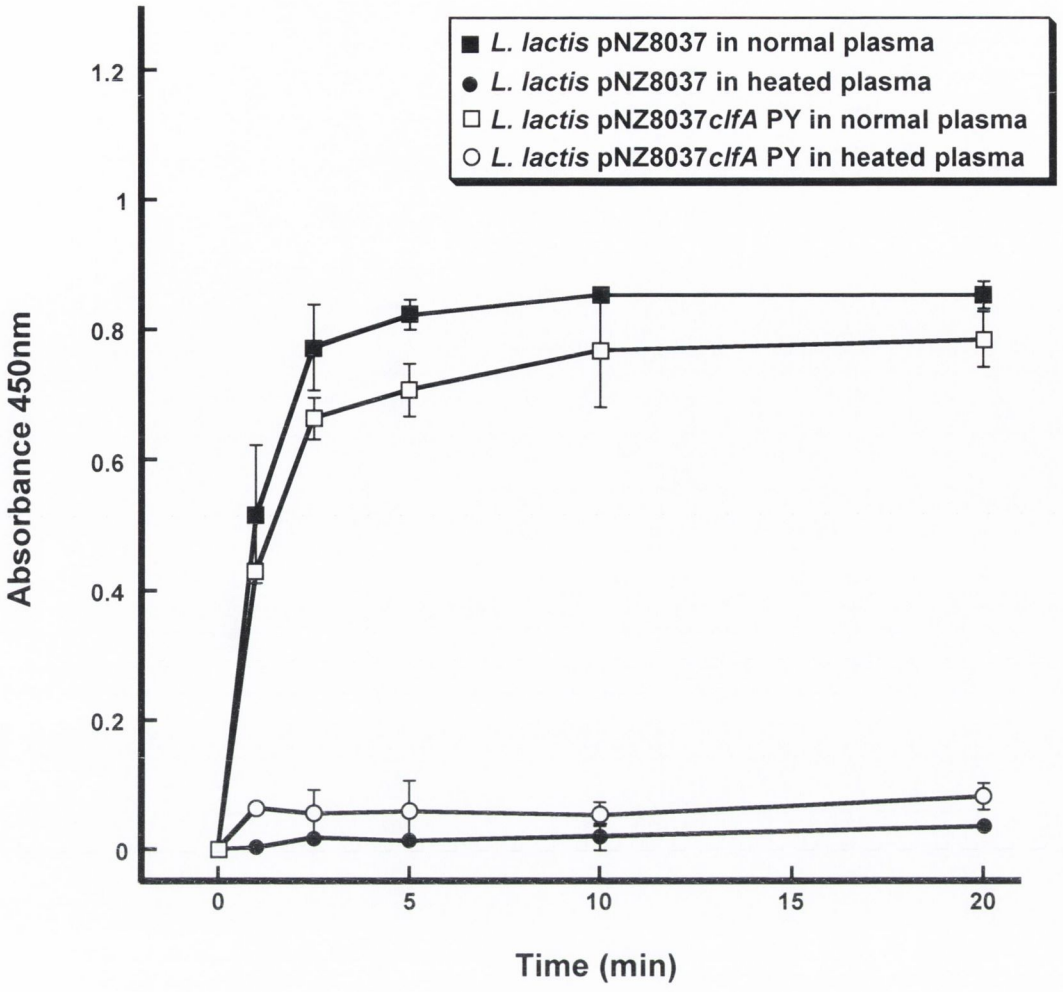


Figure 5.6. Formation of the C5b-9 complex on the surface of *L. lactis* and *L. lactis* ClfA PY cells incubated in plasma

L. lactis (pNZ8037) and *L. lactis* (pNZ8037*clfA* PY) cells were grown for 16 h in the presence of 1.6 ng/ml nisin. Cells were washed and adjusted to OD_{600} 5.0 in PBS. Cell suspensions (500 μl) were mixed with human plasma samples (500 μl) and incubated at 37°C with stirring in a platelet aggregometer. Complement-depleted plasma, prepared by heat treatment (56°C for 10 min), served as a control. At selected time points, 100 μl cell suspension was removed into 100 mM EDTA and washed extensively in PBS. Washed cells were used to coat microtitre wells. Control wells contained bacteria incubated in PBS. Complement formation was assesed by incubating the bacterial-coated wells with rabbit-anti-C5b9 antibodies (Calbiochem). Bound antibody was detected using goat-anti-rabbit antibodies conjugated to horseradish peroxidase. This experiment was performed 3 times using plasma obtained from different donors.

aggregate platelets is a result of lower levels of complement activation compared to *L. lactis* ClfA PY+.

The presence of ClfA-specific IgG was found to be essential for *L. lactis* ClfA PY+ to stimulate platelet aggregation (Figure 3.24). It is possible that the levels of specific IgG in normal donors plasma that recognize the *L. lactis* surface are insufficient to trigger activation, and that high-level expression of ClfA PY on the surface resulting in adequate binding of specific IgG is required for productive interactions with FcγRIIa, leading to activation and aggregation. ELISA experiments were performed to measure the binding of plasma IgG to washed *L. lactis* and *L. lactis* ClfA PY+ cells immobilized on microtitre wells. Serial dilutions of plasma in PBS were set up across a range of bacterial coated wells and incubated for 90 min to allow binding of specific IgG to the bacteria. Bound IgG was detected using a peroxidase-conjugated goat-anti-human antibody specific for the Fc region. Plasma samples contained antibodies that recognized *L. lactis* (pNZ8037) cells (Figure 5.7). However, significantly higher levels of antibodies recognizing *L. lactis* ClfA PY+ were present in plasma (Figure 5.7). It is reasonable to assume that the difference in antibody binding is solely due to the presence of specific IgG in donors plasma recognizing ClfA. This IgG is absolutely required for aggregation. Therefore, it is likely that expression of ClfA PY by *L. lactis* confers the ability to aggregate platelets as a result of high-level specific antibody binding to the expressed protein, allowing interaction with FcγRIIa. Although antibodies are present in plasma that recognize *L. lactis*, these do not appear to contribute to platelet aggregation by *L. lactis* ClfA PY+ (Figure 3.24). *L. lactis* does not naturally express many surface proteins, so it is possible that antibodies recognizing *L. lactis* are directed against common gram-positive surface structures, such as peptidoglycan and teichoic acids. Antibodies bound to these components may not be efficiently recognized by platelet FcγRIIa.

5.2.6 Nisin-controlled expression of SdrC, SdrD and SdrE in *L. lactis*

It has been demonstrated that slow platelet aggregation by stationary phase *S. aureus* Newman cells lacking ClfA and ClfB was mediated through a mechanism involving the serine-aspartate repeat (Sdr) protein E (O'Brien *et al.*, 2002). Constitutive high-level expression of SdrE on the surface of *L. lactis* promoted platelet aggregation in a plasma-dependent manner with lag times of 10 to 17 minutes (O'Brien *et al.*, 2002). Like ClfA PY, no plasma ligand has been identified for SdrE, and neither ClfA PY nor SdrE can promote

direct interaction with platelets (M. Brennan, personal communication). Investigation into the mechanism of platelet aggregation by bacteria expressing SdrE has demonstrated a mechanism involving IgG and complement proteins (M. Brennan, personal communication). Comparison of aggregation data for *L. lactis* expressing SdrE or ClfA PY indicate a highly similar mechanism of interacting with platelets. This raises the possibility that any *S. aureus* surface protein could stimulate platelet aggregation in a complement- and IgG-dependent manner when expressed by *L. lactis*, if it is expressed at high-enough levels and there are sufficient antibodies to that protein present in plasma.

A chromosomal locus containing three closely linked genes (*sdrC, sdrD*, and *sdrE*) is responsible for the expression of SdrE and two other homologous surface proteins, SdrC and SdrD (Josefsson *et al.*, 1998). These proteins are defined by the presence of a repeat region of Ser-Asp dipeptide repeats, which are also present in ClfA and ClfB (Figure 5.8). Unique A domains are present in SdrC, SdrD and SdrE that share less than 30 % homology in any pairwise alignment (Josefsson *et al.*, 1998). While these domains are presumed to contain ligand-binding activity, no ligands for SdrC, SdrD or SdrE have been identified to date. It is unclear why *L. lactis* cells expressing SdrE could cause slow platelet aggregation, whereas *L. lactis* expressing SdrC or SdrD could not (O'Brien *et al.*, 2002). The plasmid used for expression of the Sdr proteins in *L. lactis* (pKS80) contains a constitutive promoter driving surface protein expression (Hartford *et al.*, 2001; O'Brien *et al.*, 2002). It was possible that SdrE was expressed at higher levels than SdrC or SdrD on *L. lactis*, accounting for the ability of *L. lactis* (pKS80*sdrE*) cells to cause platelet aggregation. Alternatively, plasma from the platelet donors used in the previous study may not have contained sufficient antibodies against SdrC or SdrD to allow interaction with platelets through FcγRIIa.

