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The interaction of clumping factor B and iron regulated surface determinants of *Staphylococcus aureus* with platelets

A thesis submitted for the degree of Doctor in Philosophy

by

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September 2008

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Summary

Clumping factor B (ClfB) of *Staphylococcus aureus* is a multi-functional surface protein which binds to cytokeratin 10 and fibrinogen. Cytokeratin 10 is found on the surface of desquamated epithelial cells of the anterior nares where it provides a ligand for *S. aureus* cells expressing ClfB. ClfB contributes to nasal colonisation by *S. aureus*, which is a risk factor for infection. *S. aureus* surface proteins that bind to the blood glycoprotein fibrinogen can stimulate platelet aggregation by a fibrinogen-dependent process. Aggregation of platelets by bacteria is believed to contribute to the development of infective endocarditis of which *S. aureus* is now acknowledged to be the leading cause.

Non-fibrinogen binding mutants of ClfB were isolated based on the results of *in-silico* docking of a fibrinogen α-chain peptide into the crystal structure of ClfB. Alanine substituted variants of ClfB were expressed by *Lactococcus lactis* and their ability to promote adhesion to immobilised cytokeratin 10 and fibrinogen was assessed. Two variants of ClfB, ClfB Q235A and ClfB N526A were defective in adherence to immobilized fibrinogen and cytokeratin 10, suggesting that both ligands bind to the same or overlapping regions. Both residues are located in the putative, hydrophobic binding trench of ClfB and residue N526A is predicted to be involved in structural rearrangements that accompany ligand binding by the "dock lock latch" mechanism. Studies carried out in this laboratory identified the αC-connector of the fibrinogen α-chain as the binding site for ClfB. This region is composed of 13 tandem repeats which share a high degree of sequence similarity. Proline substitutions were introduced into the putative binding site within tandem repeat 5 to mimic the presence of proline in other repeats of the αC-connector. Introduction of a proline residue in the centre of repeat 5 resulted in an α-chain that was unable to support ClfB binding. This conclusively shows that repeat 5 is the only site in the α-chain that ClfB binds to.

The non-fibrinogen binding mutant of ClfB, ClfB Q235A was expressed by *L. lactis* and used to determine the necessity for fibrinogen in platelet adhesion and aggregation mediated by ClfB. Washed and gel filtered platelets were also used to determine the requirement for plasma proteins. *L. lactis* expressing ClfB Q235A was unable to promote fibrinogen-dependent adhesion to platelets, however it stimulated aggregation in platelet rich plasma without a significant increase in lag time. Fibrinogen was required to promote adhesion of platelets to bacteria expressing wild-type ClfB while aggregation required fibrinogen and specific anti-ClfB antibodies. Adhesion and

aggregation were inhibited by antibodies to platelet receptor GPIIb/IIIa. It seems that ClfB causes platelet aggregation by a fibrinogen-dependent mechanism. The non-fibrinogen binding ClfB mutant stimulated platelet aggregation in a complement-dependent manner which required anti-ClfB antibodies.

Bacteria circulating in the blood stream are exposed to iron-limiting conditions. Iron is sequestered in complexes with haemoproteins and is unavailable to pathogens. When iron is scarce, *S. aureus* expresses iron regulated surface determinants (Isd) which together comprise a system that captures haem from haemoproteins, transports haem across the bacterial envelope and then releases iron. The ability of *S. aureus* grown in an iron-deficient medium to promote adhesion to and activation of platelets was assessed. Strains grown in such conditions which lacked the known platelet activators ClfA and ClfB mediated direct adhesion to platelets and subsequent aggregation of platelets. The addition of iron to the growth medium inhibited expression of Isd proteins and was found to eliminate direct platelet adhesion and subsequent aggregation. Strains of *S. aureus* defective in the surface exposed Isd proteins IsdA, IsdB and IsdH were constructed to determine which mediates interaction with platelets. Eliminating IsdB from *S. aureus* cells grown in iron-restricted conditions in combination with ClfA and ClfB resulted in strains that were unable to promote adhesion to and activation of platelets. IsdA or IsdH in combination with ClfA and ClfB could be eliminated without affecting adhesion or aggregation of platelets. IsdB mediated adhesion to platelets could be inhibited with antibodies to platelet receptor GPIIb/IIIa. These results indicate that *S. aureus* grown in iron-restricted conditions which mimic conditions *in vivo* mediate platelet activation by a novel mechanism which involves direct binding of IsdB to the resting form of platelet integrin GPIIb/IIIa. Expression studies showed that in iron and nutrient-limiting conditions ClfA was expressed at low levels by *S. aureus* which may not be sufficient to stimulate platelet activation. ClfB was continuously expressed in these conditions and may contribute to interaction with platelets.

Publications

Miajlovic, H., Loughman, A., Brennan, M., Cox, D. & Foster, T. J. (2007). Both complement- and fibrinogen-dependent mechanisms contribute to platelet aggregation mediated by *Staphylococcus aureus* clumping factor B. *Infect Immun* **75**, 3335-3343.

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Wertheim, H. F., Walsh, E., Choudhurry, R., Melles, D. C., Boelens, H. A., Miajlovic, H., Verbrugh, H. A., Foster, T. & van Belkum, A. (2008). Key role for clumping factor B in *Staphylococcus aureus* nasal colonization of humans. *PLoS Med* **5**, e17.

Contents

Declaration ii

Acknowledgements iii

Summary iv

Publications vi

List of tables xiii

List of figures xiv

Key to abbreviations xviii

Chapter 1 Introduction

1.1 Biology of the staphylococci..... 1

1.1.1 Classification and identification..... 1

1.1.2 Cell-wall components..... 1

1.2 Nasal colonization and disease..... 2

1.3 Virulence factors..... 4

1.3.1 Capsule..... 4

1.3.2 Surface proteins..... 5

1.3.2.1 Sortase-mediated anchoring of cell-wall associated proteins..... 5

1.3.2.2 Fibronectin-binding surface proteins..... 6

1.3.2.3 Fibrinogen binding surface proteins..... 8

1.3.2.3.1 Clumping factor A..... 8

1.3.2.3.2 Clumping factor B..... 10

1.3.2.3.3 The dock, lock and latch model for fibrinogen binding... 11

1.3.2.4 Protein A..... 12

1.3.2.5 Collagen-binding protein..... 13

1.3.2.6 Iron regulated surface determinants..... 13

1.3.2.6.1 NEAT domains of Isd proteins..... 14

1.3.2.6.2 Haem transfer pathway..... 15

1.3.2.6.3 Other functions of Isd proteins..... 16

1.3.2.7 Serine-aspartate repeat (Sdr) and novel *S. aureus* surface (Sas) proteins..... 17

1.3.3 Secreted virulence factors..... 17

1.4 Regulation of virulence factors..... 18

1.4.1 Two-component regulatory systems..... 18
1.4.1.1 The accessory gene regulator..... 19
1.4.1.2 The haem sensor system..... 20
1.4.2 Transcription factors..... 20
1.4.2.1 SarA family..... 20
1.4.2.1.1 Staphylococcal accessory regulator..... 21
1.4.2.1.2 Repressor of toxins..... 21
1.4.2.1.3 The multiple gene regulator..... 22
1.4.2.1.4 Ferric uptake regulator..... 22
1.4.3 Sigma Factors..... 23
1.4.3.1 Sigma factor A..... 23
1.4.3.2 Sigma factor B..... 23
1.4.3.3 Sigma factor H..... 24
1.5 Immune evasion by *S. aureus*..... 24
1.5.1 Survival on skin..... 24
1.5.2 Inhibition of neutrophil chemotaxis..... 25
1.5.3 Toxins that kill leukocytes..... 26
1.5.4 Resistance to opsonophagocytosis..... 26
1.5.5 The complement system..... 27
1.5.6 Inactivation of complement by *S. aureus*..... 28
1.5.7 Survival of *S. aureus* in neutrophil phagosomes..... 29
1.5.8 Toxin superantigens..... 29
1.5.9 Major histocompatibility complex class II-analog protein..... 30
1.5.10 Lack of available iron..... 30
1.6 Bacteria, platelets and cardiovascular disease..... 32
1.6.1 Biology and function of platelets..... 33
1.6.2 Bacterial interaction with platelets..... 34
1.6.2.1 The interaction of *S. aureus* with platelets..... 35
1.6.2.2 The interaction of streptococci with platelets..... 37
1.7 Rationale for this study..... 39
1.7.1 Aims and objectives..... 40

Chapter 2 Materials and Methods

2.1 Bacterial strains and growth conditions..... 41
2.1.1 Growth of bacteria in iron and nutrient limiting conditions..... 41
2.1.2 Growth of bacteria in iron limiting conditions..... 41
2.1.3 Growth and induction of *L. lactis* strains carrying pNZ8037 derivatives. 41
2.2 Plasmids..... 42
2.3 DNA manipulations..... 42
2.3.1 Preparation of plasmid and genomic DNA..... 42
2.3.2 Polymerase chain reaction..... 42
2.3.3 Site-directed mutagenesis..... 43
2.4 Transformation..... 43
2.4.1 Preparation and electroporation of *S. aureus* RN4220..... 43
2.4.2 Preparation and electroporation of *S. epidermidis*..... 43
2.4.3 Preparation and electroporation of *L. lactis*..... 44
2.5 Phage transduction..... 44
2.6 Allelic replacement mutagenesis..... 45
2.7 Preparation of solubilised cell wall proteins..... 46
2.8 SDS-PAGE..... 46
2.9 Western immunoblotting..... 46
2.10 Whole cell immunoblots 47
2.11 Coagulase clumping test..... 47
2.12 Fibrinogen culmping test..... 47
2.13 Docking of GTWNP peptide into the 3D crystal structure of ClfB..... 47
2.14 Purification of His-tagged recombinant proteins by immobilized metal chelate affinity chromatography..... 48
2.15 Adherence of bacteria to immobilized fibrinogen and cytokeratin 10..... 49
2.16 Binding of recombinant proteins to fibrinogen..... 49
2.17 Preparation of platelet rich plasma..... 50
2.17.1 Preparation of washed platelets..... 50
2.17.2 Preparation of gel filtered platelets..... 50
2.18 Removal of contaminating IgG from commercial supplies of fibrinogen..... 50
2.18.1 Removal of IgG from human serum..... 51
2.19 Depletion of ClfB-specific antibodies from pooled human IgG..... 51

2.20 Determination of anti-clfB antibody levels in IgG samples by ELISA..... 51
2.20.1 Determination of contaminating IgG levels in fibrinogen..... 52
2.21 Heat inactivation of complement proteins in human serum 52
2.21.1 Adsorption of complement from serum by zymosan treatment..... 52
2.22 Platelet adhesion assay..... 52
2.23 Platelet aggregation..... 53
2.24 Statistical analysis..... 53

Chapter 3 Interaction of Clumping factor B of *Staphylococcus aureus* with the α-chain of fibrinogen

3.1 Introduction..... 54
3.2 Results..... 57
3.2.1 Site directed mutagenesis of DNA encoding residues in the putative binding trench of ClfB..... 57
3.2.2 Fibrinogen binding by alanine substituted variants of ClfB..... 58
3.2.3 Docking of fibrinogen peptide GTWNP into the 3D crystal structure of ClfB..... 58
3.2.4 Site-directed mutagesesis of pNZ8037 *clfB* and screening of potential ClfB mutants..... 59
3.2.5 Expression of ClfB mutants N526A and Q235A by *L. lactis*..... 60
3.2.6 Adherance of *L. lactis* expressing ClfB Q235A or ClfB N526A to fibrinogen and cytokeratin 10..... 61
3.2.7 Mutagenesis of codons within tandem repeat 5 of the fibrinogen αC-connector..... 61
3.2.8 Expression of fibrinogen αC-connector..... 62
3.2.9 ClfB-promoted bacterial adherence to Aα-S317P and Aα-T322P..... 62
3.3 Discussion..... 64

Chapter 4 Mechanism of platelet activation mediated by Clumping factor B of *Staphylococcus aureus*

4.1 Introduction..... 69

4.2 Results..... 72
4.2.1 Construction of *S. aureus* strains Newman *coa clfA* and Newman *coa clfA clfB*..... 72
4.2.2 Contribution of ClfB to strain Newman-mediated platelet aggregation... 73
4.2.3 Comparisson of fibrinogen binding by ClfB and ClfA expressed from *L. lactis*..... 73
4.2.4 Effect of ClfB-expression levels and bacterial cell density on platelet aggregation in PRP..... 74
4.2.5 ClfB-mediated platelet adhesion is fibrinogen dependent..... 75
4.2.6 Inhibition of ClfB-mediated platelet activation and adhesion using antibodies to platelet receptors..... 76
4.2.7 Fibrinogen and ClfB-specific antibodies are required for ClfB-mediated platelet activation and aggregation..... 76
4.2.8 Complement fixation is required for platelet aggregation mediated by ClfB Q235A..... 78
4.2.9 Specific anti-ClfB antibody is required for platelet aggregation mediated by ClfB Q235A..... 79
4.3 Discussion..... 80

Chapter 5 Interaction of iron regulated surface determinants of *Staphylococcus aureus* with platelets

5.1 Introduction..... 84
5.2 Results..... 86
5.2.1 Platelet adhesion and aggregation mediated by *S. aureus* strain Newman grown in iron limiting conditions..... 86
5.2.2 Aggregation of platelets by Newman *clfAclfB* grown in RPMI is not dependent on complement..... 87
5.2.3 Inhibition of platelet activation and adhesion mediated by RPMI grown Newman *clfA clfB* using antibodies to platelet receptors..... 88
5.2.4 Allelic replacement mutagenesis of *isdB*..... 89
5.2.4.1 Construction of plasmids pBlueΔ*isdB* and pHM1..... 89
5.2.4.2 Allelic replacement of *isdB* in *S. aureus*..... 90
5.2.5 Construction of *S. aureus* strains for platelet studies..... 91

5.2.5.1 Newman *clfA clfB isdA*..... 91
5.2.5.2 Newman *clfA clfB isdB*..... 92
5.2.5.3 Newman *clfA clfB isdA isdB*..... 92
5.2.5.4 Newman *clfA clfB isdH*..... 93
5.2.6 Platelet adhesion and aggregation mediated by Newman *isd* mutants..... 93
5.2.7 Plasma proteins required for platelet activation mediated by IsdB..... 93
5.2.8 Construction of plasmids pCU1 *isdB* and pCU1 *isdAB*..... 94
5.2.9 Expression of IsdA and IsdB from plasmid pCU1 and interaction with platelets..... 94
5.2.10 Expression of IsdB from *S. epidermidis*..... 95
5.3 Discussion..... 97

Chapter 6 Expression of *Staphylococcus aureus* clumping factors A and B under iron and nutrient limiting conditions

6.1 Introduction..... 102
6.2 Results..... 104
6.2.1 Growth of *S. aureus* in iron and nutrient limiting conditions..... 104
6.2.2 Expression of ClfA from RPMI-grown *S. aureus* strain Newman..... 105
6.2.3 Adherence to fibrinogen by strain Newman grown in RPMI..... 106
6.2.4 Expression of ClfA from RPMI-grown Newman cells is dependent on SigB..... 107
6.2.5 Expression of ClfB in iron limiting media..... 107
6.2.6 Adhesion of *S. aureus* Newman cells grown to exponential and stationary phase to cytokeratin 10..... 108
6.3 Discussion..... 109

Chapter 7 Discussion

7.1 Discussion..... 113

References..... 118

List of tables

Following page

2.1 Bacterial strains..... 42

2.2 Plasmids..... 42

2.3 Primers..... 42

List of figures

Following page

Figure 1.1 Surface protein anchoring in *Staphylococcus aureus*..... 6

Figure 1.2 Staphylococcal surface proteins..... 6

Figure 1.3 Tandem β-zipper mechanism of fibronectin binding..... 8

Figure 1.4 Structure of human fibrinogen..... 8

Figure 1.5 Apo-structures of domains N23 of ClfA, ClfB and SdrG and SdrG-peptide complex..... 12

Figure 1.6 Genomic regions encoding the Isd proteins *S. aureus*..... 14

Figure 1.7. The cell-wall associated Isd proteins of *S. aureus*..... 14

Figure 1.8 Structure of the apo-NEAT 1 domain of IsdH and IsdC and IsdA NEAT domain in complex with haem..... 14

Figure 1.9 Putative pathway of haem transfer between Isd proteins of *S. aureus*..... 16