The *sdrC, sdrD* and *sdrE* genes were cloned into the nisin-inducible expression vector pNZ8037 as described in section 2.5 to allow controlled expression on the surface of *L. lactis*. This was to allow experiments measuring the relationship between SdrC, SdrD or SdrE expression and the ability to cause platelet aggregation to be performed. *L. lactis* (pNZ8037*sdrC*), *L. lactis* (pNZ8037*sdrD*) and *L. lactis* (pNZ8037*sdrE*) cells were grown overnight in the presence of varying nisin concentrations. Serial dilutions of washed cell suspensions were applied to nitrocellulose membranes, which were probed with antibodies against the unique A domains of each protein. Representative blots are shown in Figure 5.9. Expression levels of SdrC, SdrD or SdrE increased approximately 60-fold across the nisin-induction range. Maximal expression was achieved at nisin concentrations of between 0.8 and

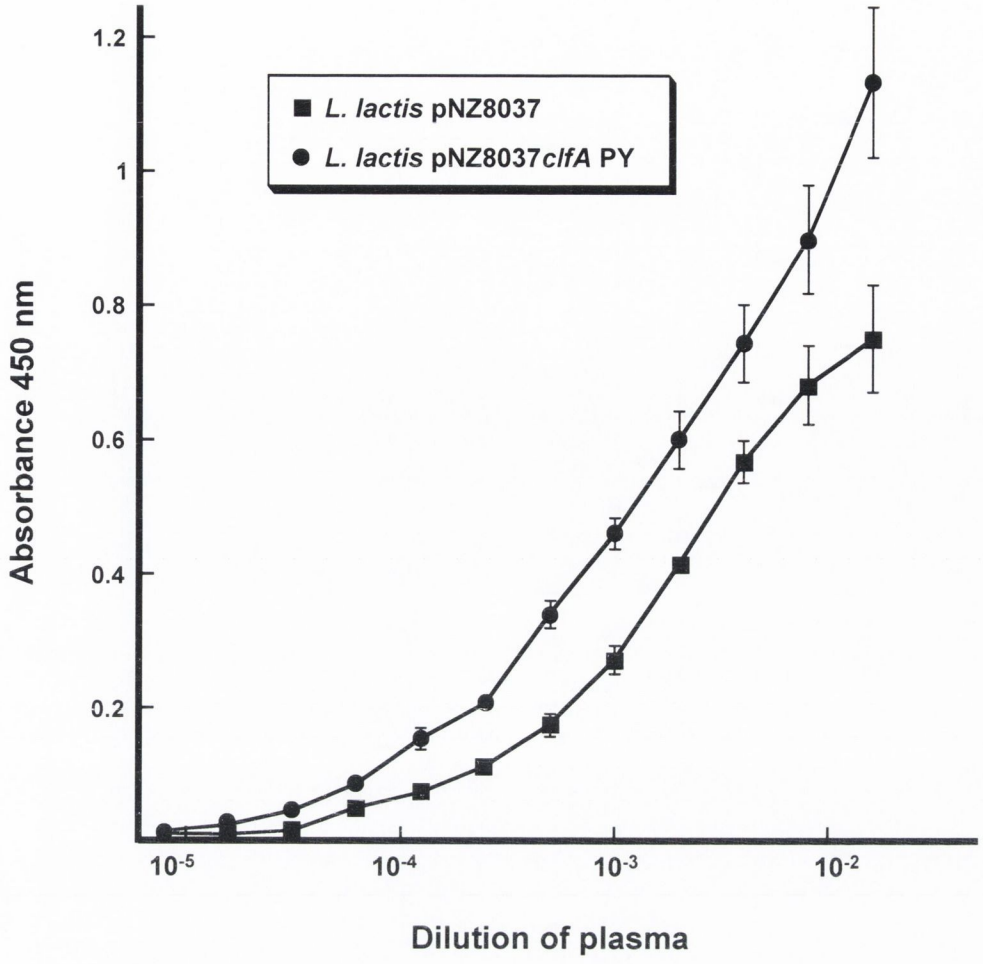


Figure 5.7. Binding of antibodies in human plasma to *L. lactis* and *L. lactis* ClfA PY

L. lactis (pNZ8037) and *L. lactis* (pNZ8037*clfA* PY) cells were grown for 16 h in the presence of 1.6 ng/ml nisin. Cells were washed and adjusted to OD_{600} 1.0 in bicarbonate buffer and 100 ml aliquots used to coat microtitre wells. Human plasma samples were serially diluted in PBS across a range of bacterial coated wells and incubated for 1.5 h. Binding of plasma-derived antibodies to the bacterial cells was measured using a goat-anti-human Fc specific antibody conjugated to peroxidase. Bound antibody was detected using goat-anti-rabbit antibodies conjugated to horseradish peroxidase. The wells were developed using a substrate for peroxidase and the absobance at 450 nm determined. This experiment was performed 3 times using plasma obtained from different donors.

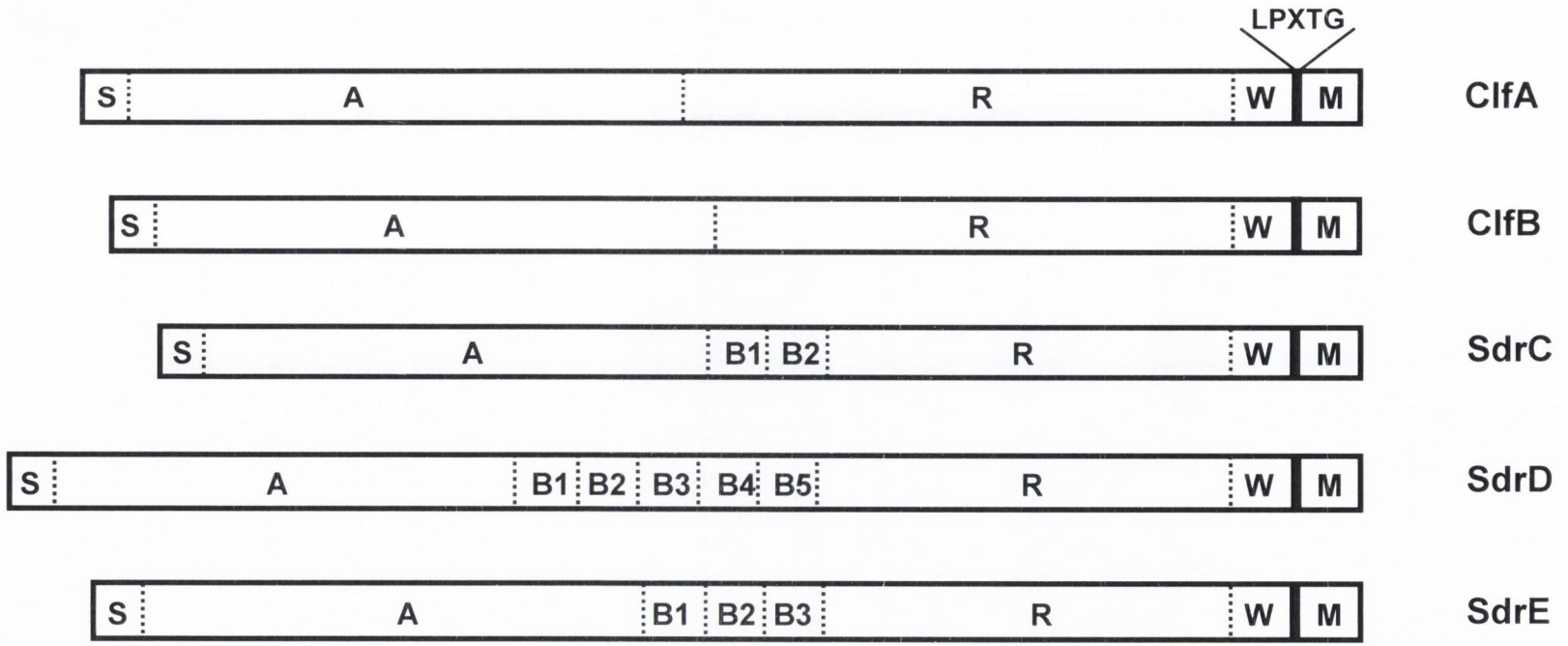


Figure 5.8. Schematic representations of the Clf-Sdr protein family of *S. aureus*

An N-terminal signal sequence (S) consisting of approximately 40 amino acids targets the protein for secretion. Domains of approximately 500 residues (A domains) follow the signal sequence and are exposed on the bacterial cell surface. The A domain of ClfA recognizes fibrinogen, and the A domain of ClfB contains fibrinogen and keratin binding activity. No ligands have been identified for the A domains of SdrC, SdrD or SdrE. SdrC contains two additional 110-113 residue B repeats. SdrD and SdrE contain 5 and 3 B repeat domains respectively. This protein family is defined by the presence of a repeat region (R) mainly composed of Ser-Asp (SD) dipeptide repeats. This region is though to function as a stalk, projecting the A domains away from the cell surface. The C-termini contain wall (W) and membrane (M) spanning domains and the LPXTG motif required for covalent attachment to the cell wall peptidoglycan by sortase.