Figure 1.10. The Agr system..... 20

Figure 1.11 Complement fixation..... 28

Figure 1.12 Platelet activation and aggregation mediated by bacteria..... 34

Figure 1.13 Mechanisms of bacterial interactions with platelets..... 36

Figure 2.1 Nisin-inducible expression of ClfB from vector pNZ8037..... 42

Figure 3.1 MSCRAMM binding sites in fibrinogen..... 55

Figure 3.2 Amino acid sequence of the N-terminal section of the fibrinogen αC-connector..... 57

Figure 3.3 Structural organisation of cytokeratin 10..... 57

Figure 3.4 3D crystal structure of domains N2 and N3 of ClfB..... 57

Figure 3.5 Binding of the recombinant A domain of ClfB and ClfB P326A F328A to immobilized fibrinogen..... 59

Figure 3.6 Binding of recombinant A domain of ClfB and ClfB R331A to immobilized fibrinogen..... 59

Figure 3.7 3D crystal structure of N2N3 of ClfB with docked GTWNP peptide..... 59

Figure 3.8 Screening of putative ClfB mutants Q235A and N526A..... 59

Figure 3.9 Position of Q235 and N526 residues in ClfB..... 61

Figure 3.10 Nisin-controlled expression of ClfB, ClfB Q235A and ClfB N526A by *L. lactis*..... 61
Figure 3.11 Expression of ClfB by *L. lactis*..... 61
Figure 3.12 Fibrinogen binding by ClfB, ClfB Q235A and ClfB N526A expressed by *L. lactis*..... 61
Figure 3.13 Cytokeratin binding by ClfB, ClfB Q235A and ClfB N526A expressed by *L. lactis*..... 61
Figure 3.14 Mutants of the Aα-chain of fibrinogen..... 63
Figure. 3.15 Adherence of Newman *clfA* to immobilised recombinant fibrinogen Aα-, AαS317P- and AαT322P-chains..... 63
Figure 3.16 Proposed model of ligand binding by ClfB..... 67
Figure 3.17 Features of the αC-connector of fibrinogen..... 67
Figure 3.18 Alignment of amino acid sequences of the αC-connector..... 67

Figure 4.1 Expression of ClfA and ClfB from *S. aureus* strain Newman..... 74
Figure 4.2 Role of ClfB in *S. aureus* mediated platelet aggregation..... 74
Figure 4.3 Nisin-dependent adherence of *L. lactis* ClfB to immobilized fibrinogen..... 74
Figure 4.4 Threshold ClfB expression and bacterial cell density necessary for platelet activation..... 76
Figure 4.5 Role of fibrinogen in ClfB-promoted platelet adhesion..... 76
Figure 4.6 Preparation of fibrinogen purified of contaminating IgG for platelet aggregation assays..... 76
Figure 4.7 Effect of platelet receptor inhibitors on ClfB-mediated platelet activation and adhesion..... 76
Figure 4.8 Role of ClfB-specific IgG in ClfB-promoted platelet activation..... 78
Figure 4.9 Detection of ClfB and ClfB Q235A expressed on the surface of *L. lactis* using pooled human IgG..... 78
Figure 4.10 Depletion of ClfB-specific antibodies from pooled human IgG..... 78
Figure 4.11 Role of plasma proteins in ClfB-promoted platelet activation..... 78
Figure 4.12 Preparation of serum purified of IgG for platelet aggregation assays..... 79
Figure 4.13 Role of ClfB-specific IgG in complement dependent platelet activation..... 79

Figure 4.14 Fibrinogen-dependent platelet activation mediated by ClfA and ClfB-expressing bacteria..... 81
Figure 4.15 Complement-dependent platelet activation mediated by ClfB Q235A..... 83

Figure 5.1 Direct adhesion to washed platelets mediated by Newman *clfA clfB* grown in RPMI..... 87
Figure 5.2 Aggregation of platelets mediated by Newman *clfA clfB* grown in RPMI..... 87
Figure 5.3 Role of complement and IgG in platelet aggregation mediated by Newman *clfA clfB* grown in RPMI..... 89
Figure 5.4 Effect of platelet receptor inhibitors on activation and adhesion mediated by RPMI-grown Newman *clfA clfB*..... 89
Figure 5.5 Construction of plasmids pBlueΔ*isdB* and pHM1..... 89
Figure 5.6 Restriction mapping of plasmids pBlueΔ*isdB* and pHM1..... 91
Figure 5.7 Allelic replacement mutagenesis of *isdB*..... 91
Figure 5.8 Screening for novel *Bam*HI site in the signal sequence of *isdB* mutants..... 91
Figure 5.9 Screening of *S. aureus* Newman strains by western immonoblotting..... 93
Figure 5.10 Expression of IsdB from Newman *clfA clfB isdA*..... 93
Figure 5.11 Expression of IsdA from Newman *isdB* and *isdH* mutants..... 93
Figure 5.12 Adhesion of washed platelets to Newman strains lacking Isd proteins..... 93
Figure 5.13 Aggregation of platelets by Newman strains lacking Isd proteins..... 93
Figure 5.14 Plasma proteins required for IsdB mediated platelet aggregation..... 95
Figure 5.15 Restriction mapping of plasmids pCU1 *isdB* and pCU1 *isdAB*..... 95
Figure 5.16 Expression of IsdA and IsdB from pCU1 in Newman strains..... 95
Figure 5.17 Complementation of Isd mutations in *S. aurues* Newman strains..... 95
Figure 5.18 Expression of IsdB from *S. epidermidis*..... 95
Figure 5.19 Adhesion to and aggregation of platelets by *S. epidermidis* strains..... 96

Figure 6.1 Expression of ClfB in exponential and post-exponential growth phases..... 103
Figure 6.2 Growth of *S. aureus* strain Newman in RPMI medium..... 105

Figure 6.3 IsdH expression from strain Newman grown in iron limiting conditions..... 105
Figure 6.4 ClfA expression from strain Newman grown in iron limiting conditions..... 107
Figure 6.5 ClfA expression from strain Newman grown in TSB depleted of iron 107
Figure 6.6 Adherence of Newman *clfB* grown in iron limiting conditions to fibrinogen..... 107
Figure 6.7 ClfA expression from strains Newman and Newman *sigB* grown in iron limiting conditions..... 107
Figure 6.8 ClfB expression from strain Newman grown in iron limiting conditions..... 107
Figure 6.9 ClfB expression from strain Newman grown in TSB depleted of iron 108
Figure 6.10 Adherence of stationary phase RPMI-grown Newman strains to cytokeratin 10..... 108
Figure 6.11 Adherence of exponential phase RPMI-grown Newman strains to cytokeratin 10..... 108

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	
Ap	Ampicillin
Erm	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
WP	washed platelets
h	hour(s)
Ig	immunoglobulin
V_H	variable immunoglobulin heavy chain
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
GFPs	gel-filtered platelets
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
BHI	brain-heart infusion medium
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 most closely related to *Enterococcus*, *Bacillus* and *Listeria*. Molecular typing and genetic analyses have placed the staphylococci in the *Bacillus-Lactobacillus-Streptococcus* cluster of the *Micrococcaceae*. Staphylococci are Gram-positive organisms (Ludwig *et al.*, 1985; Stackebrandt & Teuber, 1988). When viewed through a microscope they appear as grape-like clusters which result from cell division in more than one plane. Other characteristics of staphylococci include the low G + C content (30 – 39 %) of their genomic DNA, resistance to desiccation and extreme halotolerance (growing at up to 3.5 M NaCl).

Staphylococcus aureus is distinguished from other staphylococci by the presence of a golden pigment, the secretion of coagulase and a thermostable DNase, and by the ability to ferment mannitol. Production of coagulase separates *S. aureus* from other coagulase-negative staphylococci (CoNS) which are considered to be less virulent.

1.1.2 Cell-wall components

The cell wall acts as a physical barrier and provides a rigid exoskeleton which protects *S. aureus* from the surrounding environment. The main component of the cell wall is peptidoglycan. Peptidoglycan consists of glycan strands made of repeating disaccharide units, N-acetylglucosamine and N-acetylmuramic acid (GlcNAc - (β1→4) - MurNAc) (Ghuysen & Strominger, 1963). Glycan chains are cross-linked by short tetrapeptides to generate a rigid three-dimensional cell wall network (Ghuysen *et al.*, 1965; Tipper & Strominger, 1965). In *S. aureus* a pentaglycine bridging peptide links tetrapeptides of adjacent glycan chains. This feature makes *S. aureus* peptidoglycan susceptible to cleavage by the endopeptidase lysostaphin (Schleifer & Kandler, 1972).

Other components of the cell wall include wall teichoic acids, lipoteichoic acids and small amounts of protein. Wall teichoic acids are covalently linked to peptidoglycan and are made up of ribitol-phosphate polymers substituted with N-acetylglucosamine and D-alanine residues (Endl *et al.*, 1983; Ward, 1981). Studies have shown that wall teichoic acids promote *S. aureus* nasal colonization and may play a role in infective endocarditis (Weidenmaier *et al.*, 2004; Weidenmaier *et al.*, 2005). Lipoteichoic acids are attached to glycolipids in the plasma membrane and are

composed of glycerol phosphate polymers substituted with D-alanine (Fischer *et al.*, 1990). Resistance to defensins is partially determined by the degree of D-Alanine substitution of teichoic acids (Collins *et al.*, 2002; Peschel *et al.*, 1999).

1.2 Nasal colonization and disease

The primary habitat of *S. aureus* in humans is the moist squamous epithelium of the anterior nares. Cell types found in the nares include squamous cells, ciliated epithelial cells and mucus-secreting goblet cells. The epithelium of the anterior nares is non-ciliated which allows *S. aureus* to adhere. *S. aureus* is reported to adhere poorly to ciliated epithelium found in the rest of the nasal cavity (Shuter *et al.*, 1996). The epithelium of the anterior nares is stratified and is composed of layers of sqamous cells which mature as they progress from basal layers to the exposed surface. Maturation of squames involves a change in shape, loss of nucleus and an increase in keratinization. *S. aureus* adheres most strongly to mature squames found in the superficial epithelial layer. These cells have a flat, scale-like shape (Peacock *et al.*, 2001). Colonisation of the nares depends on the ability of *S. aureus* to adhere to desquamated epithelial cells and to avoid the host immune response.

Sevaral surface-associated proteins of *S. aureus* promote adhesion to squamous cells *in vitro*. Clumping factor B (ClfB) is believed to mediate adhesion to squamous cells through binding cytokeratin 10. Confocal microscopy and flow cytometry have shown that cytokeratin 10 is present on the surface of desquamated epithelial cells found in the anterior nares (O'Brien *et al.*, 2002b). Immunisation with ClfB was protective against nasal colonisation in a murine model. Administration of a monoclonal function-blocking antibody directed against ClfB also protected against colonization in a mouse model (Schaffer *et al.*, 2006). Higher levels of anti-ClfB antibodies have been observed in non-carriers than in individuals who have *S. aureus* present in the nares suggesting that anti-ClfB antibodies may help protect against nasal colonization (Dryla *et al.*, 2005). A recent human study found that *S. aureus clfB* mutants were eliminated from the human nares faster than wild-type strains (Wertheim *et al.*, 2008).

The nasal mucosa is an iron-restricted environment which stimulates expression of iron-regulated surface determinant (Isd) proteins by *S. aureus*. These proteins are involved in the acquisition of haem and release of iron following transport into the bacterial cytoplasm. IsdA is a component of the haem-acquisition system which has

also been shown to promote bacterial adhesion to squames. In iron-restricted conditions ClfB and IsdA were shown to contribute to adhesion of *S. aureus* to squamous cells (Clarke *et al.*, 2006). Recent studies have shown that IsdA can bind to loricrin, involucrin and cytokeratin 10 which are proteins associated with the outer envelope of cornified epithelial cells and may provide a ligand for IsdA (Clarke *et al.*, 2008b).

The serine-aspartate repeat proteins, SdrC and SdrD and the *S. aureus* surface protein G (SasG) also promote adhesion of bacteria to squamous cells although their ligands are not known (R. Corrigan and T. J. Foster personal communication). A role for wall teichoic acid (WTA) in nasal colonisation has also been proposed. Studies showed that WTA-deficient mutants had decreased adherence to nasal cells and were unable to colonize the cotton rat nares (Weidenmaier *et al.*, 2004).

S. aureus persistently colonises 20% of the population. Another 20% are never colonised while the rest of the population may intermittently carry *S. aureus*. Persistent carriers tend to have heavy loads of *S. aureus* compared to intermittent carriers and they are usually colonised by a single strain (Wertheim *et al.*, 2005). To colonise the anterior nares *S. aureus* must overcome both innate and induced immune responses controlled by nasal-associated lymphoid tissue (NALT). Therefore host factors associated with immune response are believed to play a role in determining carriage status. For example, haplotype 3 polymorphisms in glucocorticoid receptors are associated with immune enhancement and individuals with this haplotype have 68% lower *S. aureus* carriage rates. Individuals with haplotype 5 which is associated with immune suppression have an 80% increased *S. aureus* carriage rate (van den Akker *et al.*, 2006). Increased rates of persistent carriage are also seen in individuals with an interleukin 4 gene promoter polymorphism which is associated with lower IL-4 serum concentrations and reduced mucin (Emonts *et al.*, 2008). Nasal carriage of *S. aureus* is a pre-requisite for colonisation of other sites such as the skin. To survive on the skin bacteria have to overcome the acid environment and antibacterial fatty acids present in sebum. This is further discussed in section 1.5.1.

Nasal colonisation is a risk factor for *S. aureus* infections. It has been reported that 80 % of individuals with *S. aureus* skin lesions were nasal carriers (Wertheim *et al.*, 2005). Carriage of *S. aureus* is also associated with an increased risk of infection following surgery. Nosocomial *S. aureus* bacteraemia can often be attributed to an endogenous source (von Eiff *et al.*, 2001). Carriers may be immunologically adapted

to the strain of *S. aureus* that they are colonised with since *S. aureus* bacteraemia-related death was found to be significantly higher in non-carriers compared to carriers (Wertheim *et al.*, 2004).

The most common types of *S. aureus* infections are superficial skin lesions such as boils, impetigo and abscesses. If the organism gains access to the bloodstream by breach of the skin or mucosal barrier, bacteraemia may develop. Bacteraemia can result in infection of internal tissues such as bone (osteomyelitis), joints (septic arthritis), lungs (pneumonia), and heart valves (endocarditis) (Lowy, 1998). Treatment of these diseases has been complicated by the emergence of resistance to antimicrobial agents. Resistance to β-lactams such as penecillins, cephalosporins and cephamycins is associated with MRSA (Methicillin-resistant *S. aureus*). Intermediate resistance to the glycopeptide vancomycin is frequently encountered and recently isolated cases of high-level resistance have been reported (Flannagan *et al.*, 2003; Weigel *et al.*, 2003).

1.3 Virulence factors

S. aureus produces an extensive range of virulence factors which allow it to colonise a number of different sites in the body and cause a wide range of disease. Virulence factors can be classified according to their roles in pathogenesis. Adhesins are involved in bacterial adherence to the host extracellular matrix (ECM) and blood components such as fibrinogen. These proteins are called MSCRAMMS (microbial surface components recognizing adhesive matrix molecules). MSCRAMMS promote adhesion to damaged tissue and surfaces of host cells. Secreted enzymes, toxins, and proteases are involved in the later stages of infection and facilitate tissue damage and spreading. Evasins are involved in the avoidance of the host innate and adaptive immune responses. Many virulence factors have multiple functions and contribute to a number of pathogenic processes.

1.3.1 Capsule

Capsular polysaccharide is produced by a majority of *S. aureus* clinical isolates (O'Riordan & Lee, 2004; Roghmann *et al.*, 2005). *In vitro* capsular polysaccharide is expressed in the stationary phase of growth and has been detected *in vivo* in endocardial vegetations (Lee *et al.*, 1993).

Expression of serotype 5 and 8 capsule (CP) is associated with increased virulence in animal infection models (Baddour *et al.*, 1992; Lee *et al.*, 1997; Luong & Lee, 2002; Nilsson *et al.*, 1997; Thakker *et al.*, 1998). Expression of capsule inhibits binding of antibodies to *S. aureus* cell surface components impeding opsonisation and subsequent phagocytosis (Thakker *et al.*, 1998). High titres of specific anti-capsular polysaccharide antibodies overcome this effect and promote opsonophagocytosis (Lee *et al.*, 1997; O'Riordan & 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). Immunization of haemodialysis patients with StaphVax reduced the incidence of infection over an 8 month period (Fattom *et al.*, 2004).