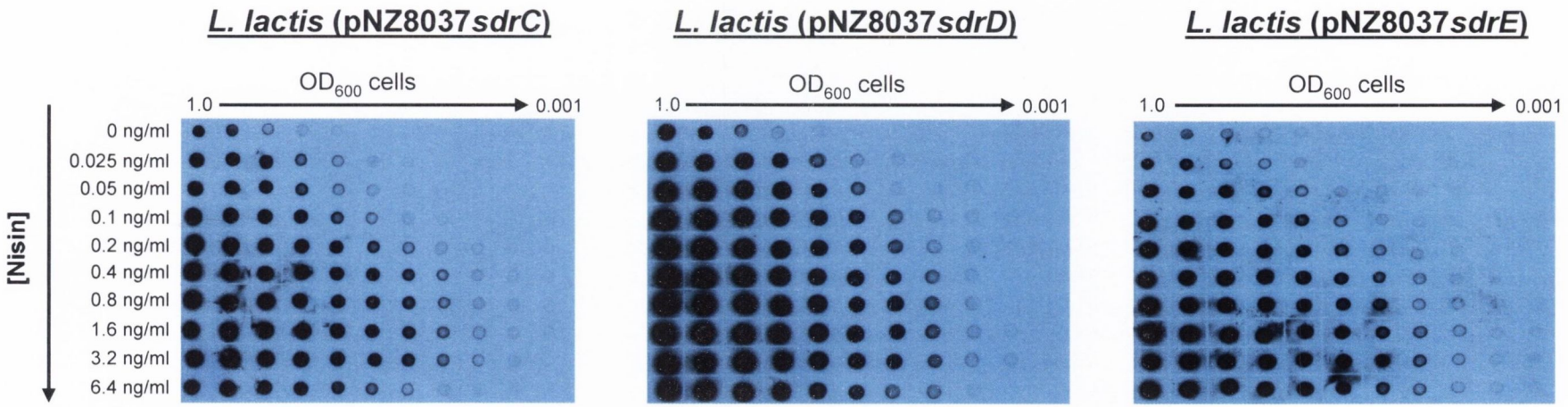


Figure 5.9. Nisin-controlled expression of SdrC, SdrD and SdrE on the surface of *L. lactis*

L. lactis (pNZ8037*sdrC*), *L. lactis* (pNZ8037*sdrD*) and *L. lactis* (pNZ8037*sdrE*) cells were grown for 16 h in the presence of varying nisin concentrations as indicated. Cells were washed twice in PBS and suspended to OD_{600} of 1.0 in PBS. Serial dilutions were made in PBS in 96-well microtitre dishes. 5 μl of each dilution was applied to nitrocellulose membranes and blocked in 10 % skimmed milk solution for 16 h. Membranes were then incubated with polyclonal antibodies recognizing the unique A domains of SdrC (1:2000 dilution), SdrD (1:5000 dilution) or SdrE (1:2000 dilution). Bound antibodies were detected using goat-anti-rabbit horseradish peroxidase-conjugated antibodies (1:2000). Membranes were developed by chemiluminescence and exposed to autoradiographic film.

3.2 ng/ml for each protein. Concentrations of nisin greater than 3.2 ng/ml resulted in variable expression (Figure 5.9) and affected cell growth, possibly due to toxic effects of nisin at high concentrations.

Similar dot-immunoblot experiments were preformed to compare SdrC, SdrD and SdrE expression from the constitutive expression level plasmid pKS80 with expression from the nisin-inducible pNZ8037 vector. *L. lactis* cells carrying pNZ8037 and its derivative plasmids were grown in the presence of 1.6 ng/ml nisin to achieve maximal expression of the plasmid-encoded surface protein. Antibodies against the A domains of SdrC, SdrD or SdrE did not react with *L. lactis* (pNZ8037) cells (Figure 5.10). Maximal expression of SdrC from pNZ8037*sdrC* was approximately 2-fold higher than that expressed from pKS80*sdrC* (Figure 5.10). SdrD expression levels from pNZ8037*sdrD* and pKS80*sdrD* were equivalent (Figure 5.10). Expression of SdrE from pNZ8037*sdrE* was 4 to 8 fold higher than expression achieved from pKS80*sdrE* (Figure 5.10). These data indicate that expression of the Sdr proteins could be controlled on the surface of *L. lactis*. Expression levels could be achieved that were comparable to, or higher than the expression levels from the pKS80 expression plasmids.

5.2.7 Platelet aggregation by *L. lactis* cells expressing SdrC, SdrD or SdrE

L. lactis cells containing pNZ8037 derivative plasmids were grown overnight in the presence of 1.6 ng/ml nisin concentrations to induce maximal expression of SdrC, SdrD or SdrE. Cells were washed in PBS and adjusted to OD_{600} of 1.6. Fifty µl of cell suspension was added to 450 µl PRP and platelet aggregation monitored. *L. lactis* cells maximally expressing SdrE stimulated aggregation of PRP with lag times 7.5 ± 0.3 min (n = 2). *L. lactis* (pNZ8037*sdrC*) cells and *L. lactis* (pNZ8037*sdrD*) cells caused aggregation after lag times of 6.1 ± 1 min and 7.8 ± 0.2 min respectively (n = 2). A representative aggregation trace is shown in Figure 5.11. This demonstrates that SdrC and SdrD, in addition to SdrE, can cause platelet aggregation if expressed at sufficient levels in *L. lactis*.

The ability to control Sdr protein expression on the surface of *L. lactis* allowed the relationship between surface protein expression level and the ability to cause aggregation to be determined. *L. lactis* (pNZ8037*sdrC*), *L. lactis* (pNZ8037*sdrD*) and *L. lactis* (pNZ8037*sdrE*) cells were induced with increasing concentrations of nisin. The ability of washed cell suspensions to trigger aggregation of PRP samples was determined. A minimum threshold

level expression of SdrC, SdrD or SdrE was required to cause aggregation. Expression levels achieved at 0.2 ng/ml were sufficient for *L. lactis* expressing SdrC, SdrD and SdrE to cause platelet aggregation, whereas no aggregation of platelets was caused by cells induced with 0.1 ng/ml after 25 min incubation (Figure 5.12). For *L. lactis* (pNZ8037*sdrC*), aggregation occurred with lag times of 15.7 ± 3.9 min (n = 2) at 0.2 ng/ml nisin, which decreased to 6.1 ± 1 min at 1.6 ng/ml nisin (n = 2). Similarly, increasing expression levels of SdrD or SdrE on the *L. lactis* surface resulted in a progressive shortening of the lag time, although lag times were always greater than 5 min (Figure 5.12).

Expression levels of the Sdr proteins from the pKS80 constitutive expression vectors (Figure 5.10) appears to be at or above the threshold expression levels required for platelet aggregation determined above. *L. lactis* cells expressing SdrC, SdrD or SdrE from pKS80 were tested for the ability to cause aggregation of PRP samples. *L. lactis* (pKS80*sdrC*), *L. lactis* (pKS80*sdrD*) and *L. lactis* (pKS80*sdrE*) caused platelet aggregation with lag times of 7.7 ± 1.4 min, 10.4 ± 2.7 min and 8 ± 0.8 min respectively (n = 2). It is unclear why *L. lactis* cells expressing SdrC or SdrD did not cause platelet aggregation in previous studies (O'Brien *et al.*, 2002). Perhaps the platelet donors did not contain sufficient levels of surface protein-specific IgG in their plasma.

5.2.8 Platelet aggregation by *S. aureus* Newman mutants lacking multiple surface proteins

The finding that SdrC and SdrD can independently stimulate platelet aggregation extends the list of *S. aureus* proteins that are known to be capable of doing this. While not demonstrated here, it is presumed that SdrC and SdrD interact with platelets in a complement- and IgG-dependent manner in a similar manner to SdrE (M. Brennan, personal communication) and ClfA PY; these interactions are characterized by extended lag times (> 5 min). Long lag phases before the onset of aggregation has been observed for complement- and IgG-dependent platelet aggregation by *Strep. sanguis* (Ford *et al.*, 1996, 1997) and was suggested to be the result of the time taken to accumulate complement on the bacterial surface. It is feasible that slow aggregation stimulated by *S. aureus* mutants lacking the major pro-aggregatory surface proteins (ClfA, ClfB, FnBPA and FnBPB) is the result of the expression of a number of other surface components that promote sufficient levels of plasma IgG binding to the bacterial cell. Bacterial-bound IgG, in conjunction with complement proteins, can

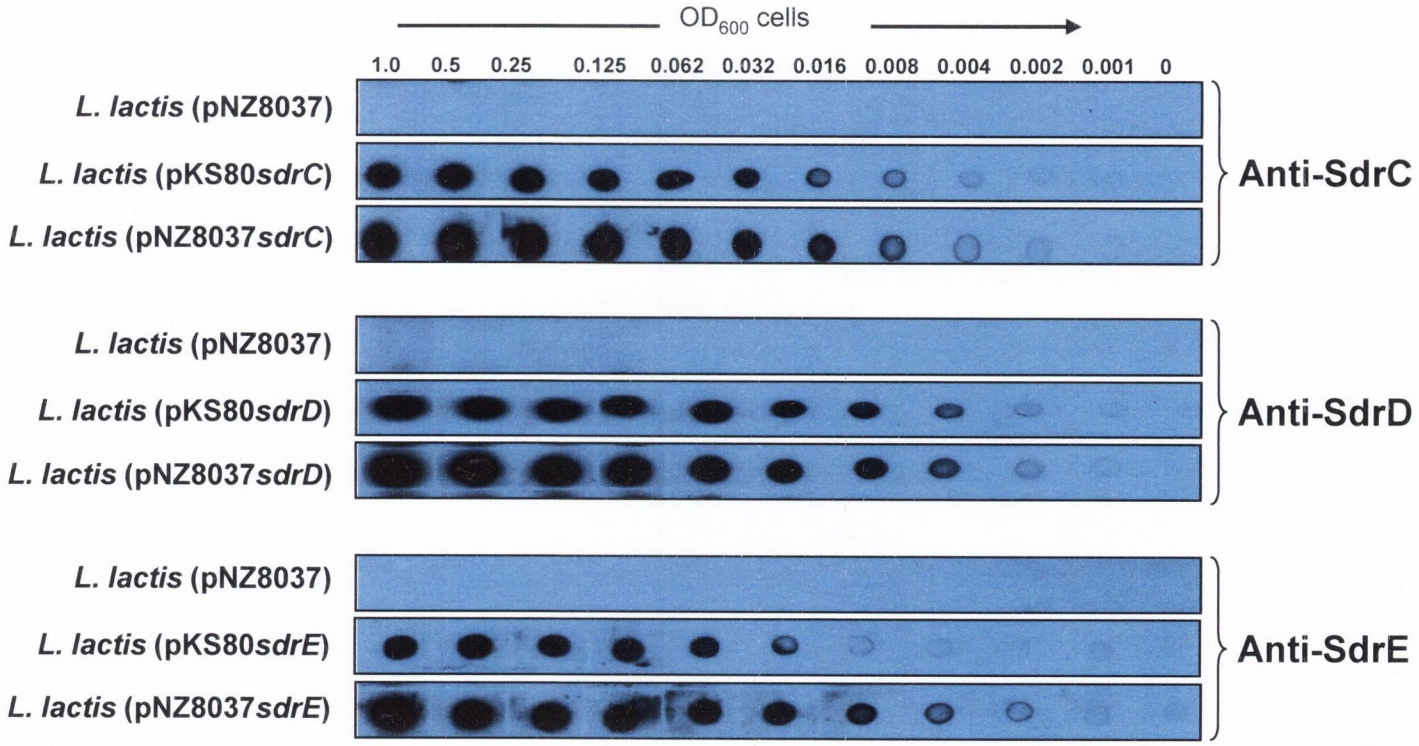


Figure 5.10. Expression of SdrC, SdrD and SdrE in *L. lactis* from constitutive and inducible promoters