Expression of capsule may mask *S. aureus* surface adhesins. In a recent study expression of capsule inhibited *S. aureus* clumping factor A-mediated binding to fibrinogen and platelets (Risley *et al.*, 2007).

1.3.2 Surface proteins

1.3.2.1 Sortase-mediated anchoring of cell-wall associated proteins

S. aureus expresses numerous surface proteins which are covalently linked to the peptidoglycan layer of the cell wall by a process referred to as 'sorting'. The N-terminal domains of these proteins consist of a signal sequence which directs the protein into the secretory (Sec) pathway. Membrane anchored signal peptidase enzymes cleave the signal sequence upon translocation across the cytoplasmic membrane (Cregg *et al.*, 1996; Mazmanian *et al.*, 2001). A C-terminal hydrophobic, membrane-spanning domain followed by a number of positively charged residues anchor the cleaved peptide and allow subsequent covalent attachment to the cell wall by sortase (Fischetti *et al.*, 1990; Mazmanian *et al.*, 2001; Navarre & Schneewind, 1999).

Sortase enzymes are membrane anchored transpeptidases that covalently attach surface proteins to peptidoglycan. *S. aureus* has two sortase (Srt) enzymes, SrtA and SrtB (Mazmanian *et al.*, 2001; Pallen *et al.*, 2001). SrtA anchors the majority of *S. aureus* surface proteins and recognises the LPXTG motif. SrtB anchors the iron-regulated surface determinant C (IsdC) and recognises the C-terminal NPQTN motif of this protein (Mazmanian *et al.*, 2002). Cleavage of the LPXTG motif by SrtA occurs between threonine and glycine residues. SrtA covalently attaches the cleaved

protein via threonine to the pentaglycine bridge of peptidoglycan (Ton-That *et al.*, 2000). The C-terminal membrane spanning domain and positively charged tail is then released and degraded (Figure 1.1). X-ray structures of SrtA in complex with an LPETG peptide and NMR analysis of sortase in the presence or absence of ligand provided insight into the detailed action of sortase (Liew *et al.*, 2004; Zong *et al.*, 2004).

SrtA plays a crucial role in the correct anchoring of surface proteins, many of which are virulence factors. *S. aureus srtA* mutants can not correctly anchor LPXTG-motif surface proteins and are attenuated in animal infection models of septic arthritis and endocarditis (Jonsson *et al.*, 2002; Weiss *et al.*, 2004). Recognition of the LPXTG motif is highly stringent and altering residues 1, 2, 4 or 5 prevents correct sorting of surface proteins (Kruger *et al.*, 2004).

1.3.2.2 Fibronectin-binding surface proteins

Fibronectin is an extracellular matrix glycoprotein found in blood plasma at high concentrations. The fibronectin molecule is a dimer composed of two covalently linked 250 kDa subunits. It contributes to a number of cell processes including adhesion, migration, growth and differentiation (Pankov & Yamada, 2002).

S. aureus has two closely related fibronectin-binding proteins (FnBP), A and B which are expressed on the cell surface in the exponential growth phase. Both proteins promote adherence to immobilized fibronectin (Greene *et al.*, 1995). FnBPA and FnBPB are encoded by two closely linked but separately transcribed genes *fnbA* and *fnbB* (Jonsson *et al.*, 1991; Signas *et al.*, 1989). The majority of *S. aureus* strains contain both genes (Peacock *et al.*, 2000).

FnBPA and FnBPB have structurally similar N-terminal A domains which mediate binding to fibrinogen and elastin (Roche *et al.*, 2004; Wann *et al.*, 2000). The FnBPA A domain varies substantially between strains despite retaining ligand-binding functions. Antigenic variation of the A domain may be involved in evasion of the host immune response (Loughman *et al.*, 2008). The A domains of FnBPA and FnBPB are 45 % identical and consist of N1, N2 and N3 sub-domains (Figure 1.2; Jonsson *et al.*, 1991). The N2N3 domains are predicted to bind to fibrinogen by the "dock lock latch" model described in section 1.3.2.3.3. FnBPA and FnBPB bind to the same region of fibrinogen as ClfA, the flexible C terminus of the γ-chain (Wann *et*

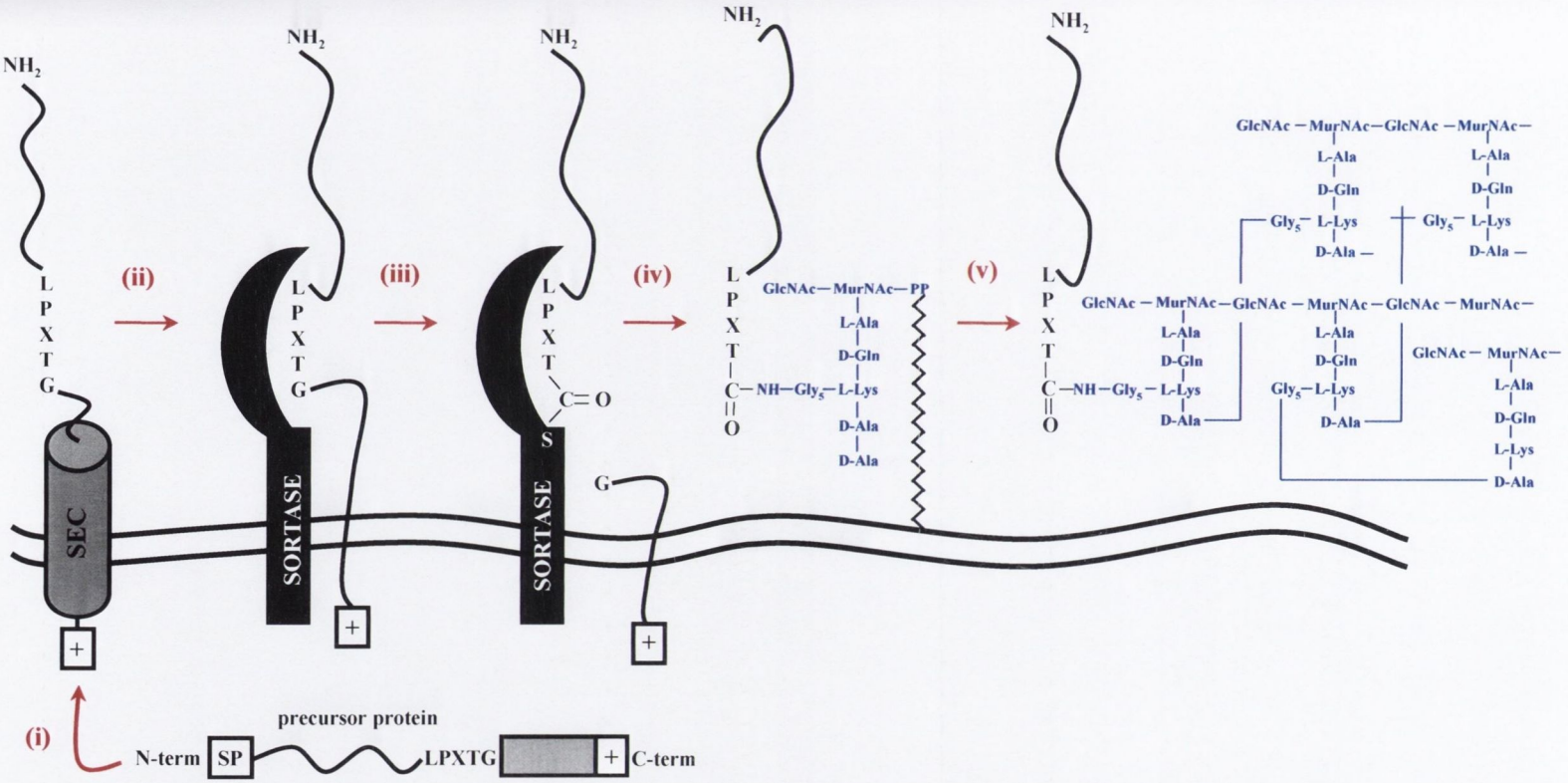


Figure 1.1. Surface protein anchoring in *Staphylococcus aureus*.

(**i**) Export. Precursor proteins are directed into the Sec pathway where the signal peptide (SP) is removed. (**ii**) Retention. The C-terminal sorting signal retains polypeptides within the secretory pathway. (**iii**) Cleavage. Sortase cleaves between residues T and G of the LPXTG motif forming a thioester enzyme intermediate. (**iv**) Linkage. The acyl-enzyme intermediate is resolved, and an amide bond is formed between the surface protein and the uncross-linked pentaglycine bridge on lipid II (**v**) Cell wall incorporation. Lipid-linked surface protein is incorporated into the cell wall peptidoglycan by transglycosylation. The murein pentapeptide subunit with attached surface protein is cross-linked other cell wall peptides, generating the mature murein tetrapeptide.

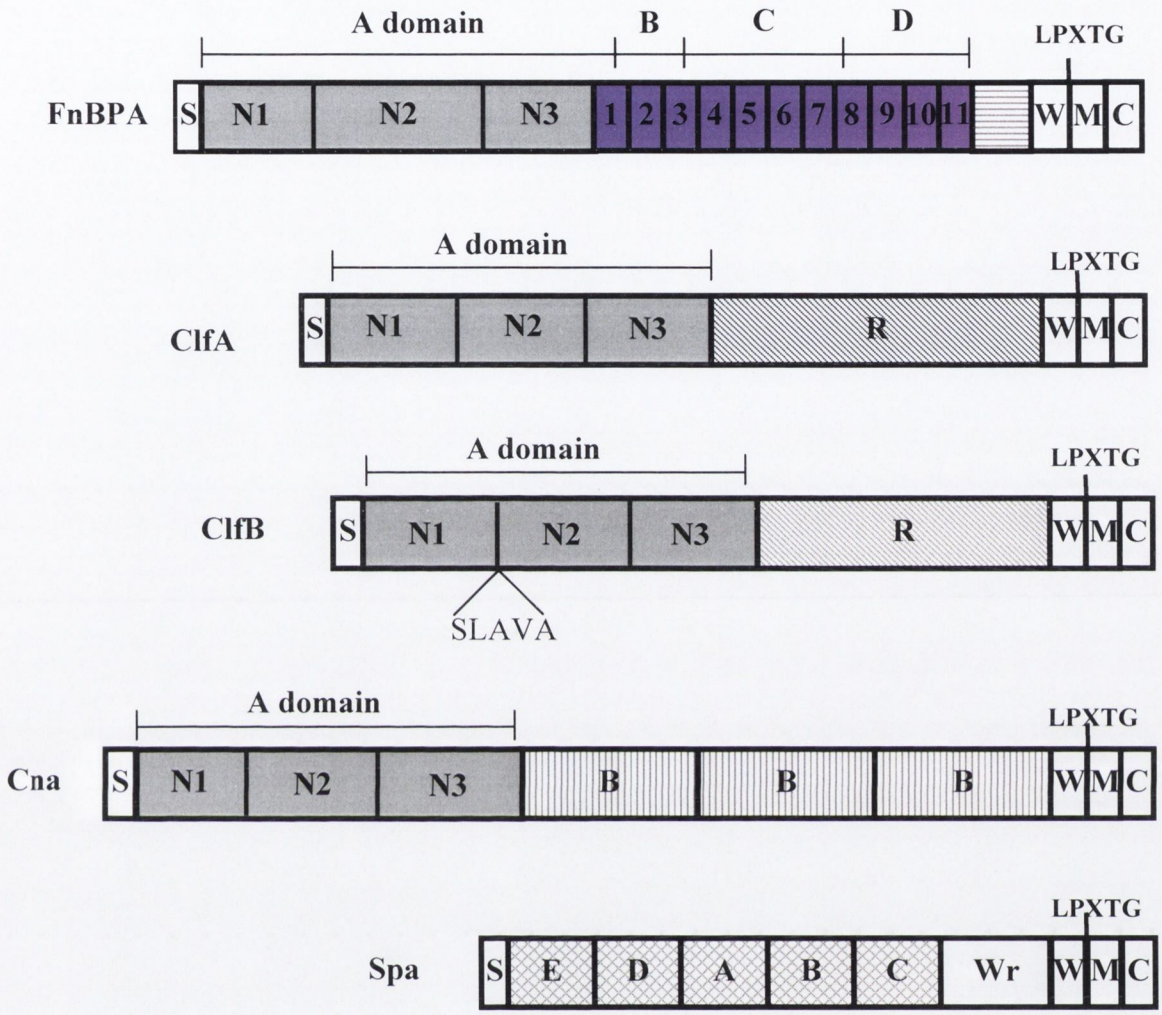


Figure 1.2. Staphylococcal surface proteins

Structural organisation of surface proteins from *S. aureus.* The A domains of FnBPA, ClfA, ClfB and Cna are ligand binding domains. Recently the domain organisation of FnBPA has been revised and the BCD region is now known to consist of 11 repeated fibronectin binding domains. Spa domains E, D, A, B and C are homologous ligand-binding repeats. The relative sizes of the signal sequence (S), A domain (A), B-repeat region (B), SD-repeat region (R), and wall/membrane/cytoplasmic spanning regions (WMC) are shown. LPXTG sortase A recognition motifs are indicated.

al., 2000). Elastin is also bound between the N2N3 domains and is thought to interact with FnBPA in a similar manner to fibrinogen (Keane *et al.*, 2007).

The BCD domains of FnBPA and FnBPB are 95 % identical and mediate binding to fibronectin (Jonsson *et al.*, 1991; Signas *et al.*, 1989). Fibronectin contains five N-terminal type I modules, which are the primary binding site for FnBPs (Sottile *et al.*, 1991). A second binding site in fibronectin has been identified in the heparin-binding type III module 14 (Bozzini *et al.*, 1992). Eleven fibronectin-binding repeats each with type I module-binding motifs have been identified in FnBPA. These repeats do not have an ordered structure until ligand binding takes place (House-Pompeo *et al.*, 1996; Schwarz-Linek *et al.*, 2003).

Strep. dysgalactiae also produces a fibronectin binding protein, Sfbl. Based on the solved structure of the B3 peptide of Sfbl in complex with two type I modules from fibronectin, a tandem β-zipper mechanism of binding has been proposed. The binding motifs in the B3 peptide form additional antiparallel β-strands on adjacent type I modules of fibronectin (Schwarz-Linek *et al.*, 2003). FnBPs also interact with fibronectin by a tandem β-zipper mechanism. Recently the crystal structure of two fibronectin binding repeats of FnBPA in complex with type I modules of fibronectin has been solved (Figure 1.3, Bingham *et al.*, 2008). The fibronectin binding repeats form anti-parallel strands along four adjacent type 1 modules of fibronectin. The disordered nature of the fibronectin binding repeats is believed to facilitate the formation of large intermolecular interfaces allowing one FnBP molecule to bind up to 9 molecules of fibronectin (Bingham *et al.*, 2008; Matsuka *et al.*, 2003).

The ability of *S. aureus* to adhere to and to invade endothelial and epithelial cells is mediated by FnBPs (Dziewanowska *et al.*, 1999; Ogawa *et al.*, 1985). The FnBPs from a fibronectin-bridge between *S. aureus* and the $\alpha_5\beta_1$ integrin on host cells (Fowler *et al.*, 2000; Sinha *et al.*, 1999). Invasion of cells may provide a means of evading the host immune response and of spreading to tissues such as bones and joints.

The FnBPs are believed to be important in establishing infections by promoting adhesion of *S. aureus cells* to implanted devices coated in fibronectin (Arrecubieta *et al.*, 2006; Vaudaux *et al.*, 1993). Bacterial cells expressing FnBPA promoted heart valve colonization and invasion of surrounding endothelium in experimental endocarditis models (Que *et al.*, 2001; Que *et al.*, 2005). Patients recovering from

invasive *S. aureus* infections have been shown to have higher anti-FnBPA antibody titres than healthy individuals (Dryla *et al.*, 2005). FnBPs are also involved in the intracellular accumulation phase of biofilm formation by MRSA strains. Methicillin-sensitive *S. aureus* strains mediate biofilm formation by a different mechanism (O'Neill *et al.*, 2008). FnBPs play an important role in platelet aggregation by exponential growth phase *S. aureus*. This is discussed further in section 1.6.2.1.

1.3.2.3 Fibrinogen binding surface proteins

Fibrinogen is a 340 kDa plasma protein that is synthesised by hepatocytes. It plays a crucial role in hemostasis following vascular damage. Once cleaved by thrombin, fibrinogen is converted to fibrin, a major component of fibrin clots. Fibrinogen is required for platelet aggregation and thombus formation (Herrick *et al.*, 1999).

Fibrinogen is made up of two subunits which both consist of three polypeptide chains Aα, Bβ and γ. The N-termini of the Aα-, Bβ- and γ-chains form an N-terminal disulphide knot which makes up the central E-domain. Fibrinopeptides A and B are released from the N termini of the Aα and Bβ chains upon cleavage by thrombin. Two distal D domains consist of the globular C-termini of the Bβ- and γ-chains and the middle section of the Aα-chain (Figure 1.4). These are linked to the central E domain by triple helical coiled-coils, held together by disulphide bonds (Herrick *et al.*, 1999).

The remaining C-terminal of each Aα-chain consists of the αC-connector and the globular αC-domain (Burton *et al.*, 2006; Rudchenko *et al.*, 1996; Tsurupa *et al.*, 2002). The globular αC-domains can interact with each other and the central E domain via fibrinopeptide B (Litvinov *et al.*, 2007). During fibrin assembly fibrinopeptide B is cleaved from fibrinogen and the self interaction of the αC-domains is abolished. The αC-domains are then able to participate in intermolecular interactions in which they promote lateral aggregation of fibrin (Medved *et al.*, 1985). The αC-domains also contribute to fibrin clot stability by activation of transglutaminase factor XIII which cross-links fibrin clots (Collet *et al.*, 2005; Credo *et al.*, 1981; Gorkun *et al.*, 1994).

1.3.2.3.1 Clumping factor A

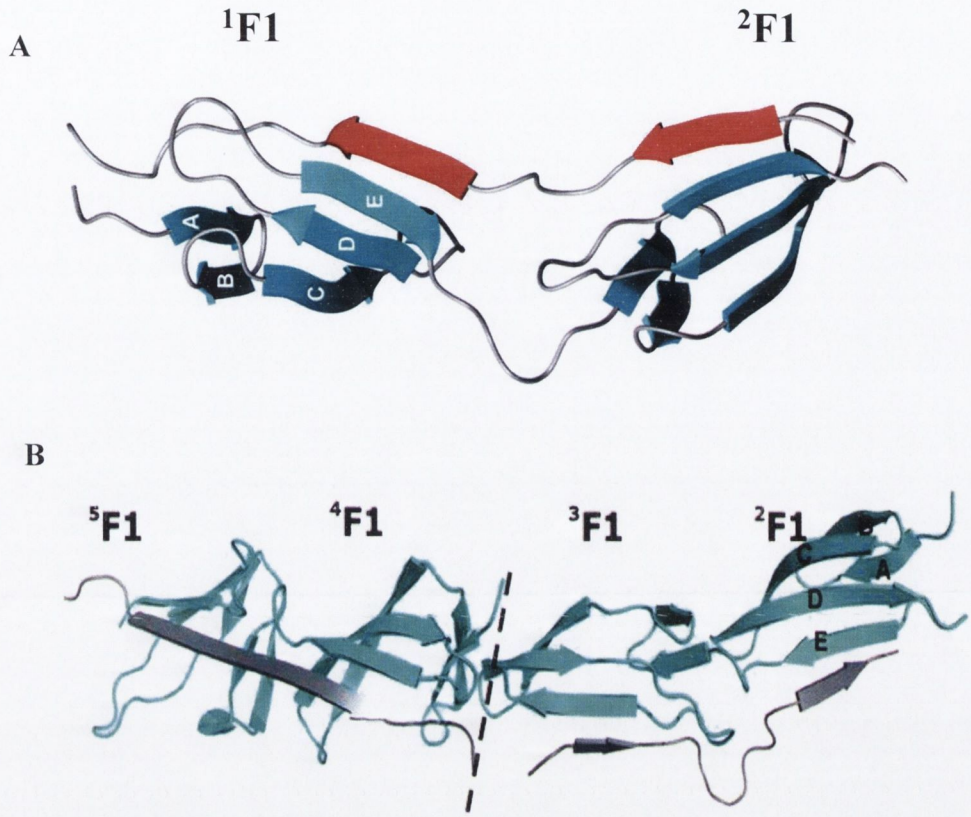


Figure 1.3. Tandem β-zipper mechanism of fibronectin binding

A. Ribbon diagram of *S. dysgalactiae* B3 peptide in complex with ^{1}F1^{2}F1 type I fibronectin modules. Strands of the fibronectin F1 modules are shown in cyan and the fourth strand formed by B3 is shown in red. Taken from Schwarz-Linek *et al.*, 2003.

B. Ribbon diagram of two *S. aureus* fibronectin binding repeats each in complex with ^{2}F1^{3}F1 and ^{4}F1^{5}F1 fibronectin modules. Strands of fibronectin are shown in cyan and the fibronectin binding repeats in gray. Taken from Bingham *et al.*, 2008.

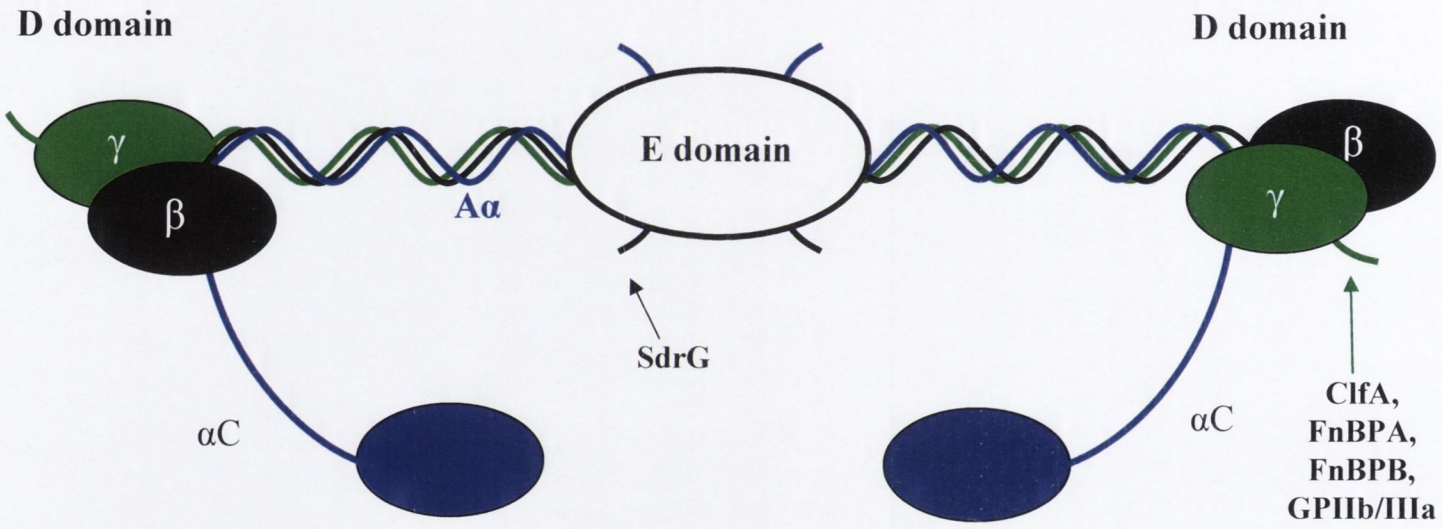


Figure 1.4. Structure of human fibrinogen.

Fibrinogen consists of two identical disulfide-linked subunits, each composed of three non-identical polypeptide chains, Aα, Bβ and γ. Binding sites for *S. aureus* surface proteins and the *S. epidermidis* SdrG protein are indicated. The platelet integrin GPIIb/IIIa binds to the same site as ClfA and the FnBPs. Fibrinogen can be divided into 4 major regions, the central E region, 2 identical terminal D regions and the αC-domains.

The fibrinogen-binding protein Clumping factor A (ClfA) was identified by McDevitt *et al.* ClfA is expressed predominantly in the stationary phase of growth by *S. aureus* cells in complex broth media (Wolz *et al.*, 1996). *S. aureus* cells expressing ClfA promote clumping in soluble fibrinogen and adherence to immobilised fibrinogen (McDevitt *et al.*, 1994). Previous studies have shown that in the stationary phase of growth ClfA is expressed from a SigB dependent promoter (Bischoff *et al.*, 2004; Nicholas *et al.*, 1999). Weaker expression of ClfA occurs in the exponential growth phase and is dependent on transcription from a SigA-dependent promoter (Higgins, 2005).

The structure of ClfA is characteristic of Gram-positive bacterial surface-anchored proteins (Figure 1.2). A 40 residue N-terminal signal sequence is followed by the fibrinogen-binding A domain. The A domain is projected from the cell wall surface by the serine-aspartate (SD) repeat region which varies in length between strains. The C-terminal wall-spanning region, membrane-spanning region and LPDTG motif are involved in anchoring and sorting of ClfA (McDevitt *et al.*, 1994).

The A domain of ClfA consists of N1, N2 and N3 sub domains. Binding of fibrinogen is proposed to occur between the N2 and N3 domains by the "dock lock latch" model as described in section 1.3.2.3.3. ClfA binds to a flexible unfolded peptide at the C terminal of the γ-chain of fibrinogen (McDevitt *et al.*, 1997). The same region of fibrinogen is recognized by the GPIIb/IIIa receptor on platelets (Farrell *et al.*, 1992; Hettasch *et al.*, 1992). Binding of fibrinogen by ClfA can be inhibited by Ca^{2+} (O'Connell *et al.*, 1998).

Several studies have reported a role for ClfA in infective endocarditis. *S. aureus clfA* mutants were less infective in endocarditis models than parental strains (Moreillon *et al.*, 1995). Function blocking antibodies to ClfA have been shown to sterilize vegetations on heart valves when administered with vancomycin in experimental endocarditis (Vernachio *et al.*, 2003). ClfA is also 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* and has been shown to be expressed *in vivo* late in infection (Dryla *et al.*, 2005; Peacock *et al.*, 2002). It is the target of several novel strategies to combat *S. aureus* infections. Inhibitex Inc.

(Georgia, USA) have developed Veronate, a hyperimmunoglobulin against ClfA and the fibrinogen-binding *S. epidermidis* SdrG protein (Vernachio *et al.*, 2003). A humanised monoclonal antibody against the A domain of ClfA (Aurexis) has also been developed by Inhibitex (Patti, 2004). In combination with vancomycin, it reduced bacterial densities in infected vegetations, kidneys and spleens in an infective endocarditis model (Patti, 2004).

1.3.2.3.2 Clumping factor B

Clumping factor B (ClfB) was identified as a fibrinogen binding protein by Ní Eidhin *et al.* Unlike ClfA which is maximally expressed in the stationary growth phase ClfB was only found on the surface of exponential growth phase cells (McAleese *et al.*, 2001; Ni Eidhin *et al.*, 1998). Studies carried out by McAleese *et al.* determined that transcription of ClfB ceases in the late exponential growth phase after which ClfB protein is degraded by the metalloprotease aureolysin. Cleavage occurs at the SLAVA motif at the end of the N1 domain of ClfB (Figure 1.2). Degradation by aureolysin results in the loss of ClfB ligand binding ability. Loss of ClfB in the post-exponential growth phase is also mediated by the dilution of existing protein as the cells grow and divide while transcription of *clfB* has ceased and by shedding of ClfB into the growth medium by autolysis (McAleese *et al.*, 2001).

Transcription of *clfB* is not directly affected by Agr or the staphylococcal accessory regulator, SarA. However, SarA represses expression of aureolysin which cleaves ClfB. The regulator of toxins, Rot is a global regulator which activates certain cell wall-associated proteins expressed in the exponential growth phase including ClfB (McAleese & Foster, 2003; McNamara *et al.*, 2000; Said-Salim *et al.*, 2003). Regulation of ClfB is further discussed section 1.4 and in Chapter 6.

Unlike ClfA and the FnBPs which bind to the γ-chain of fibrinogen, ClfB binds to the α-chain (Walsh *et al.*, 2008). Elucidation of the precise binding site in fibrinogen is discussed in Chapter 3. ClfB shares similar domain organization with ClfA. The A domain possesses fibrinogen-binding activity and shares 26 % sequence identity with the A domain of ClfA (Ni Eidhin *et al.*, 1998). The binding of ClfB to fibrinogen can be inhibited by Ca^{2+} (Ni Eidhin *et al.*, 1998).

ClfB also binds to cytokeratin 10. Cytokeratins belong to the family of intermediate filaments (IF) which provide mechanical strength to vertebrate epithelial cells. ClfB binds specifically to the tail region of cytokeratin 10 which is rich in

glycine/serine repeats and is believed to form structures known as Ω loops (Walsh *et al.*, 2004). Cytokeratin10 is found in the differentiated layer of the human epidermis and is believed to act as a receptor for ClfB on desquamated epithelial cells of the anterior nares (see section 1.2).

1.3.2.3.3 The dock, lock and latch model for fibrinogen binding

The ligand binding A domains of *S. aureus* fibrinogen-binding proteins vary considerably in sequence. However their structural organisation is similar (Figure 1.5). Crystal structure studies of ClfA revealed that the N2N3 domains are organised into a novel immunoglobulin-type fold which was named the D-variant or DEv-type IgG fold (Deivanayagam *et al.*, 2002). Sequence alignments and secondary structure predictions indicated that the N2N3 domains of ClfB, FnBPA and FnBPB are also organised into DEv-type IgG folds. The DEv-type IgG fold is also found in the A domain of the *S. epidermidis* fibrinogen-binding protein SdrG. SdrG binds to the N terminus of the fibrinogen β-chain (Davis *et al.*, 2001). Solution of the crystal structure of the N2N3 domains of SdrG in complex with a synthetic fibrinogen peptide provided information on the structural changes that occur upon ligand binding (Ponnuraj *et al.*, 2003).

The "dock lock latch" model has been proposed as a common mechanism for fibrinogen binding by staphylococcal surface associated proteins. Docking of the fibrinogen peptide occurs in the hydrophobic trench that separates the N2 and N3 folded domains. Protein-protein interactions between residues in the trench and the ligand stabilise the docked peptide. Binding triggers structural rearrangements at the C-terminus of the N3 domain (β-strand G″), also known as the latching peptide. Upon ligand docking, the G″ strand undergoes a directional change and crosses over the binding trench. The peptide becomes locked in place by the β-sheet which covers the binding trench and is secured by hydrogen bonding that takes place between the bound fibrinogen peptide and β-strand G′/linker regions. The C-terminal β-strand G″ of the N3 domain then 'latches' in 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. This β-strand complementation stabilizes the overall structure (Ponnuraj *et al.*, 2003). A conserved motif (TYTFTDYVD) at the back of the latching cleft in SdrG, ClfA

and ClfB is likely to be involved in binding of the latching peptide to the latching cleft in domain N2 (Ponnuraj *et al.*, 2003).

1.3.2.4 Protein A

Over 95 % of *S. aureus* strains express the immunoglobulin-binding surface protein, protein A (Spa) (Forsgren & Nordstrom, 1974). The surface-exposed segment of this protein consists of five homologous repeats (EDABC) of 58 – 62 amino acids (Figure 1.2). Each of the repeats is composed of three anti-parallel α-helices that pack together to form a compact helical bundle stabilized by hydrophobic interactions in the bundle interior (Gouda *et al.*, 1992; Starovasnik *et al.*, 1996). Each repeat is capable of binding the Fcγ region of IgG (Inganas *et al.*, 1981). *S. aureus* cells expressing Spa become coated with IgG molecules in the incorrect orientation to allow recognition by the neutrophil Fc receptor (Gemmell, 1991). This inhibits phagocytosis and stimulation of complement fixation by the classical pathway. *S. aureus spa* mutants are phagocytosed more efficiently *in vitro* and exhibit decreased virulence in several animal infection models (Gemmell, 1991; Palmqvist *et al.*, 2002; Patel *et al.*, 1987).

The 5 repeat domains of protein A also bind to the variable region of the Fab heavy chain of V_H3 class antibodies, including subsets of IgM molecules (Hillson *et al.*, 1993). Fab binding occurs on the opposite face of Spa to that mediating Fc binding (Graille *et al.*, 2000). Binding of Spa to the Fab region of V_H3-class IgM molecules on B-cells is believed to cause their activation, proliferation and subsequent apoptotic destruction (Goodyear & Silverman, 2003). A reduction in antibody-secreting cells from the spleen and bone marrow may account for the immunosuppressive activity of Spa (Goodyear & Silverman, 2004).