L. lactis strains containing pKS80-derivative constitutive expression plasmids were grown for 16 h. Strains containing pNZ8037 and its derivative plasmids were grown for 16 h in the presence of 1.6 ng/ml nisin. Cells were washed twice in PBS and suspended to OD_{600} of 1.0 in PBS. Serial dilutions were made in PBS in 96-well microtitre dishes. 5 μl of each dilution was applied to nitrocellulose membranes and blocked in 10 % skimmed milk solution for 16 h. Membranes were then incubated with polyclonal antibodies recognizing the unique A domains of SdrC (1:2000 dilution), SdrD (1:5000 dilution) or SdrE (1:2000 dilution). Bound antibodies were detected using goat-anti-rabbit horseradish peroxidase-conjugated antibodies (1:2000). Membranes were developed by chemiluminescence and exposed to autoradiographic film.

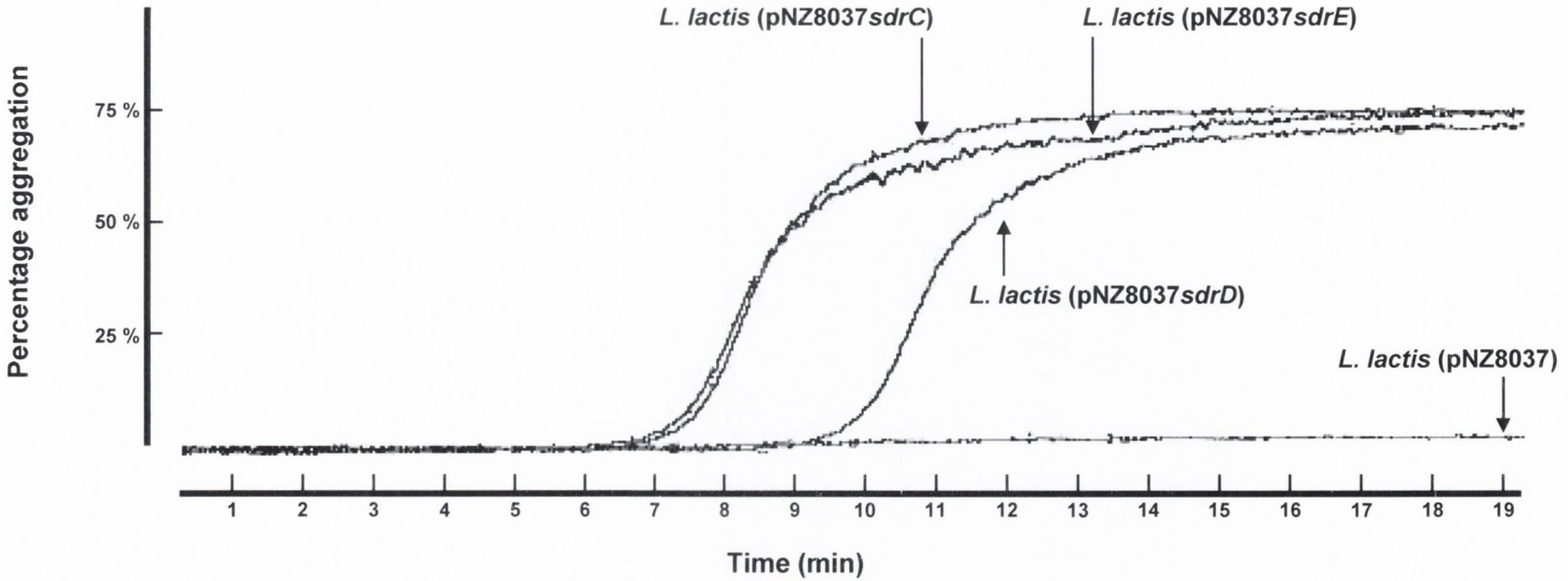


Figure 5.11. Aggregation of human platelets in plasma by *L. lactis* expressing SdrC, SdrD or SdrE

L. lactis cells were grown for 16 h in the presence of 1.6 ng/ml nisin. Cells were washed twice in PBS and suspended to OD_{600} of 1.6 in PBS. Fifty μl cell suspension was added to 450 μl platelet-rich-plasma (PRP) and platelet aggregation was monitored by light transmission at 37°C with stirring. The aggregation trace shown is a representative of 3 different experiments.

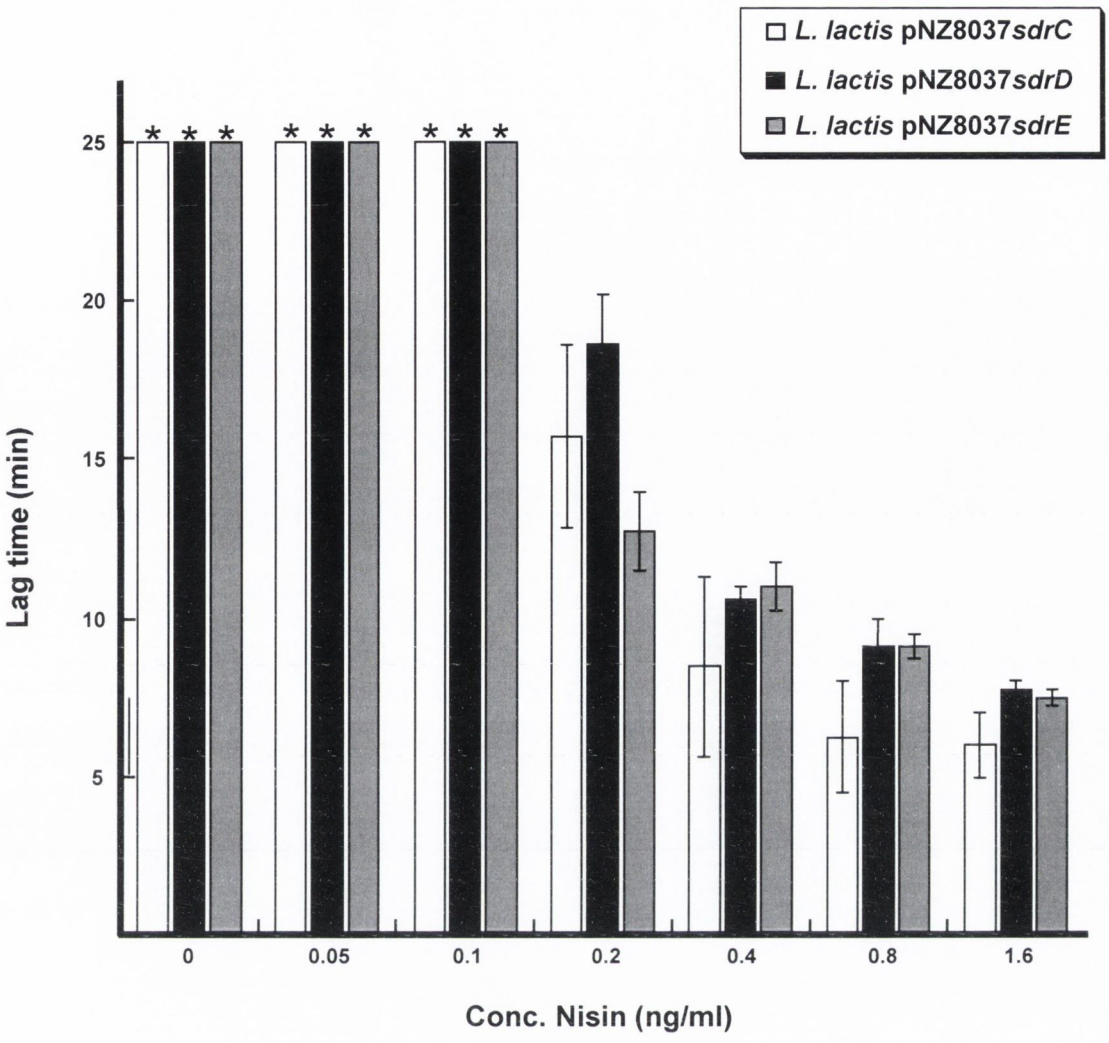


Figure 5.12. Platelet aggregation by *L. lactis* expressing SdrC, SdrD or SdrE

L. lactis (pNZ8037*sdrC*), *L. lactis* (pNZ8037*sdrD*) and *L. lactis* (pNZ8037*sdrE*) cells were grown for 16 h in the presence of varying concentrations of nisin as indicated. Fifty μl washed cell suspension (OD_{600} 1.6) was added to 450 μl PRP and platelet aggregation was monitored by light transmission at 37°C. Data is presented as mean percentage aggregation ± SD after 20 min incubation of bacteria with platelets. This experiment was performed twice using platelets from 2 different donors. *L. lactis* (pNZ8037) cells grown in the presence of 1.6 ng/ml nisin were used as a control and did not cause aggregation after 25 min incubation.

promote interactions with platelet receptors (such as FcγRIIa) leading to platelet activation and aggregation.