Each of the EDABC repeats of Spa is capable of binding to domains A1 and D'-D3 of von Willebrand factor (vWF). Binding to these regions of vWF occurs on the same face of Spa as that mediating Fc binding (O'Seaghdha *et al.*, 2006). Binding of protein A to vWF contributes to bacterial adhesion to platelets under shear conditions (Hartleib *et al.*, 2000). This is described in more detail in section 1.6.2.1. The IgG binding domains of Protein A have also been shown to activate the receptor for tumour necrosis factor-α, (TNFR1) on epithelial cells. It is postulated that this

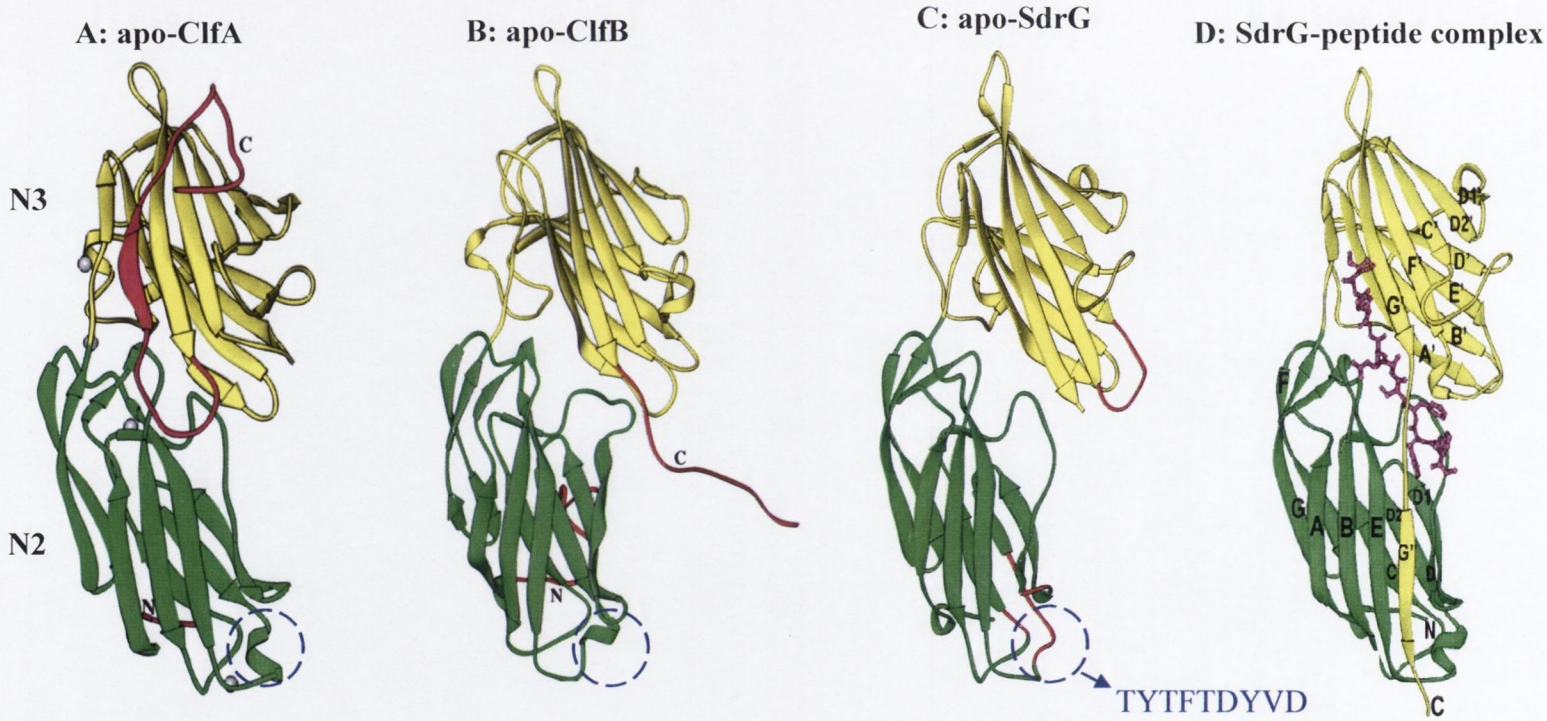


Figure 1.5. Apo-structures of domains N23 of ClfA, ClfB and SdrG and SdrG-peptide complex

Ribbon diagrams N2N3 domains of ClfA, ClfB and SdrG. **A.** The C-terminal latching peptide (shown in red) of ClfA loops back and folds into the N3 domain, partially blocking the proposed ligand-binding cleft. This may be an artifact of crystallization. **B.** In ClfB the latching peptide is located in the latching cleft of another rClfB molecule (M. Hook unpublished data). **C.** In apo-SdrG the peptide (G") is free in solution but interacts with N2 in the SdrG-peptide complex. The location of the conserved motif TYTFTDYVD is indicated. **D:** Fibrinogen β-chain in complex with SdrG is shown in ball and stick form. The SdrG-peptide complex is taken from Ponnuraj *et al.*, 2003.

interaction may be important in the initiation of infection and the induction of inflammation during *S. aureus* pneumonia (Gomez *et al.*, 2004; Gomez *et al.*, 2006).

1.3.2.5 Collagen-binding protein

The *cna* gene encodes a collagen-binding adhesin (Cna) found in the genome of 30 to 50 % of *S. aureus* strains (Arciola *et al.*, 2005; Peacock *et al.*, 2002; Smeltzer *et al.*, 1997). Collagen is a component of the extracellular matrix of connective tissue and is also found in bone tissue. Cna mediates adhesion to collagen substrates and collagenous tissues and is a virulence factor in animal models of septic arthritis, endocarditis and osteomyelitis (Elasri *et al.*, 2002; Hienz *et al.*, 1996; Patti *et al.*, 1994).

The A domain of Cna is responsible for collagen binding (Figure 1.2). The central binding segment of Cna is structurally organised into a DEv-IgG-type fold and flanking regions also participate in collagen binding (Patti *et al.*, 1993; Xu *et al.*, 2004). A surface trench in one of β-sheets of the DEv-IgG fold provides the binding site for the collagen triple helix (Deivanayagam *et al.*, 2000; Symersky *et al.*, 1997). The mechanism of collagen binding is similar to the dock, latch and lock model and is known as the "collagen hug" model (Zong *et al.*, 2005).

1.3.2.6 Iron regulated surface determinants

In humans the majority of iron is sequestered by haem-containing proteins and is unavailable to invading pathogens. Iron is essential for bacterial survival and growth inside the human host. The *isd* locus is involved in the capture and subsequent transport of haem-iron across the cell envelope of *S. aureus* (Skaar & Schneewind, 2004).

The *isd* genes are located on five transcriptional units (*isdA*, *isdB*, *isdCDEFsrtBisdG*, *isdH* and *isdI*) and are regulated by the iron-dependent transcriptional repressor Fur (Figure 1.6). IsdA, IsdB and IsdH (also known as HarA) are surface-located proteins containing C terminal LPXTG motifs. They are anchored to cell wall peptidoglycan by sortase A. IsdC is partially buried in the cell wall and has an atypical cell-wall sorting signal, NPQTN (Figure 1.7). It is cross linked to an unknown component of the cell wall envelope by sortase B, a unique sortase encoded on the transcription unit *isdCDEFsrtBisdG* (Mazmanian *et al.*, 2002).

1.3.2.6.1 NEAT domains of Isd proteins

S. aureus expresses secreted haemolysins which lyse red blood cells and release haemoglobin. Free haemoglobin is toxic and rapidly cleared from plasma by the haemoglobin carrier molecule, haptoglobin. Free haem is also highly reactive and is rapidly cleared from circulation by haemoplexin. The cell surface exposed IsdH, IsdB and IsdA components of the Isd system can bind to haem-containing proteins. IsdB binds to haemoglobin, IsdH binds to haemoglobin and to haptoglobin-haemoglobin complexes while IsdA can bind to haemin (oxidised form of haem) and haemoglobin (Clarke *et al.*, 2004; Dryla *et al.*, 2007; Torres *et al.*, 2006).

The surface exposed Isd proteins have variable numbers of NEAT (NEAr iron Transporter) domains which are involved in binding to haem and haem-containing proteins. These 125 residue domains were originally identified in genome searches as domains located near to iron transporters (Andrade *et al.*, 2002). IsdH has three NEAT domains, IsdB has two while IsdA and IsdC have one NEAT domain. Based on primary sequence NEAT domains can be divided into four groups. The NEAT 1 and 2 domains of IsdH and NEAT 1 of IsdB are Type I NEAT domains that bind haemoglobin and haemoglobin-haptoglobin complexes. They do not bind haem. Type II NEAT domains include NEAT 3 of IsdH and NEAT 2 of IsdB. Type III and IV NEAT domains are found in IsdA and IsdC respectively (Figure 1.7).

The three-dimensional structure of the NEAT 1 domain of IsdH has been reported. It is structurally related to the immunoglobulin fold family. Based on sequence comparisons, all NEAT domains were predicted to form IgG-type folds. The IsdH NEAT 1 domain is organised into a β sandwich fold composed of two five-stranded antiparallel beta sheets. Aromatic residues in the loop connecting strands β1b to β2 are predicted to bind haemoglobin. Other surface loops that connect strands β7 to β8 and β3 to β4 are also predicted to contact haemoglobin (Figure 1.8A). The negative charge of the putative binding surface in NEAT domain 1 of IsdH is implicated in hemoglobin binding (Pilpa *et al.*, 2006).

The crystal structure of IsdC in complex with haem has been solved. The NEAT domain of IsdC is structurally organised into an IgG-type fold. Haem is bound near the hydrophobic core of the β-sandwich structure and rests against a prominent β-hairpin structure (Figure 1.8B). Tyrosine residues located on the β-hairpin coordinate the iron atom of haem. Opposite to the β-hairpin a short helical peptide acts as a lip and is predicted to undergo conformational changes resulting in haem being locked in

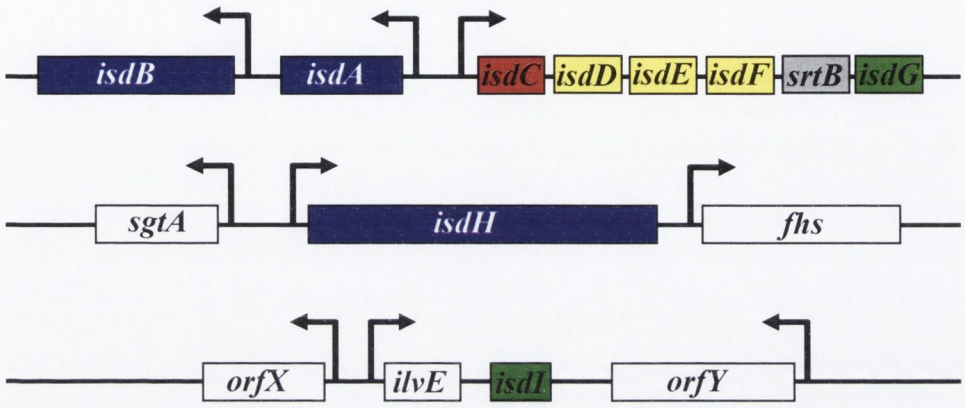


Figure 1.6. Genomic regions encoding the Isd proteins *S. aureus*

The Isd system is encoded on five transcriptional units located in three distinct regions of the *S. aureus* chromosome. Genes represented in blue encode cell surface-exposed proteins that are anchored by sortase A. Genes encoding sortase B substrates are shown in red. Genes that encode the haem transport system are shown in yellow and cytoplasmic haem-degrading enzyme genes in green. The genes shown in white are uncharacterized. Taken from Skaar *et al.*, 2004.

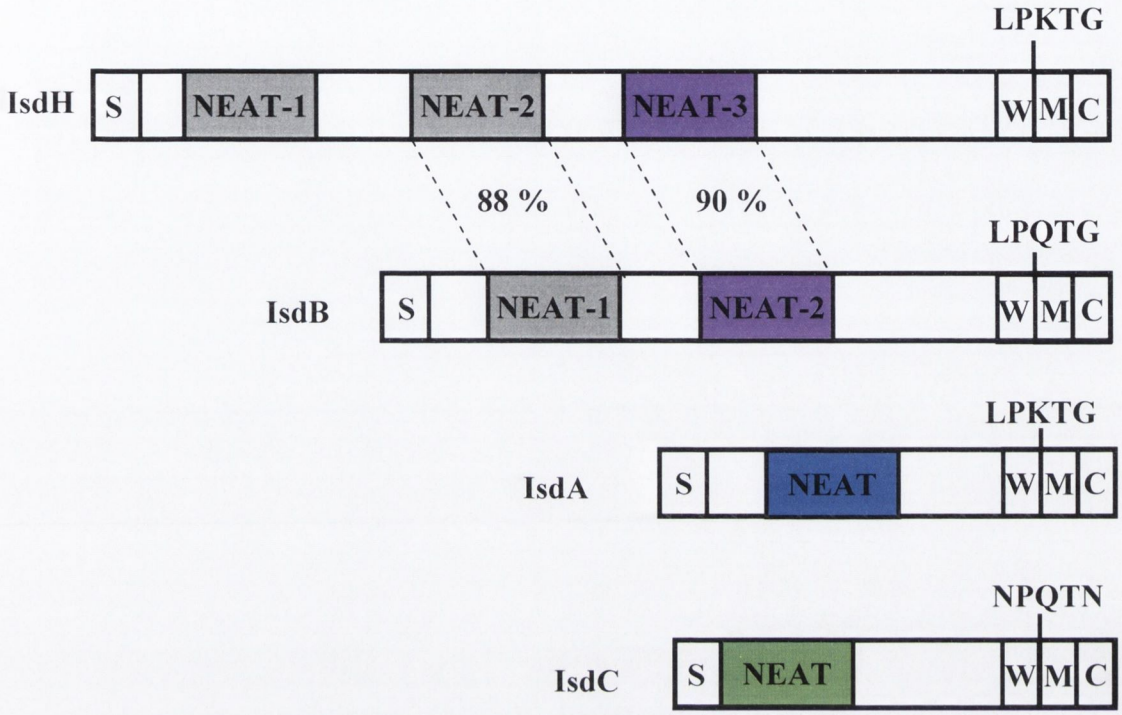


Figure 1.7. The cell-wall associated Isd proteins of *S. aureus*

The surface exposed Isd proteins have variable numbers of NEAT domains. Type I NEAT domains are shown in grey, typeII in purple, type III in blue and type IV in green. NEAT domains 2 and 3 of IsdH are highly homologous to NEAT domain 1 and 2 of IsdB. The C terminal LPXTG SrtA recognition motifs are indicated for IsdH, IsdB and IsdA. IsdC has an atypical NPQTN motif which is recognised by SrtB.

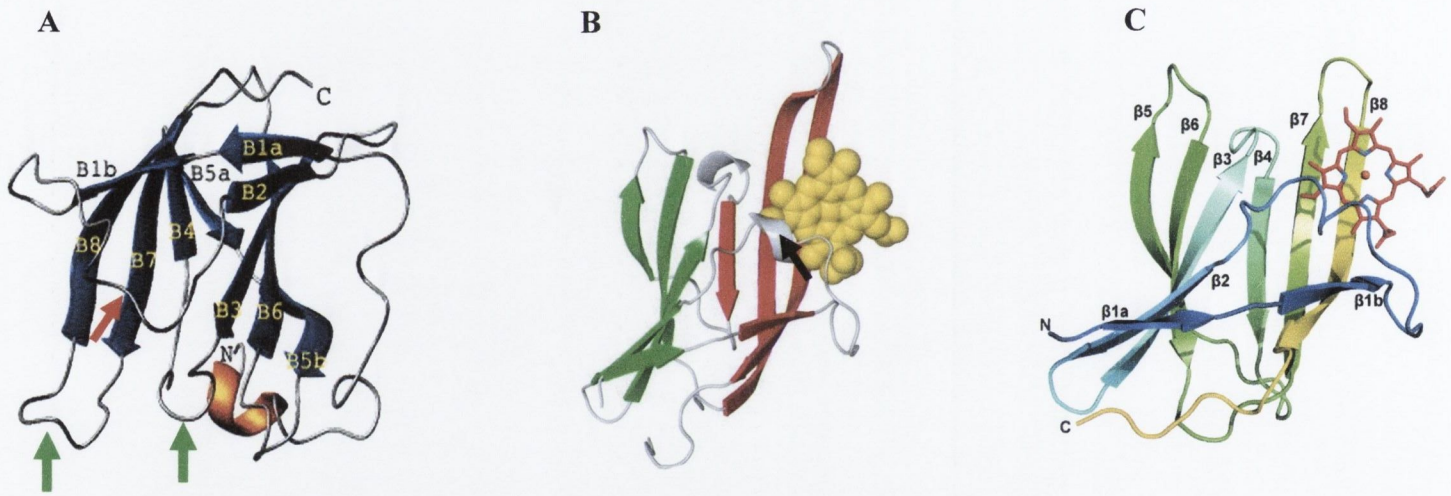


Figure 1.8 Structure of the apo-NEAT 1 domain of IsdH and IsdC and IsdA NEAT domain in complex with haem

(A). Ribbon diagram of the structure of IsdH NEAT domain 1. The loop connecting strands β 1b to β 2 is predicted to be involved in haemoglobin binding (red arrow). Loops connecting strands β 7 to β 8 and β 3 to β 4 are also thought to contact haemoglobin (green arrow). Taken from Pilpa *et al*., 2006. (B). Ribbon structure of IsdC in complex with haem (yellow). Haem rests on a β-hairpin structure and is locked in place by a helical peptide that acts as a lip (indicated by black arrow). Taken from Sharpe *et al* 2007. (C). Ribbon diagram of IsdA NEAT domain haem complex. Haem carbon, nitrogen and iron atoms are shown in red, blue and orange respectively. Taken from Grigg et al 2007.

a closed conformation. The tyrosine residues which coordinate the haem-iron atom in IsdC are conserved in IsdA and the Type II NEAT domains of IsdB and IsdH. A non-conserved tryptophan residue in IsdC also contributes to haem binding (Sharp *et al.*, 2007; Villareal *et al.*, 2008).