Mutants of *S. aureus* defective in the expression of surface proteins known to be capable of causing aggregation (ClfA, FnBPA, FnBPB, ClfB, SdrC, SdrD, SdrE and protein A) were tested in aggregation assays with PRP. Strain Newman was chosen for this study as it is naturally defective in fibronectin-binding protein (FnBP)-expression (Grundmeier *et al.*, 2004) making the construction of multiple mutants technically easier. *S. aureus* Newman wild type and its surface protein-deficient mutants were grown to both exponential and stationary phase. Washed cells were added to PRP samples and aggregation was monitored. As previously observed, wild-type *S. aureus* Newman stimulated rapid platelet aggregation (lag time 1.5 ± 0.2 min; n = 3) when grown to stationary phase, and caused aggregation with longer lag times (3.6 ± 0.2 min; n = 3) when grown to exponential phase (Figure 5.13). The absence of FnBPs from the surface of *S. aureus* Newman could explain the relatively long lag time to platelet aggregation for exponentially growing wild-type cells (Fitzgerald *et al.*, 2005). *S. aureus* Newman defective in ClfA and ClfB expression caused aggregation after 9.9 ± 4.2 min lag times (n = 3) when grown to exponential phase. Lag times of 19.7 ± 4.2 min (n = 3) were observed for *clfAclfB* mutants grown to stationary phase (Figure 5.13). Newman *clfAclfBsdrCsdrDsdrEspa* mutants caused platelet aggregation with lag times of 11.2 ± 5.6 min (exponential phase) and 23.9 ± 1 min (stationary phase) (Figure 5.13).

Exponentially-growing bacteria lacking the expression of ClfA, ClfB, SdrC, SdrD, SdrE and protein A caused aggregation after an average of 12 min incubation with platelets. The lag time to aggregation for the multiple mutant was doubled when cells were grown to stationary phase (approximately 24 min). This is in contrast to previous observations, where a mutant of strain Newman deficient in the same factors did not cause aggregation after 25 min incubation when grown to stationary phase (O'Brien *et al.*, 2002). This may reflect variability in platelet preparations from different donors. Another explanation may involve the length of time that bacterial preparations were kept in PBS before use in platelet aggregation assays. The progressive loss in the capability of *L. lactis* cells expressing ClfB to cause platelet aggregation has been observed in this laboratory with increasing lengths of time that cells were kept suspended in PBS prior to use (H. Miajlovic, personal communication). In this study, cells were washed in PBS and used immediately for aggregation assays. These data suggest that other surface components are expressed by *S. aureus* Newman (in addition to ClfA, ClfB, SdrC, SdrD, SdrE and protein A) that can cause platelet aggregation, and that

these are mainly expressed in the exponential growth phase. The expression of SdrC, SdrD, SdrE or protein A did not significantly contribute to platelet aggregation by exponentially growing bacteria. Expression of these factors was associated with reduced lag times for bacteria grown to stationary phase. Although it was not demonstrated here, it is suggested that slow aggregation by surface protein-deficient *S. aureus* mutants is the result of complement- and IgG-dependent interactions with platelets. The collective expression of a number of antigenic surface components allows sufficient levels of IgG binding which, in conjunction with complement-dependent processes, stimulate platelet activation and aggregation.

5.3 Discussion

A crucial involvement of the complement system in platelet aggregation by bacteria expressing ClfA PY was demonstrated here. Depletion of either complement or IgG from serum samples abolished aggregation, indicating multiple stimuli are involved in triggering aggregation, such as the ClfA-specific IgG binding to FcγRIIa. IgG and complement proteins apparently become bound to the surface of *L. lactis* ClfA PY+ where they are presented to the platelet surface, as cells that were incubated in plasma and then washed caused the aggregation of washed platelets suspended in fibrinogen. Furthermore, plasma-incubated cells triggered aggregation with significantly shorter lag times than cells in buffer alone, indicating that the characteristic lag phase (> 5 min) associated with platelet aggregation by *L. lactis* ClfA PY+ was due to the time taken to accumulate sufficient levels of plasma proteins on the bacterial surface. This could be due to the time taken for complement assembly, as has been suggested for complement-dependent platelet aggregation by *Strep. sanguis* NCTC 7863 (Ford *et al.*, 1996). Indeed, it was demonstrated that maximal C5b-9 formation on the surface of *L. lactis* ClfA PY+ cells occurred after 5 min incubation in plasma. However, the rate and extent of complement deposition did not differ from that observed for the non-aggregating *L. lactis* host strain. ELISA experiments demonstrated significantly higher level of antibodies recognizing *L. lactis* ClfA PY+ compared to the *L. lactis* host strain in plasma samples, which represented the presence of antibodies specific for ClfA. This suggests that the expression of a surface determinant by *L. lactis* against which there are significant levels of antibodies in plasma is sufficient to trigger the aggregation of resting platelets. Aggregation proceeds by a

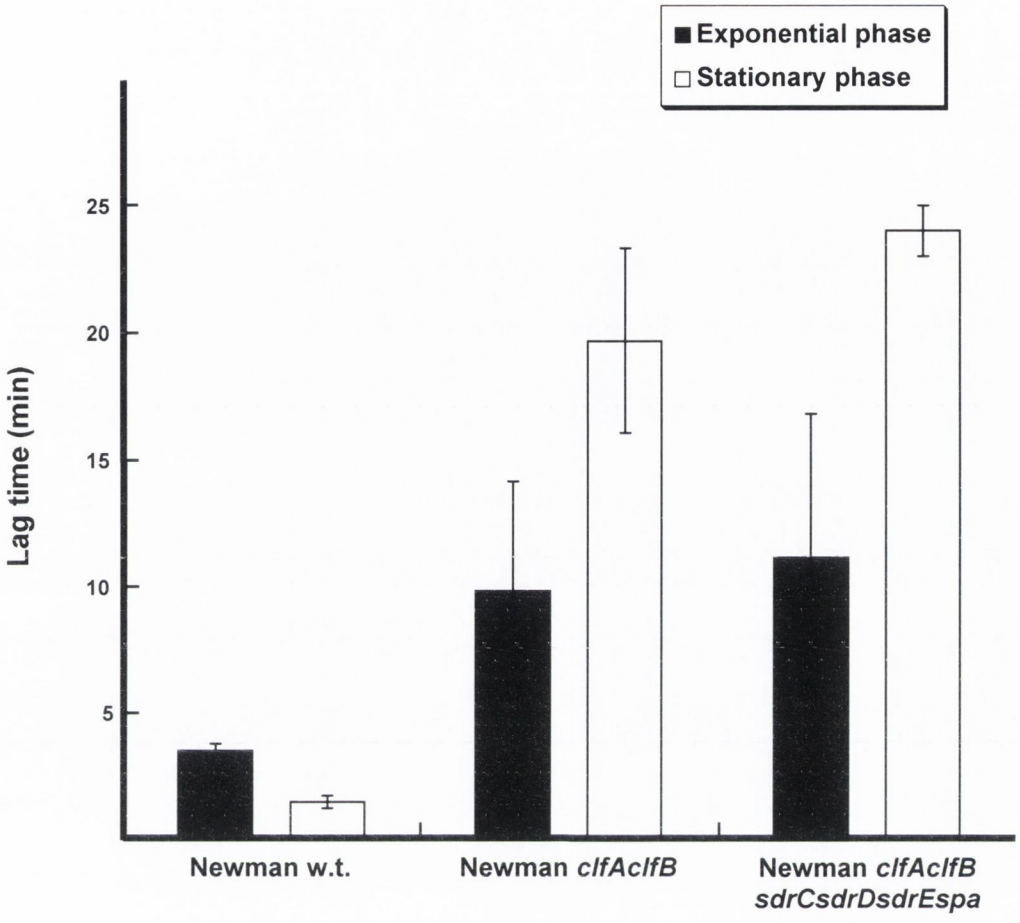


Figure 5.13. Platelet aggregation by surface protein-deficient mutants of *S. aureus* Newman

S. aureus strains were grown either to exponential phase or to stationary phase as indicated. Fifty μl washed cell suspension (OD_{600} 1.6) was added to 450 μl PRP and platelet aggregation was monitored by light transmission at 37°C. Data is presented as mean lag time ± SD to aggregation after incubation of bacteria with platelets. This experiment was performed 3 times using platelets from 3 different donors.

mechanism involving multiple stimuli, including surface protein-bound IgG binding to FcγRIIa and the interaction of cell-bound complement components with platelet receptors. Although plasma samples contained antibodies that recognized *L. lactis*, these did not seem to participate in platelet aggregation by *L. lactis* ClfA PY+.

The classical complement pathway (CCP) appeared to be the predominant activation mechanism leading to complement assembly and platelet aggregation by *L. lactis* ClfA PY+. This is consistent with the absolute requirement of ClfA-specific IgG which, in addition to linking ClfA PY and FcγRIIa as part of the platelet activation process, may serve as the activation stimulus for the CCP. Human serum depleted of C1q, the recognition molecule of the CCP, did not support aggregation of platelets suspended in fibrinogen by bacteria expressing ClfA PY. Also, depletion of C4 from PRP samples, specific for the classical and lectin complement pathways, abolished aggregation by *L. lactis* ClfA PY+, suggesting a role for the formation of the C4b2a C3 convertase complex. This is supported by the finding that C3 depletion from PRP samples abolished aggregation. It is likely that the C3 convertase generated through the CCP cleaves plasma C3 molecules, leading to the covalent attachment of C3b to the bacterial surface. Processed derivatives of C3b that remain surface-bound (iC3b / C3dg) may play a direct role in the interaction with platelets. This is supported by the finding that an antibody against the iC3b / C3dg receptor CR4 (CD11c) inhibited platelet aggregation by *L. lactis* ClfA PY+.

Alternatively, C3b may be involved in formation of the C5 convertase complex (C4b3b2a), resulting in C5 cleavage and the eventual formation of the C5b-9 terminal membrane-attack-complex on the cell surface. Depletion of C5 from PRP samples inhibited aggregation suggesting an important role downstream of C5 cleavage, possibly the formation of the C5b-9 complex on the cell surface. Formation of this complex was detected on bacterial cells upon incubation in plasma samples. However C5b-9 formation did not appear to be crucial for *L. lactis* ClfA PY+ to cause platelet aggregation, as full aggregation was observed in the presence of fibrinogen and C9-depleted human serum. The lag time to aggregation was delayed upon C9 depletion, suggesting that C5b-9 formation on the bacterial surface may enhance the interaction with platelets. C5b-9 assembly is regulated by vitronectin (serum spreading factor) (Johnson *et al.*, 1997). SC5b-7 is the complex formed upon binding of vitronectin (S) to the C5b-7 complex (Preissner *et al.*, 1989). This normally functions to regulate the assembly of mature terminal attack complexes by preventing polymerization of

C9 molecules (Johnson *et al.*, 1997), but binding of C8 and a low level of C9 to SC5b-7 results in the formation of SC5b-9 (Preissner *et al.*, 1989; Cooper, 1999). The interaction of SC5b-9 with activated GPIIb/IIIa has been demonstrated (Røger *et al.*, 1995). This may be the result of GPIIb/IIIa recognition of the RGD adhesion motif in vitronectin (Preissner and Jenne, 1991). It was suggested that complement-mediated platelet aggregation by *Strep. sanguis* NCTC 7863 was due to elevated levels of bacteria-bound SC5b-9 in comparison to a non-aggregating strain (Ford *et al.*, 1996). It is possible that vitronectin, in association with cell-bound C5b-7, C5b-8 or C5-b9, aids the interaction of *L. lactis* ClfA PY+ with resting platelets through engagement of integrins with vitronectin-binding activity (GPIIb/IIIa and/or $\alpha_v\beta_3$). This could be addressed in future studies using antibodies to deplete vitronectin from PRP samples.

The finding that full aggregation occurred in the presence of fibrinogen and factor B-depleted serum indicates that the alternative pathway of complement activation (ACP) is not essential. In concurrence with this, polyclonal anti-factor B antibodies failed to inhibit aggregation of PRP by *L. lactis* ClfA PY+. Serum adsorbed against the yeast polysaccharide zymosan, which activates complement through the ACP, could not support platelet aggregation by *L. lactis* ClfA PY+. While this may indicate an important role for the ACP in platelet aggregation by *L. lactis* ClfA PY+, it probably reflects an overall depletion of complement factors common to all complement pathways (C3, C5, C6-9). However, elimination of the alternative pathway through factor B-depletion was associated with prolonged lag phases before the onset of aggregation. The alternative pathway may serve to amplify the classical pathway through the C3b amplification loop. The generation of the classical pathway C3 convertase (C4b2a) would result in C3 cleavage and C3b deposition on the bacterial surface. Recognition of cell-bound C3b by factor B would allow formation of the alternative pathway C3 convertase (C3bBb). This would accelerate the overall level of C3 cleavage and the subsequent steps of the complement pathway. Increased levels of the C3-degradation products iC3b / C3dg on the bacterial cell could facilitate stronger interactions with CR4 on platelets, resulting in shorter lag times to activation and aggregation. Increased synthesis of C5b-9 on the bacterial surface may facilitate bacterial interaction with GPIIb/IIIa.

Platelet activation by *L. lactis* ClfA PY could possibly involve multiple interactions between several bacterial-bound complement components (such as iC3b/C3dg and SC5b-9) interacting with platelet receptors (CR4 and GPIIb/IIIa / $\alpha_v\beta_3$) in conjunction with the crucial

interaction of ClfA PY-bound IgG with FcγRIIa. This is consistent with the interaction of opsonized bacteria with phagocytes leading to phagocytosis. Engagement of C3 receptors on phagocytes by pathogen-bound C3b derivatives does not directly lead to phagocytosis, which requires a second, cellular activation stimulus such as engagement of Fc receptors by IgG (Cooper, 1999). It is also possible that complement-dependent platelet activation results in the activation-dependent surface expression of the gC1qR/p33 receptor (Peerschke *et al.*, 2003). This could allow the recognition of bacterial-bound C1q by platelets and thus enhance signaling and activation. The proposed interactions involved in complement-dependent platelet aggregation are depicted schematically in Figure 5.14.

A possible role for complement-mediated platelet aggregation by *S. aureus* is highlighted in aggregation assays using isogenic mutants defective in the expression of the major platelet activating factors (ClfA, FnBPA and FnBPB). While the ability to stimulate aggregation is impaired upon removal of these factors from the cell surface, it is not eliminated. Characteristic long lag times to aggregation (greater than 7 minutes) are normally observed for these mutant strains. SdrE was identified as a factor mediating platelet aggregation by stationary phase *S. aureus* Newman cells deficient in ClfA expression (O'Brien *et al.*, 2002). The mechanism by which SdrE stimulates platelet aggregation has been shown to involve IgG and complement proteins in a manner analogous to ClfA PY (M. Brennan, personal communication). It was shown here that SdrE and two related surface proteins (SdrC and SdrD) could independently trigger platelet aggregation, characterized by lag times greater than 5 min, if expressed at sufficiently high-levels on the surface of *L. lactis*. It is proposed that SdrC and SdrD trigger platelet aggregation in a complement- and IgG-dependent manner, although this was not demonstrated. Complement-dependent platelet aggregation appears to be a general mechanism, in that the bacterial factors mediating such aggregation do not have to possess ligand-binding activity or a specified function. Rather, it seems that the expression of *S. aureus* surface proteins in *L. lactis* is sufficient to trigger platelet aggregation, given that the protein is expressed at high-levels on the cell surface and that antibodies to that protein are present in plasma. Binding of specific-IgG to the expressed surface protein which, along with complement proteins assembled on the bacterial surface, allow interaction with multiple receptors on the platelet leading to activation and aggregation.

It is postulated that *S. aureus* mutants defective in ClfA, FnBPA and FnBPB expression trigger slow platelet aggregation through complement-dependent processes. The collective expression of a number of antigenic surface components allows sufficient levels of

IgG binding which, in conjunction with time-dependent complement-assembly, stimulate platelet activation and aggregation. Surface protein-deficient mutants of *S. aureus* Newman triggered faster aggregation when grown to exponential phase than when grown to stationary phase, which may reflect the general notion that the expression of *S. aureus* surface proteins is maximal in the exponential growth phase (Lowy, 1998). Increased surface protein expression may permit higher levels of IgG binding to bacteria, thus facilitating a stronger interaction with platelets. *S. aureus* has the potential to express 21 LPXTG-surface anchored proteins (Roche *et al.*, 2003) so systematic elimination of all surface proteins by allelic replacement to determine those that are involved in platelet aggregation by *S. aureus* is not technically feasible.

More work is needed to definitively identify the specific complement components required to stimulate aggregation, and the platelet receptors involved in recognizing these. It remains to be demonstrated that slow aggregation by *S. aureus* mutants is complement-dependent. Such studies are needed to fully understand the interaction of *S. aureus* with platelets if novel therapeutics are to be developed to inhibit such interactions.