The crystal structure of the IsdA NEAT domain in complex with haem revealed that haem was bound in a hydrophobic pocket within a β-sandwich structure (Figure 1.8.C). The conserved tyrosine residues were crucial for haem-iron coordination (Grigg *et al.*, 2007). The solution of the crystal structure of the NEAT domain 3 of IsdH in complex with haem revealed a similar method of interaction (Watanabe *et al.*, 2008). However, Wantanabe *et al.* predicted that IsdH NEAT 3 can bind multiple haem molecules and that haem binding involves protein multimerisation. This process is predicted to increase efficiency of haem transport.

1.3.2.6.2 Haem transfer pathway

The Isd proteins are thought to function together to acquire and transport haem into the cytoplasm of *S. aureus* where it can be degraded to release iron. Recent studies have reported a pathway for transfer of haem between Isd proteins. Muryoi *et al.* demonstrated that haem can be passed in a unidirectional manner from IsdB NEAT domain 2 to the NEAT domain of IsdA. Haem is passed from the NEAT domain of IsdA to IsdC. Alternatively haem can be transferred from the NEAT domain 3 of IsdH to the NEAT domain of IsdA. IsdC acts as a central conduit and transfers haem to IsdE a component of the ABC transporter made up of IsdD, IsdE and IsdF (Muryoi *et al.*, 2008). Once inside the cell, IsdG and IsdI bind the haem and degrade it, releasing biliverdin and free iron, which is used as a nutrient source (Figure 1.9). IsdG and IsdI are differentially regulated by iron and haem and are required for growth of *S. aureus* when haem is the sole iron source available (Reniere & Skaar, 2008).

Following erythrocyte lysis by *S. aureus* toxins, haemoglobin is released and rapidly associates with haptoglobin. Since free haem is toxic and is found at very low concentrations, the haemoprotein-binding NEAT domains found in IsdB and IsdH are likely to be involved extracting haem from haemoglobin and haemoglobin-haptoglobin complexes. Haem can then be transferred to the haem-binding NEAT domains of IsdA or IsdC. The model of haem transfer proposed by Muryoi *et al.* is supported by data from a similar study. Zhu *et al.* reported that IsdB bound to

methehaemoglobin (oxidised form of haemoglobin), extracted haemin and directly passed it to IsdA which transferred it to IsdC. Neither IsdA nor IsdC could obtain haemin directly from methehaemoglobin (Zhu *et al.*, 2008).

The NEAT domain 2 of IsdB and NEAT 3 of IsdH were also reported to transfer haem directly to IsdC and IsdE (Muryoi *et al.*, 2008). On *S. aureus* cells IsdC is partially embedded in the cell wall while IsdE is completely buried. Direct transfer of haem to these proteins from the NEAT domains of the surface exposed IsdB and IsdH proteins seems unlikely. Instead sequential transfer of haem from IsdB and IsdH to IsdA seems more probable. Protein-protein interactions are predicted to be involved in haem transfer suggesting that the Isd proteins need to be located close enough for their functional domains to interact (Watanabe *et al.*, 2008; Zhu *et al.*, 2008).

Inactivation of components of the *isd* system does not eliminate the ability of *S. aureus* to utilise haem as an iron source, indicating that other pathways of haem acquisition exist (Skaar *et al.*, 2004).

1.3.2.6.3 Other functions of Isd proteins

As well as binding to hemin and haemoglobin, IsdA interacts which an array of host proteins. IsdA binds to transferrin and has been suggested to be the specific cell wall transferrin receptor. Studies carried out by Clarke *et al.* demonstrated that IsdA could also interact with feutin, fibronectin and fibrinogen. The Bβ and γ-chain of fibrinogen appear to be the binding site for IsdA (Clarke *et al.*, 2004). A role for IsdA in nasal colonisation has been identified (section 1.2). Vaccination of cotton rats with IsdA and IsdH was found to be protective against nasal carriage (Clarke *et al.*, 2006). Expression of IsdA by *S. aureus* also confers resistance to the innate defences of the human skin (Clarke *et al.*, 2007). This is discussed further in section 1.5.1.

IsdH has been shown to play a role in the evasion of phagocytosis. An *S. aureus isdH* mutant was engulfed more rapidly by human neutrophils, survived poorly in fresh whole human blood and was less virulent in a mouse model of sepsis. The protective mechanism promoted by IsdH seems to be a result of accelerated degradation of the serum opsonin C3b by capture and activation of complement regulatory protein factor I (Visai *et al.*, 2008). Immunisation with recombinant IsdB was highly immunogenic in rhesus macaques and was also protective in a mouse sepsis model (Kuklin *et al.*, 2006).

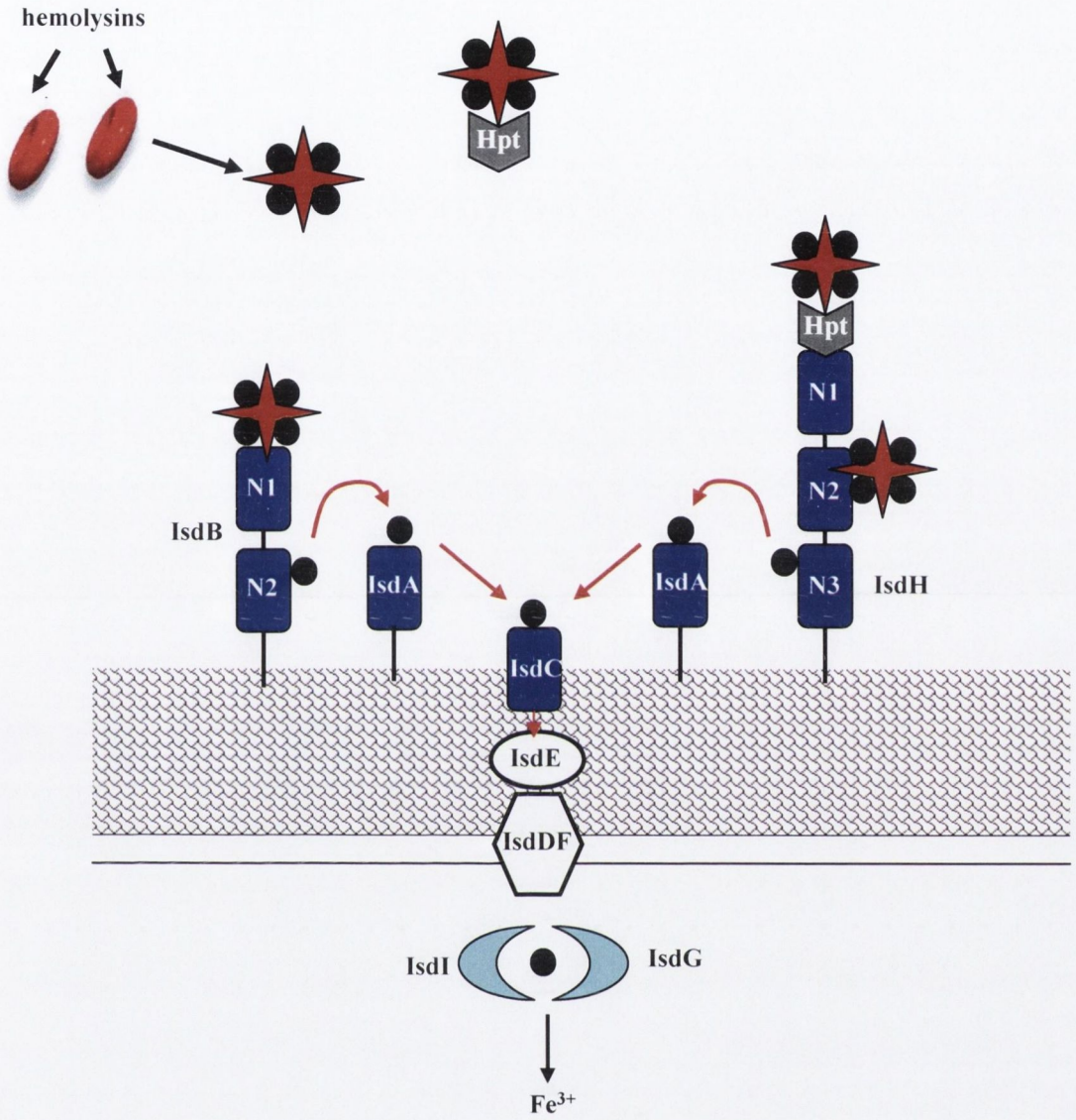


Figure 1.9. Putative pathway of haem transfer between Isd proteins of *S. aureus*

Lysis of erythrocytes by *S. aureus* toxins releases haemoglobin which rapidly associates with haptoglobin. IsdB and IsdH NEAT1 domains can bind to haemoglobin and haemoglobin-haptoglobin complexes, respectively. Haem is extracted and transferred to the IsdA NEAT domain. IsdA transfers haem to IsdC. Haem is then transferred to IsdE a component of the ABC transporter which transports haem into the cytoplasm. Haem is then degraded by haem-monoxygenases IsdG and IsdI and free iron released.

1.3.2.7 Serine-aspartate repeat (Sdr) and novel *S. aureus* surface (Sas) proteins

Serine-aspartate repeat (Sdr) proteins C, D and E are LPXTG-anchored proteins. The *sdrC*, *sdrD*, and *sdrE* genes are closely linked in the *S. aureus* chromosome although not all strains contain all three genes (Josefsson *et al.*, 1998). Expression of SdrC and SdrD by *S. aureus* was recently shown to contribute to adhesion of bacteria to squamous cells, although the ligands involved are not known (R. Corrigan and T. J. Foster, personal communication). SdrE may also contribute to platelet aggregation by *S. aureus*. Expression of SdrE by *Lactococcus lactis* promoted platelet aggregation in previous studies (O'Brien *et al.*, 2002a). Immunization with recombinant SdrD and SdrE in combination with IsdA and IsdB provided high level of protection in a murine renal infection model (Stranger-Jones *et al.*, 2006).

Analysis of *S. aureus* genome sequences identified 10 putative LPXTG-proteins (Mazmanian *et al.*, 2001; Roche *et al.*, 2003a). These were named *S. aureus* surface (Sas) proteins. SasE, SasI and SasJ have since been renamed IsdA, IsdH and IsdB, respectively. Antibodies to Sas proteins have been detected in sera from patients recovering from *S. aureus* infections indicating that these proteins are expressed *in vivo* (Roche *et al.*, 2003a). SasG is homologous to the Pls (plasmin-sensitive) surface protein present in certain MRSA strains. Pls impairs bacterial adhesion to ligands such as fibrinogen and promotes adherence to nasal epithelial cells (Savolainen *et al.*, 2001). Similar roles have been identified for SasG (Corrigan *et al.*, 2007; Roche *et al.*, 2003b). Expression of SasG masked the ability of *S. aureus* to bind to IgG, cytokeratin 10, fibrinogen and fibronectin. SasG also promoted adhesion to desquamated nasal epithelial cells and contributed to the formation of PIA independent biofilm (Corrigan *et al.*, 2007). SasA (also known as SraP) can promote *S. aureus* binding to platelets (Siboo *et al.*, 2005).

1.3.3 Secreted virulence factors

Many of the potential virulence determinants produced by *S. aureus* are secreted extracellular proteins. The cytolytic toxins α-, β- and γ-haemolysin are produced at later stages of infection. They form pores in erythrocytes, leukocytes and platelets which results in destruction of host cells (Bhakdi *et al.*, 1988; Montoya & Gouaux, 2003). Panton-Valentine leukocidan (PVL) is a 2-component, cytolytic toxin highly specific for leukocytes (Kaneko & Kamio, 2004). The *pvl* gene is located on a lysogenic bacteriophage which is present in a minority of strains, but is strongly

associated with community acquired (CA)-MRSA. Expression of PVL is associated with severe skin infections and necrotizing pneumonia (Labandeira-Rey *et al.*, 2007; Peacock *et al.*, 2002). The role of leukocytes in immune evasion is discussed in section 1.5.3.

Secreted enzymes and proteases produced by *S. aureus* such as aureolysin and staphopain A/B are involved in tissue destruction and may facilitate spread of infection to adjoining tissue (Shaw *et al.*, 2004). Coagulase is a secreted protein that binds fibrinogen and also activates prothrombin to initiate the host blood coagulation pathway (Boden & Flock, 1992; Phonimdaeng *et al.*, 1990). Another extracellular fibrinogen binding protein, Efb has been shown to block platelet aggregation and inhibit complement fixation (Lee *et al.*, 2004b; Shannon & Flock, 2004)(section 1.5.6 and 1.6.). Expression of superantigen toxins by *S. aureus* is associated with toxic shock syndrome and food poisoning (section 1.5.8; Bohach *et al.*, 1990; McCormick *et al.*, 2001).

1.4 Regulation of virulence factors

Expression of cell surface adhesins from *S. aureus* is generally accepted to take place during the exponential phase of growth while secreted proteins and toxins are expressed as cells approach stationary phase (Chan & Foster, 1998; Novick, 2003). Expression of adhesins early in infection is believed to facilitate colonisation. Secreted exoproteins are produced later in infection and are involved in damage to host tissues, evading the host immune response and facilitating detachment and spreading throughout the body (McAleese *et al.*, 2001; McGavin *et al.*, 1997).

The regulatory network in *S. aureus* is complex and depends on the interplay of sigma factors, transcription factors and signal transduction via two-component regulatory systems. The interactions between regulators are likely to differ *in vivo* from those that have been described *in vitro*.

1.4.1 Two-component regulatory systems

Two-component regulatory systems consist of a sensor, which is often an integral membrane protein, and a response regulator, which is cytoplasmic. In response to particular stimuli the enzymatic domain of the sensor becomes phosphorylated. This in turn activates the response regulator which acts on target genes, either directly or indirectly to initiate transcription (Stock *et al.*, 1989).

Analysis of the genome sequence of *S. aureus* has identified up to 16 potential two-component regulatory systems. The best characterised is the accessory gene regulator (Agr), which responds to increasing cell density during exponential growth and is largely responsible for the temporal switch in protein expression in the post-exponential phase of growth. The stimuli to which each of the two-component systems of *S. aureus* respond to has not been fully elucidated. Those encoded by *saeRS*, *arlRS*, *ssrAB*, *lytRS*, *yycGF*, *vraSR* and *yhcSR* have been characterised to some degree (Bronner et al., 2004).

1.4.1.1 The accessory gene regulator

The Agr system is a two-component cell density-sensing system which is activated in the late-exponential growth phase. Agr upregulates many secreted proteins of *S. aureus* while downregulating surface-associated proteins (Dunman *et al.*, 2001).