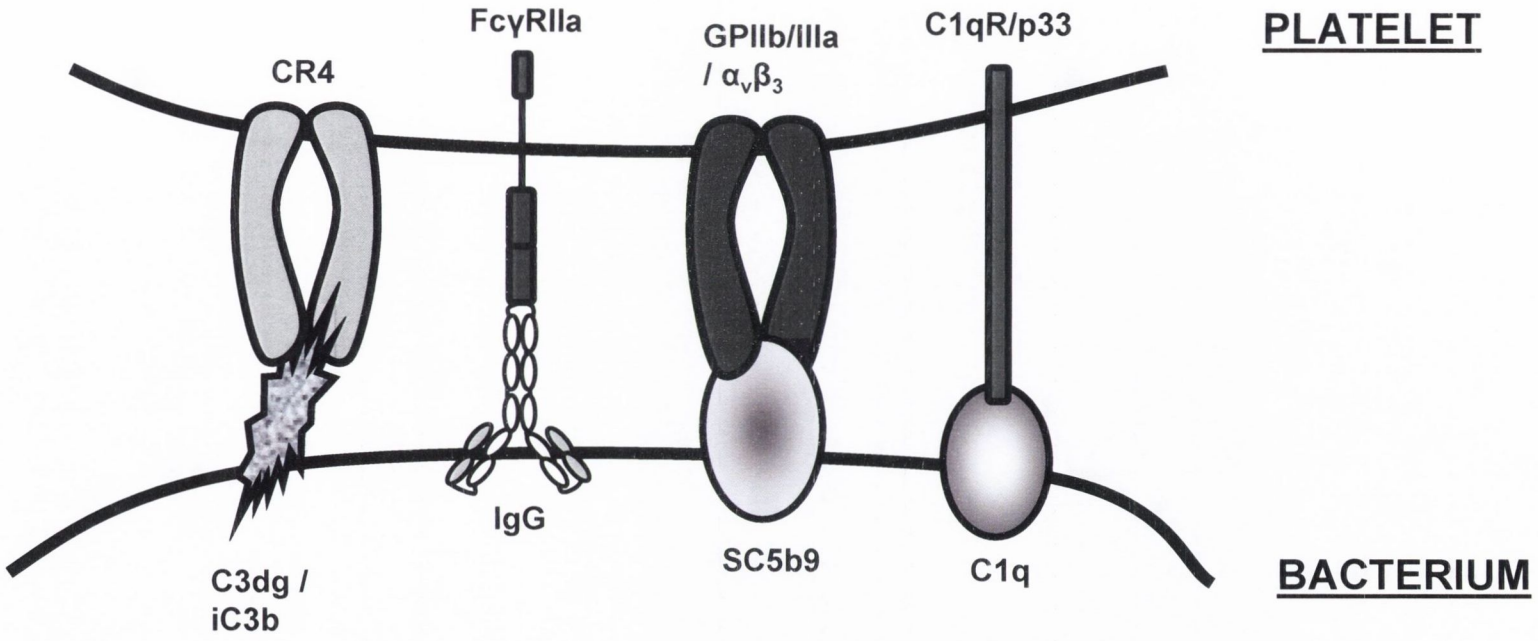


Figure 5.14. Putative interactions between bacteria and platelets leading to complement-dependent platelet aggregation

Specific IgG binding to *S. aureus* adhesins is required for a crucial interaction with FcγRIIa on platelets. Degradation products of covalently-bound C3b (iC3b / C3dg) may be involved in an interaction with CR4 (CD11c) on platelets. The C5b-9 complex in association with vitronectin could possibly be recognized by platelet vitronectin receptors (GPIIb/IIIa or $\alpha_v\beta_3$). Cell-bound C1q could serve as a ligand for the platelet C1q receptor C1qR/p33.

Chapter 6

Discussion

6.1 Discussion

It is remarkable that *S. aureus* has the capacity to cause a wide range of diverse, and sometimes fatal, clinical conditions given that it is a normal human commensal. This reflects its ability to produce a wealth of virulence factors with diverse functions that collectively contribute to the progression of different infections. A serious complication of *S. aureus* bacteraemic infections is infective endocarditis (IE). *S. aureus* is the most common cause of IE, which is often fatal even with aggressive antibiotic therapy (Moreillon and Que, 2004; Fowler *et al.*, 2005). The emergence of multiply resistant *S. aureus* strains that cannot be treated with clinically available antibiotics will no doubt result in increasing mortality rates in the near future. Alternative, non-antibiotic based therapies for treating IE caused by *S. aureus* are required. The development of novel therapeutics requires a detailed understanding of the pathogenesis of this infection.

IE is characterized by the formation of macroscopic vegetations on heart valves consisting of bacteria, fibrin and aggregated platelets (Mylonakis and Calderwood, 2001). These vegetations provide a focus of infection, withstanding the shear forces of flowing arterial blood allowing the bacteria to settle at the site of infection. It may also shield the bacteria against the host immune response and antibiotics. Reseeding of bacteria into the bloodstream can occur through septic embolisms. The interaction of pathogenic bacteria with platelets is felt to be an important step in the initiation of IE. Bacteria that have become attached either to nascent developing thrombi or constituents of the extracellular matrix that become exposed upon valvular damage may capture circulating platelets from the bloodstream through specific surface components that recognise platelet receptors. This binding can then result in signalling events causing platelet activation and aggregation, leading to the development of an infected thrombus. Similarly, the interactions of circulating bacteria and platelets in the bloodstream may result in the formation of micro-aggregates that subsequently become deposited on the endocardium surface, which develop into mature vegetations. Bacteria capable of activating platelets leading to their aggregation *in vitro* are more virulent in experimental endocarditis that non-aggregating strains (Herzberg *et al.*, 1992).

The mechanisms by which various *Streptococcus* species (another major cause of IE) interact with platelets leading to their activation and aggregation have been studied intensely. In contrast, relatively little is known about the mechanisms employed by *S. aureus* to cause platelet activation. It was previously determined that ClfA was the predominant factor

mediating platelet activation by *S. aureus* cells grown to stationary phase (O'Brien *et al.*, 2002a). Further investigations in this laboratory demonstrated that the fibronectin-binding proteins FnBPA and FnBPB were the major mediators of platelet activation by exponentially growing *S. aureus* (J.R. Fitzgerald, personal communication). During the course of this study, an article was published demonstrating a role for FnBPA in promoting platelet aggregation, but the molecular mechanisms involved were not elucidated (Heilmann *et al.*, 2004). Both ClfA and FnBPA are ubiquitous amongst clinical invasive isolates (100% and 97% prevalence respectively; Peacock *et al.*, 2002), and may be the major platelet activating factors expressed by invasive *S. aureus* strains.

The aim of this study was to determine the nature of the interaction(s) between bacteria expressing ClfA or FnBPA and human platelets that leads to their activation and aggregation. Surface proteins were expressed in the surrogate host *L. lactis*, which does not naturally activate platelets, to study the role of each factor in isolation. Isogenic *S. aureus* strains defective in the surface protein of interest were also used to verify the findings of studies performed with *L. lactis*. By combining a panel of function-blocking antibodies to individual platelet receptors with aggregation assays where washed platelets were supplemented with purified plasma components, the molecular interactions between bacteria expressing ClfA and human platelets leading to their activation were elucidated. Preliminary work in this laboratory indicated that bacteria expressing FnBPA stimulated platelet activation in an analogous manner to ClfA (J.R. Fitzgerald, personal communication). A number of experiments were performed to clarify the mechanism of activation by bacteria expressing FnBPA. Two distinct, independent mechanisms of platelet activation promoted by bacteria expressing FnBPA were elucidated, which closely parallel the model suggested for ClfA. A general mechanism by which *S. aureus* cells induce platelet activation is proposed, which is applicable to both stages of the growth cycle.

The expression of surface proteins that are capable of binding a platelet-reactive factor from blood, such as fibrinogen (ClfA, FnBPA) or fibronectin (FnBPA), promote bacterial-platelet binding through specific plasma protein bridging to GPIIb/IIIa on resting platelets. Although GPIIb/IIIa is in the low-affinity "non-adhesive" state on resting platelets and cannot bind its soluble ligands, it can bind to fibrinogen and fibronectin immobilized on surfaces (Savage *et al.*, 1996; Shattil and Newman, 2004). Presumably, bacteria expressing FnBPA and ClfA rapidly become coated with plasma proteins through their high-affinity interactions and behave as surfaces promoting efficient platelet adhesion. This is followed by recognition

of specific antibodies bound to the activating bacterial protein by the platelet low-affinity IgG Fc receptor FcγRIIa. This triggers signalling events resulting in platelet activation. Conformational changes in GPIIb/IIIa increase its affinity for fibrinogen, and fibrinogen-dependent cross-linking of GPIIb/IIIa receptors on adjacent platelets results in the formation of platelet aggregates. The rapidity associated with platelet activation and aggregation promoted by bacteria expressing ClfA and FnBPA likely reflects high-affinity plasma protein-dependent adhesion to platelet GPIIb/IIIa, thus allowing efficient interactions of bound IgG with platelet FcγRIIa causing activation.

Isogenic *S. aureus* mutants lacking the major pro-aggregatory surface proteins (ClfA, FnBPA and FnBPB) still caused activation, but with significantly longer times (> 8 min) compared to the rapid activation promoted by ClfA, FnBPA or FnBPB (1 – 2 min lag time). Such mutants lack fibrinogen and fibronectin binding activity, suggesting a different activation mechanism is promoted by these strains. One such factor mediating 'slow' activation is SdrE (O'Brien *et al.*, 2002a). It was demonstrated by our collaborators in the Royal College of Surgeons that SdrE-mediated platelet activation is dependent on IgG and complement deposition on the bacterial surface (M. Brennan, personal communication). A complement- and IgG-dependent mechanism promoted by a ClfA mutant defective in fibrinogen-binding was identified here. In addition, SdrC and SdrD were identified as factors that stimulate platelet aggregation with long (> 7 min) lag times, which may also be complement dependent. The precise nature of the interactions by which bacteria coated with complement proteins and IgG trigger platelet activation are not entirely clear, but recognition of specific IgG bound to *S. aureus* antigens by platelet FcγRIIa is crucial, as it is for the rapid activation promoted by ClfA and FnBPA. It is suggested that slow activation promoted by *S. aureus* mutants lacking ClfA, FnBPA and FnBPB proceeds through complement- and specific IgG-dependent processes, yet this remains to be determined. The collective expression of a number of antigenic surface components is sufficient to permit high-level binding of specific antibodies present in plasma which, in conjunction with assembled complement factors, can interact with platelets leading to activation. The long lag times to platelet aggregation may reflect the time taken for complement assembly on the bacterial surface. Interestingly, the lag time to aggregation for the multiple surface protein-deficient mutants of *S. aureus* was growth phase dependent, and was approximately double for stationary phase cells compared to exponentially growing cells. This may reflect either (1) increased surface protein expression in the

exponential growth phase compared to stationary phase, resulting in increased levels of IgG binding promoting stronger interactions with the platelet or (2) masking of antigenic surface determinants by capsular polysaccharide expressed in stationary phase, somewhat inhibiting recognition of bound IgG and complement proteins by platelet receptors. These issues need to be addressed with further experimental work.