The *agr* locus consists of two transcription units, driven by promoters P2 and P3. The P2 operon encodes AgrACDB, components of the sensing system, while the P3 transcript is RNAIII, the effector molecule. AgrC is a transmembrane protein and sensor of the Agr system (Lina *et al.*, 1998). It is activated upon extra cellular accumulation of the secreted auto-inducing peptide (AIP). AIP is the product of AgrD and is post-translationally modified by AgrB (Zhang *et al.*, 2002). AIP binds to the extracellular N-terminal domain of AgrC and at a specific concentration activates AgrC (Ji *et al.*, 1995). This leads to phosphorylation of the response regulator AgrA, which then binds to both the P2 and P3 promoters, inducing production of AIP and transcription of RNAIII (Figure 1.10, Novick *et al.*, 1995). An additional RNAIII activating pathway induced by RNAIII activating protein, RAP has been reported (Korem *et al.*, 2003).

As well as acting as a global regulator, RNAIII encodes the toxin δ-haemolysin. RNAIII is thought to act either by binding the RNA encoding target proteins and inhibiting translation or by binding regulatory proteins and effectively neutralising them. RNAIII positively regulates α-haemolysin by binding to *hla* mRNA. Complementary sequences at the 5′ end of RNAIII prevent *hla* mRNA folding into an untranslatable configuration, thus promoting translation (Novick, 2003). RNAIII negatively regulates Rot. The 3′ domain of RNAIII is believed to associate with the 5′ untranslated region of *rot* mRNA through loop-loop interactions. Translation of *rot* is

inhibited by the base pairing of RNAIII with the ribosome binding region of *rot* mRNA. Subsequent cleavage of *rot* mRNA is mediated by RNase III (Boisset *et al.*, 2007; Geisinger *et al.*, 2006). A similar mechanism inhibits translation of *spa* mRNA (Huntzinger *et al.*, 2005). Other studies have suggested that RNAIII acts to neutralise Rot protein rather than inhibiting translation of *rot* mRNA (McNamara *et al.*, 2000).

The *agr* system of *S. aureus* has 4 specificity groups in which the AIP and the sensor region of AgrC vary in sequence. AIP of one group can block autoinduction and virulence gene expression of other groups. The *agr* system has been shown to play a role in pathogenesis of several animal infection models and is associated with increased persistence *in vivo* (Abdelnour *et al.*, 1993; Gillaspy *et al.*, 1995).

1.4.1.2 The haem sensor system

The majority of iron inside the human host is sequestered in complexes with haem-containing proteins. Iron is essential for the growth and survival of bacteria and *S. aureus* has several mechanisms of capturing haem and extracting iron from this complex (section 1.3.2.6 and 1.5.10). The accumulation of excess haem in the cytoplasm is potentially toxic. *S. aureus* has a haem sensor system (HssRS) which responds to exess haem in the cytoplasm. This system consists of haem sensor HssS and the response regulator HssR. Upon activation HssR binds to the *hrtAB* promoter and induces the expression of HrtAB which excretes excess haem from the cytoplasm (Torres *et al.*, 2007).

1.4.2 Transcription factors

1.4.2.1 SarA family

Transcription factors interact with target gene DNA to increase or decrease the rate of transcription. In *S. aureus* transcription factors share sequence and structural homology with the staphylococcal accessory regulator (SarA). A conserved winged-helix structure is common to SarA and its homologues (Cheung *et al.*, 2004). The proteins in this family can be categorised on the basis of their structure: single-domain proteins that occur as homodimers and two-domain proteins that occur as monomers.

SarA and its homologues have a canyon-like structure and a concave DNA-binding surface at their centre. Based on this structure several mechanisms of regulation have been proposed. SarA and its homologues have been proposed to hold promoter sequences in optimal or sub-optimal positions to increase or decrease

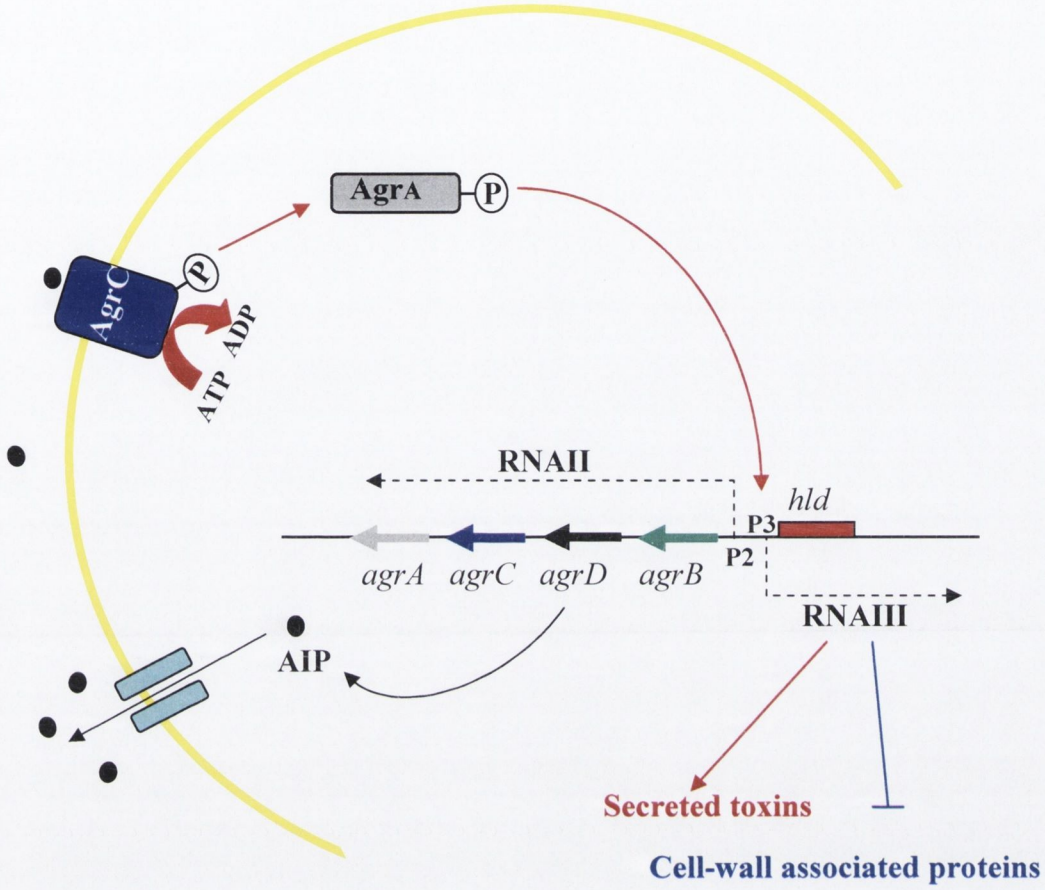


Figure 1.10. The Agr system

AgrD is processed by AgrB to form the secreted auto-inducing peptide, AIP. Binding of AIP to the sensor AgrC results in phosphorylation of AgrA, which then binds to the P2 and P3 promoter regions causing auto-induction of the system. The effector molecule of *agr* is RNAIII, produced from P3. It positively regulates secreted toxins and enzymes and negatively regulates cell-wall associated proteins in the post-exponential growth phase

transcription. Binding of multiple dimers to promoter sequences may bend the DNA into a closed configuration, encircling the Sar proteins and preventing transcription in a similar manner to mammalian histones. Formation of heterodimers between one-domain SarA homologs may interfere with the function of homodimers, resulting in new patterns of expression under certain growth conditions (Cheung *et al.*, 2004).

1.4.2.1.1 Staphylococcal accessory regulator

SarA is a global regulator of *S. aureus* virulence factors which is predominantly expressed in late exponential growth phase (Manna & Cheung, 2001). It is encoded on three overlapping transcripts from promoters P1, P2 and P3. Promoters P1 and P2 are dependent on the housekeeping sigma factor, SigA for transcription while P3 is dependent the stationary growth phase accessory sigma factor, SigB.

SarA is a single-domain protein that occurs as a homodimer. It binds to an AT-rich consensus sequence in the promoter regions of target genes (Chien *et al.*, 1999; Sterba *et al.*, 2003). SarA upregulates transcription of *agr* by binding between the *agr* P2 and P3 promoters. It also upregulates expression of FnBPs and haemolysins. Expression of protein A, aureolysin and the cysteine protease SspB are inhibited by SarA (Dunman *et al.*, 2001). SarA activates its own expression and is downregulated by another single domain transcription factor, SarR (Bayer *et al.*, 1996; Manna & Cheung, 2001).

1.4.2.1.2 Repressor of toxins

The repressor of toxins (Rot) is a large one-domain SarA homologue. It was identified by McNamara *et al.* as a repressor of α-toxin in the exponential growth phase. Later studies found that Rot is a global regulator which repressed expression of secreted toxins and enzymes while also activating certain cell wall-associated proteins expressed in the exponential growth phase (Said-Salim *et al.*, 2003). Rot therefore seems to be an antagonist of *agr* regulation and is believed to be important in the early steps of infection.

Many adhesins including ClfB are positively regulated by Rot. The two domain SarA homologue, SarS, is positively regulated by Rot and it in turn positively regulates Spa. Increased expression of adhesins facilitates colonisation early in infection. Rot negatively regulates factors associated with the later stages of infection

such as lipase, α- and β-haemolysins and the *spl* and *ssp* protease operons (Said-Salim *et al.*, 2003).

Transcription of Rot occurs throughout all growth phases. However, it is believed to be inhibited by RNAIII post-transcriptionally as described in section 1.4.1.1. In the post-exponential growth phase when the *agr* system is active, Rot is inactivated partly through RNAIII. This leads to increased exotoxin production and repression of cell wall-associated factors.

1.4.2.1.3 The multiple gene regulator

The multiple gene regulator (MgrA) is a global regulator that belongs to the SarA family. It contains a helix-turn-helix DNA binding motif and regulates certain virulence genes by binding directly to their promoter regions. Microarray studies and RT-PCR have indicated that MgrA acts in a similar way to Agr, by upregulating exoproteins and downregulating surface-associated proteins (Luong *et al.*, 2006). MgrA upregulates production of capsular polysaccharide, SrtA, serine proteases, leukotoxins and α-hemolysin. It downregulates expression of Spa via SarS (Ingavale *et al.*, 2005). MgrA is involved in autolysis and antibiotic resistance (Ingavale *et al.*, 2003; Truong-Bolduc *et al.*, 2003). Recently it has been reported that MgrA contributes to the severity of experimental sepsis and septic arthritis (Jonsson *et al.*, 2008).

1.4.2.1.4 Ferric uptake regulator

The ferric uptake regulator (Fur) of *S. aureus* is homologous to Fur of *B. subtilis*. Fur has conserved metal binding domains and motifs at its C terminus. A DNA binding motif located at the N terminus of Fur is characteristic of metalloregulatory proteins. When environmental iron levels are sufficiently high, Fur forms a complex with ferrous iron and acts as a transcriptional repressor. Genes regulated by Fur have a concensus sequence known as the Fur box located within their promoter regions. The Fur box sequence consists of a 19-bp inverted repeat which is highly homologous to the Fur box of *E. coli* and *B. subtilis* (Xiong *et al.*, 2000).

Fur regulates many systems involved in iron homeostasis. Expression of genes encoding the haem transport system (*htsABC*), the Isd proteins and *S. aureus* siderophores are repressed by Fur. *S. aureus* has two Fur homologues PerR and Zur. PerR is a manganese dependent repressor involved in oxidative stress response and

iron storage (Horsburgh *et al.*, 2001a). PerR represses transcription of Fur and regulates transcription of *katA* in coordination with Fur (Horsburgh *et al.*, 2001b). Studies have determined that Zur mediates zinc homeostasis (Lindsay & Foster, 2001). Another metalloregulatory protein of *S. aureus* is MntR. MntR regulates expression of *mntABC* and *mntH*, which encode putative manganese transporters (Horsburgh *et al.*, 2002b).

1.4.3 Sigma Factors

Bacterial sigma factors are involved in the initiation of transcription in response to various stimuli. Association of sigma factor with the core RNA polymerase is required to allow recognition of promoter elements and to form the RNA polymerase holoenzyme which initiates transcription. *S. aureus* has three known σ-factors, SigA, SigB and SigH which recognise different promoter sequences (Bronner *et al.*, 2004).

1.4.3.1 Sigma factor A

Sigma factor A (SigA) is the vegetative sigma factor that is involved in the transcription of essential housekeeping genes (Deora & Misra, 1996). SigA is necessary for cell growth and disruption of the gene encoding SigA is lethal in *S. aureus* strains. There is significant homology between SigA and the primary sigma-factors of *B. subtilis* and *E. coli* (Deora & Misra, 1996).

1.4.3.2 Sigma factor B

The accessory sigma-factor, SigB is involved in response to environmental stress. Microarray analysis reported that SigB is activated when cells are exposed to heat shock, alkaline shock or when grown in the presence of NaCl or MnCl (Pane-Farre *et al.*, 2006). It is homologous to SigB of *B. subtilis* which is also involved in the stress response (Hecker *et al.*, 2006). In *S. aureus* expression of SigB-dependent genes is enhanced in stationary growth phase cells *in vitro*. *In vivo* studies have also demonstrated that markers of SigB activity are expressed late in infection (Goerke *et al.*, 2005).

The *sigB* loci of *B. subtilis* and *S. aureus* encode post-translational regulators of SigB. The genes *rsbU*, *rsbV* and *rsbW* are located directly upstream of the *sigB* gene in *S. aureus*. RsbW is an anti-sigma-factor which binds to and sequesters SigB (Miyazaki *et al.*, 1999). RsbU is a phosphatase which acts on RsbV. In its

dephosphorylated form RsbV can bind competitively to RsbW and release SigB (Palma & Cheung, 2001). Differential transcription from the *rsbUVWsigB* operon has been proposed to regulate the level of free SigB in *S. aureus*. In the exponential growth phase transcription of the *rsbUVWsigB* operon results in basal expression of *sigB* from *S. aureus*. In the stationary growth phase a short transcript that encodes *sigB* only is induced at high levels and raises the concentration of free SigB in cells (Kullik & Giachino, 1997). *S. aureus* strains 8325 and RN6390 have an 11-base-pair deletion in *rsbU* (Kullik et al., 1998). This deletion affects pigmentation, protease and haemolysin activity and the response to cellular stress (Giachino *et al.*, 2001; Horsburgh *et al.*, 2002a).

In the stationary growth phase SigB upregulates *sarA*, *sarS* and *arlRS* (Bischoff *et al.*, 2001; Bischoff *et al.*, 2004). Expression of ClfA in the stationary growth phase is dependent on SigB. Genes regulated by SigB have a consensus SigB-binding site (GttTaa – N_{12-15} – gGGTAt, Gertz *et al.*, 2000; (Homerova *et al.*, 2004)). Mutation of *sigB* results in attenuation of virulence in the murine model of sepsis and septic arthritis but has no apparent effect on the outcome of experimental rat endocarditis (Entenza *et al.*, 2005; Jonsson *et al.*, 2004).

1.4.3.3 Sigma factor H

S. aureus has an additional accessory sigma-factor, SigH. It is homologous to SigH of *B. subtilis* which is involved in regulation sporulation and the development of natural genetic competence (Grossman, 1995). SigH of *S. aureus* may also regulate of the development of natural DNA competence (Morikawa *et al.*, 2003).

1.5 Immune evasion by *S. aureus*

1.5.1 Survival on skin

The outer layer of human skin (stratum corneum), provides a physical barrier composed of tightly associated desquamated epithelial cells. To survive on the skin, bacteria have to overcome acidic conditions as well as antimicrobial peptides (AMPs) and fatty acids. Fatty acids and AMPs bind the bacterial cell surface by hydrophobic interactions. Expression of IsdA makes the *S. aureus* cell surface hydrophilic and confers resistance to lipids and cationic AMPs. This activity was localised to the C terminal domain of IsdA (Clarke *et al.*, 2007). Clarke *et al.* demonstrated that IsdA was required for survival of *S. aureus* on the skin of healthy human volunteers.

S. aureus is also protected from antimicrobial peptides on the surface of skin by modifications to teichoic acids and phosphatidyl glycerol. Teichoic acids are modified by substitutions with D-Alanine mediated by the Dlt proteins while the MprF enzyme adds L-lysine residues to phosphatidylglycerol exposed on the cytoplasmic membrane (Collins *et al.*, 2002; Peschel *et al.*, 1999). These modifications reduce the negative charge at the cell surface which repulses cationic antimicrobial peptides such as defensins. The secreted *S. aureus* proteins staphylokinase and aureolysin also inactivate human defensin peptides (Jin *et al.*, 2004; Sieprawska-Lupa *et al.*, 2004).

Community-associated MRSA strain USA-300 has an arginine catabolic mobile element (ACME) integrated directly adjacent to the type IV SCC*mec* cassette. ACME encodes for enzymes of the arginine deiminase pathway which convert arginine into carbon dioxide, ATP and ammonia. It has been suggested that production of ammonia aids bacterial survival on the acid environment of the skin and may in fact be responsible for the rapid spread of CA-MRSA (Diep *et al.*, 2008).