The proposed interactions between *S. aureus* and platelets that result in platelet activation are highly similar to mechanisms described for various species of streptococci. *Strep. sanguis* strains that express the serine-rich glycoprotein SrpA adhesin can bind directly to the GPIb complex on platelets which, in conjunction with FcγRIIa, is required for platelet activation (Kerrigan *et al.*, 2002; Plummer *et al.*, 2005). *Strep. sanguis* strains that don't express an SrpA-like protein do not adhere directly to platelets and cause platelet aggregation with prolonged lag times (Kerrigan *et al.*, 2002; Plummer *et al.*, 2005). This was due to a complement- and IgG-dependent process, requiring FcγRIIa (Ford *et al.*, 1996, 1997). Signalling events leading to platelet activation caused by *Strep. sanguis* strains were induced by the interaction with FcγRIIa (Pampolina and McNicol, 2005). Fibrinogen-binding surface proteins expressed by some streptococci mediate platelet activation in a virtually identical manner to ClfA and the FnBPA A domain. The FbsA surface protein of *Strep. agalactiae* induced activation in a fibrinogen- and IgG-dependent manner through GPIIb/IIIa and FcγRIIa (Pietrocola *et al.*, 2005). Platelet activation by *Strep. pyogenes* M proteins proceeded through a fibrinogen bridge to GPIIb/IIIa and antigen bound anti-M protein IgG interacting with FcγRIIa (Sjöbring *et al.*, 2002).

It appears that vascular pathogens utilize a common mechanism for inducing platelet activation. Initially, bacterial surface components mediate adhesion to platelets. The mechanisms by which this occurs are variable between different species depending on the profile of expressed surface proteins. It generally involves either direct bacterial binding to a platelet receptor or binding of a platelet-reactive plasma protein that acts as a bridging molecule to its cognate platelet receptor. Regardless of the nature of the adhesion event, it appears to be required for an interaction of IgG bound to bacterial antigens with platelet Fc receptor FcγRIIa, which results in intracellular signals leading to activation.

One major drawback of *in vitro* platelet aggregation assays is that they are performed in relatively static conditions, which neglect the role of hydrodynamic shear forces experienced in the vasculature *in vivo*. Shear forces depend both on the rate of blood flow and

the diameter of the blood vessel. High shear forces are experienced in rapidly flowing blood in narrow blood vessels such as small arteries and arterioles. Lower shear forces occur in veins and around the heart valves. Shear forces regulate the interactions of some platelet receptors with their ligands, such as the interaction of GPIb with von Willebrand factor which only occurs under high shear forces (Jackson *et al.*, 2003). This raises the possibility that *S. aureus* interacts with platelets *in vivo* in a different manner to that observed in aggregation assays *in vitro*. Very few studies have examined platelet activation by *S. aureus* under shear forces experienced in the circulatory system, and none have examined activation under shear caused by exponentially growing cells. ClfA was the dominant factor causing activation under a wide range of shear forces when *S. aureus* cells were mixed with platelets in suspension, and this was dependent on GPIIb/IIIa (Pawar *et al.*, 2004). Immobilized *S. aureus* supported thrombus development when whole blood was perfused over the cells under a range of shear forces (Sjöbring *et al.*, 2002; S. Kerrigan, personal communication). In both instances, maximal aggregation occurred at lower shear forces representative of the conditions found in heart valves. Importantly, FcγRIIa was crucial for *S. aureus* induced thrombus formation in flowing blood (Sjöbring *et al.*, 2002). This strongly supports the mechanisms of platelet activation by *S. aureus* surface proteins suggested here, and indicates that thrombus development *in vivo* induced by *S. aureus* probably proceeds through the same mechanisms.

Platelet activation by *S. aureus* (and other bacteria such as streptococci) is dependent on the presence of IgG in plasma that is specific for antigenic surface expressed proteins. This may be considered as another immune subversion mechanism employed by *S. aureus* to hijack the host immune response to further its pathogenic potential. Both *S. aureus* and oral streptococci such as *Strep. sanguis* are normal human commensals, so it is not unexpected that antibodies against these bacteria are present in persons sera. Both carriers and non-carriers contain variable, low-level antibody titres against a range of *S. aureus* proteins (Colque-Navarro *et al.*, 2000; Dryla *et al.*, 2005), which probably reflects exposure to *S. aureus* at some stage in their lifetimes. Platelets from virtually all individuals tested in this study produced highly reproducible activation responses upon exposure to bacteria, which indicates that the low-level antibodies present in sera from normal individuals are sufficient to drive activation.

For ClfA- or FnBPA-promoted activation, IgG against the protein interacting with the resting platelet was required for maximal responses. Depletion of IgG recognizing the *S. aureus* surface protein engaging the resting platelet dramatically reduced the level of

activation observed. It was observed that platelet activation stimulated by *Strep. pyogenes* M-proteins was drastically reduced in blood from persons with naturally low titres of anti-M protein antibody (Sjöbring *et al.*, 2002). This suggests that individuals who have low serum antibody titres against vascular pathogens may be protected to some degree from developing infections dependent on bacterial-mediated platelet activation like infective endocarditis. However such individuals appear to be in the minority. It may be that IgG bound to the protein engaging the platelet is more efficiently recognized by FcγRIIa than IgG bound to other surface components. Alternatively, the surface protein engaging the resting platelet is likely a major antigenic component on the bacterial cell surface, and removal of IgG recognizing that protein will result in an overall reduction in IgG binding to the cell surface, compromising the interaction with FcγRIIa. Further experimental work is needed to clarify this.

The utilization of specific IgG as a pathogenic mechanism for promoting platelet activation has clear implications for the development of targeted immunotherapies for treating infective endocarditis caused by *S. aureus*. There is an obvious danger of increasing the risk of or exacerbating disease by promoting platelet activation through increasing plasma levels of specific anti-staphylococcal IgG. Increased plasma anti-staphylococcal antibody titres generated by immunization of rabbits with whole *S. aureus* provided no protection against subsequent challenge in experimental endocarditis studies (Greenberg *et al.*, 1987). However, a polyclonal IgG preparation containing elevated levels of anti-ClfA antibodies was effective in eliminating *S. aureus* from infected vegetations when used in combination with vancomycin (Vernachio *et al.*, 2003). This IgG preparation was shown to block fibrinogen-binding by *S. aureus in vitro* (Vernachio *et al.*, 2003). If an immunotherapeutic approach was to be developed to block *S. aureus*-platelet interactions, it should exhibit function-blocking activity (i.e inhibit ligand binding) so as to prevent plasma-protein dependent adhesion of *S. aureus* to platelets, and thus reduce platelet activation and thrombus development. This should target both ClfA and the fibronectin-binding proteins to be fully effective. However, there are a number of complications that would arise from such an approach. Firstly, the immune response against the fibronectin-binding domains of FnBPs is primarily directed at the neo-epitopes formed upon binding of these domains to the type I modules of fibronectin (Casolini *et al.*, 1998). Therefore, the generation of function-blocking antibodies against this domain would be extremely difficult. In addition, these studies have highlighted antigenic diversity in

the A domains of FnBPA and FnBPB. This may be an immune evasion mechanism, and the development of immunotherapies against the A domains of these proteins would have to take into account this antigenic diversity in the design of a product that recognizes FnBPs expressed by the majority of clinical strains. Finally, it has been shown here that *S. aureus* mutants defective in ClfA and FnBPs still cause platelet activation, postulated to involve complement proteins and IgG, although this mechanism is noticeably less efficient than that promoted by wild-type cells. Nevertheless, it may play an accessory role *in vivo* and should be considered in the design of novel therapeutic strategies.

Perhaps a more attractive strategy would be the development of anti-platelet therapies to prevent bacterial-mediated platelet activation, thus possibly reducing the incidence of endocarditis in high-risk groups, such as hemodialysis patients and patients with intravascular devices. Aspirin, an inhibitor of platelet function through its inhibition of cyclooxygenase activity, has been shown to reduce *S. aureus* titres within vegetations in experimental endocarditis (Nicolau *et al.*, 1993; Kupferwasser *et al.*, 1999). However, this effect may be pleiotrophic, as salicylic acid (the *in vivo* metabolite of aspirin) has been demonstrated to downregulate FnBPA and FnBPB expression *in vitro* (Kupferwasser *et al.*, 2003), which may reduce platelet activation and valve colonization by *S. aureus*. Such strategies are not ideal, as they may lead to secondary problems resulting from the inhibition of platelet function such as excess bleeding. The identification of FcγRIIa as the major platelet receptor mediating activation by *S. aureus* is consistent with its role in platelet activation by other endovascular pathogens. The crystal structure of FcγRIIa in complex the Fc portion of IgG is known (Maxwell *et al.*, 1999), which may be used in the design of small molecule inhibitors that block this interaction. This may provide a specific mechanism to block platelet activation by *S. aureus* and other vascular pathogens without affecting normal platelet function. Molecules that have the potential to fulfil this function need to be characterized both *in vitro* and in appropriate animal infection models. Such therapeutic molecules which could be used in future treatment of infective endocarditis, possibly in combination with classical antimicrobial therapy, to reduce the incidence and severity of infective endocarditis caused by a broad range of pathogenic bacteria.

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