Lysozyme and lactoferrin are present in many body fluids and are important components of innate defenses against bacterial infections. A membrane-bound O-acetyltransferase modifies muramic acid and confers lysosyme resistance to peptidoglycan of *S. aureus* (Bera *et al.*, 2005). The surface protein IsdA binds lactoferrin and neutralizes its anti-bacterial activity (Clarke & Foster, 2008).

1.5.2 Inhibition of neutrophil chemotaxis

A strong inflammatory response is induced at sites were the skin and mucous surfaces are breached. Neutrophils and macrophages migrate to the site of infection to engulf and dispose of invading *S. aureus*. Phagocytes are attracted by formylated peptides released by growing bacteria as a by-product of bacterial translation and by chemo-attractant molecules released during complement activation (C3a and C5a) (Gasque, 2004).

The secreted chemotaxis inhibitory protein of *S. aureus* (CHIPS) is expressed by 60% of clinical isolates (de Haas *et al.*, 2004). It binds to receptors for complement fragment C5a (C5aR) and the formylated peptide receptor (FPR) on neutrophils and monocytes. This inhibits chemotaxis and leukocyte migration to sites of infection (Haas *et al.*, 2004; Postma *et al.*, 2005). The formylated peptide receptor (FPR) has a homologue, FPR-like 1 (FPRL1). *S. aureus* produces a protein with 28% identity to

CHIPS called FLPR-1 inhibitory protein (FLIPr). This protein binds to FPRL-1 and inhibits neutrophil responses to FPR-like agonists (Prat *et al.*, 2006). CHIPS and FLIPr are likely to function together to protect *S. aureus* from early detection by the innate immune system.

Other *S. aureus* proteins involved in inhibition of neutrophil migration include the staphylococcal superantigen-like 5 (SSL5) protein and Eap. SSL5 binds to the P-selectin ligand on neutrophils and prevents capture of phagocytic cells at sites of inflammation (Bestebroer *et al.*, 2007). Eap binds the intercellular adhesion molecule-1 (ICAM-1) on the surface of endothelial cells. This disrupts receptor-mediated leukocyte adhesion to endothelial cells which is essential for recruitment of inflammatory cells to the site of infection. (Chavakis *et al.*, 2002).

1.5.3 Toxins that kill leukocytes

S. aureus expresses cytolytic leukotoxins that kill neutrophils. Bi-component leukotoxins are composed of two subunits that assemble into oligomers in the membranes of leucocytes resulting in pore formation (Kaneko & Kamio, 2004). Bi-component leucotoxins include γ-haemolysin (Hlg), Panton-Valentine leucocidin (PVL), leucocidin E/D and leucocidin M/F-PV-like. The γ-toxin lyses both erythrocytes and leucocytes whereas PVL only lyses leucocytes (Menestrina *et al.*, 2003).

1.5.4 Resistance to opsonophagocytosis

Expression of capsule and anti-opsonic proteins by *S. aureus* interferes with the deposition of antibodies and complement on the bacterial cell surface. This prevents efficient phagocytosis by neutrophils (Thakker *et al.*, 1998).

Protein A binds the Fc-region of IgG, coating bacteria with IgG molecules in the incorrect orientation to trigger complement fixation and to be recognized by neutrophil Fc-receptors. *S. aureus spa* mutants are phagocytosed more efficiently *in vitro* and exhibit decreased virulence in several animal infection models (Palmqvist *et al.*, 2002; Patel *et al.*, 1987). ClfA also displays anti-phagocytic properties. Coating of bacteria expressing ClfA with fibrinogen inhibits deposition of opsonins (Higgins *et al.*, 2006; Palmqvist *et al.*, 2004).

Expression of capsular polysaccharide reduces phagocytosis *in vitro* and is associated with increased virulence in animal infection models (Luong & Lee, 2002; Thakker *et al.*, 1998). Capsule inhibits binding of antibodies to *S. aureus* cell surface components which hinders opsonisation (Thakker *et al.*, 1998). It also obstructs access of phagocyte complement receptors to complement components assembled beneath the capsule layer (Cunnion *et al.*, 2003). Reduced O-acetlylation of capsular polysaccharide decreases antiphagocytic activity, due to increased antibody penetration and cell surface recognition (Bhasin *et al.*, 1998).

S. aureus also secretes a number of proteins that can interfere with complement fixation on the bacterial cell surface and thus inhibit phagocytosis by neutrophils. These proteins are discussed in section 1.5.6.

1.5.5 The complement system

The complement system is part of the innate immune response and can also initiate the adaptive immune response (Carroll, 2004). Complement activation results in a cascade of enzymatic cleavage and protein-protein interactions of complement components which are deposited on the bacterial surface to form the membrane-attack-complex (MAC). Insertion of the MAC complex into the cell membranes of certain bacteria creates trans-membrane pores and causes osmotic lysis of cells (Cooper, 1999). *S. aureus* is protected from the action of the MAC complex by the peptidoglycan cell wall. Instead deposition of complement components on the *S. aureus* cell surface marks cells for destruction by phagocytic cells (Rooijakkers *et al.*, 2005b).

There are three pathways which trigger complement activation. Recognition of antigen-bound IgG triggers the classical complement pathway (CCP). Components C1q and two molecules of C1r and C1s make up the C1 complex found in plasma. Binding of C1q to antibody-antigen complexes results in activation of C1r and C1s. Complement component C4 is cleaved by activated C1s into two fragments, C4a and C4b. Fragment C4a diffuses from the cell surface while fragment C4b covalently binds to the target surface and clusters around the bound C1 molecules. Complement component C2, binds to immobilized C4b and is cleaved by activated C1s into fragments C2a and C2b. Fragment C2b diffuses away while C2a remains bound to C4b forming the C4b2a complex which acts as the C3 convertase of the CCP (Figure 1.11).

The mannose-binding lectin (MBL) pathway of complement activation is triggered by conserved sugar residues found on microbe cell surfaces. The MBL associated proteins MASP-1 and MASP-2 act in a similar manner to C1r and C1s of the classical pathway to cleave C4 and C2 and facilitate formation of the C3 convertase, C4b2a.

Unlike the CCP and MBL pathways of complement activation the alternative pathway (ACP) lacks a specific pathogen recognition molecule. Instead low-level cleavage of component C3 by plasma enzymes generates C3b which binds covalently to the pathogen surface. If no pathogens are present the cleavage products of C3 are deactivated. Factor B binds to C3b on the surface of pathogens and is cleaved by Factor D, a serine-protease resulting in fragment Bb which remains bound to C3b and fragment Ba which diffuses away. Surface bound C3bBb acts as the C3 convertase of the ACP.

The three pathways of complement activation converge after the formation of C3 convertase. The most abundant component of complement, C3, is cleaved by the C3 convertase complexes resulting in fragments C3a and C3b. Fragment C3a, diffuses away from the cell and is involved in stimulating inflammation while C3b molecules become attached to the bacterial surface. Bound C3b and its degradation products act as opsonins and promote phagocytosis. C3b can bind to C3 convertase complexes to form a C5 convertase complex (C4b2a3b or $C3b_2Bb$). The final events of the complement cascade are triggered by C5 convertases. Binding of C3b to the pathogen surface also provides a substrate for generation of C3 convertases by the ACP and accelerates complement fixation.

The cleavage of complement component C5 into fragments C5b and C5a is catalyzed by C5 convertase. Fragment C5a induces potent pro-inflammatory responses, and triggers chemotaxis. Fragment C5b is involved in a cascade of protein-protein interactions that result in the formation of C5b-9, the membrane-attack-complex (MAC). C5b binds to complement component C6, which is followed by binding of C7 to C5b6. The resulting complex C5b-7, inserts into target membranes and subsequently binds one C8 molecule. The C5b-8 complex becomes inserted further into the membrane. Finally polymerization of component C9 on C5b-8 complex produces the MAC (Hu *et al.*, 1981).

1.5.6 Inactivation of complement by *S. aureus*

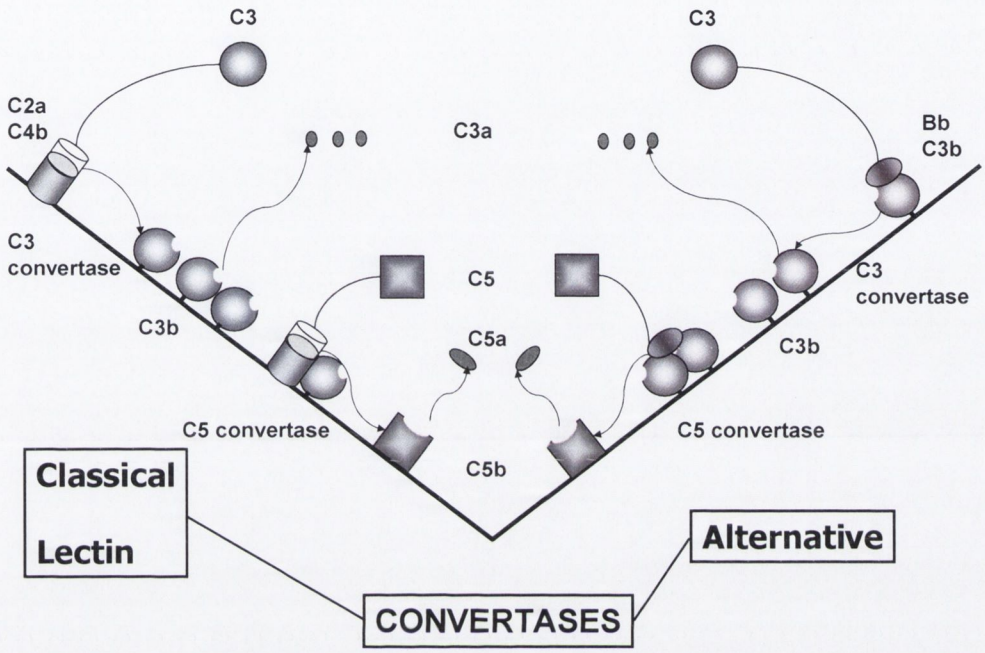


Figure 1.11. Complement fixation

Recognition of antigen-bound IgG triggers the classical complement pathway. The mannose-binding lectin pathway of complement activation is triggered by mannose sugar residues on microbe cell surfaces. The cleavage of C3 results in fragments C3a and C3b. The neutrophil chemoattractant C3a diffuses away while C3b is covalently linked to the cell surface. Accumulation of C3b results in the formation of C5 convertases that cleave C5 releasing the neutrophil chemoattractant C5a.

S. aureus secretes a small protein called staphylococcus complement inhibitor (SCIN) which binds to and stabilizes both C4bC2a and C3bBb. This blocks further C3b deposition by all complement pathways and inhibits phagocytosis of *S. aureus* by neutrophils (Rooijakkers *et al.*, 2005a).

The secreted extracellular fibrinogen-binding protein, Efb binds to complement factor C3 and blocks its deposition on the cell surface (Lee *et al.*, 2002; Lee *et al.*, 2004a; Lee *et al.*, 2004b). Blockage of C3 deposition by Efb inhibits all pathways of complement activation and deposition (Lee *et al.*, 2004b).

Staphylokinase is secreted plasminogen activator of *S. aureus*. Conversion of plasminogen to plasmin by Sak occurs on the staphylococcal surface. Plasmin cleaves the entire Fc fragment of IgG including the glycosylation site essential for recognition by C1q. Surface bound C3b is also cleaved, resulting in inhibition of complement fixation (Rooijakkers *et al.*, 2005c).

Complement factors I and H are natural down-regulators of complement fixation. Recently ClfA was shown to trigger the capture and activation of factor I, which may contribute to the inhibition of phagocytosis by surface-bound ClfA (Hair *et al.*, 2008).

1.5.7 Survival of *S. aureus* in neutrophil phagosomes

If *S. aureus* becomes phagocytosed it has mechanisms to survive within phagosomes. *S. aureus* is partially protected from toxic O_2^- and $NO^{\cdot}$ free radicals by expression of the yellow carotenoid pigment (Liu *et al.*, 2005). It can also express enzymes to remove and inactivate free radicals (Richardson *et al.*, 2008; Singh & Moskovitz, 2003). Resistance to cationic antimicrobial peptides found in the phagosome is mediated by modifications to the bacterial cell surface which reduce net negative charge (see section 1.5.1). The secreted *S. aureus* proteins aureolysin and staphylokinase also neutralize cationic peptides (section 1.5.1).

1.5.8 Toxin superantigens

Many *S. aureus* strains secrete superantigen toxins that are associated with food poisoning and toxic shock syndrome (Fraser *et al.*, 2000; Michie & Cohen, 1998). Superantigens bind to invariant regions of the MHC class II molecules on the surface of antigen-presenting cells and link them to the T-cell receptor on the surface of T helper cells. This process is antigen-independent. When expressed at high levels, superantigen toxins cause the unrestricted expansion of T-cells and massive release of

cytokines from macrophages and T cells. Release of cytokines causes toxic shock resulting in tissue damage and multi-organ dysfunction (Marrack & Kappler, 1990). Low-level expression of superantigen toxins can cause immune suppression by the localized depletion of T cells.

1.5.9 Major histocompatibility complex class II-analog protein

The major histocompatibility complex class II-analog protein (Map, also known as Eap) is composed of 6 repeated domains, each of which contains a 30-amino acid motif with strong homology to the MHC class II β-chain (Jonsson *et al.*, 1995). This motif allows Map/Eap to bind to T cell receptors and inhibits antigen presentation resulting in reduced T cell proliferation (Lee *et al.*, 2002). Map has been shown to inhibit clearance of bacteria from abscesses in chronically infected mice (Lee *et al.*, 2002). By directing the immune system towards a Th2-type response, Map may decrease phagocytic uptake of bacteria and enhance intracellular survival of *S. aureus* (Harraghy *et al.*, 2003).

1.5.10 Lack of available iron

An important innate defence mechanism against bacterial survival and growth inside the human host is the lack of available iron. Iron is required by bacteria as a structural component of cytochromes and is essential as a cofactor for bacterial antioxidant defence enzymes such as catalase and peroxidise. The majority of iron is sequestered by haem-containing proteins in the host while a small percentage is also found in iron binding proteins such transferrin and lactoferrin. At physiological pH iron has a low solubility which also contributes its unavailability. *S. aureus* expresses an array of proteins to capture and extract iron from host proteins.

Siderophores are secreted, high affinity iron chelators which bind to free iron and can also remove iron from host proteins. *S. aureus* produces four siderophores including staphyloferrin A, staphyloferrin B, aureochelin and staphylobactin (Courcol *et al.*, 1997; Dale *et al.*, 2004a; Drechsel *et al.*, 1993; Konetschny-Rapp *et al.*, 1990). Specific receptors and ABC transporters actively transport siderophores with bound iron back into the cell.

The *sirABC* operon is regulated by the iron-responsive transcriptional regulator Fur and encodes an iron transport system essential for staphylobactin uptake (Dale *et al.*, 2004b). This operon is divergently transcribed from the *sbn* operon and encodes

for SirA (a lipoprotein) while SirB and SirC make up an ATP-dependent ABC transporter. Mutating *sbn*, *sirA* and *sirB* genes in *S. aureus* resulted in growth deficiencies in iron restricted media (Dale *et al.*, 2004b).

Other siderophore uptake systems are encoded by the iron-regulated locus *sstABCD* locus and the *fhu* (ferric hydroxamate uptake) operon. The *fhu* operon is involved in uptake of hydroxamate siderophores and encodes 2 lipoproteins that direct siderophores to an ATP-binding ABC transporter system made up of FhuC, FhuB and FhuG (Sebulsky *et al.*, 2003).

Approximately 6-7.5 % of iron in humans is found in complex with the extracellular iron carrier transferrin. Transferrin can be utilised as an iron source by *S. aureus* (Taylor & Heinrichs, 2002). Capture of iron from transferrin may occur through acquisition by siderophores and subsequent uptake by siderophore receptors. Another mechanism of uptake involving a specific cell wall transferrin receptor is also thought to exist. IsdA has transferrin binding activity and has been suggested as the specific transferrin receptor (Clarke *et al.*, 2004). In conditions of iron starvation an increase in production of acidic end-products occurs as a result of redirection of central metabolic pathways (Friedman *et al.*, 2006). Friedman *et al.* reported that this decrease in pH facilitates the release of iron from transferrin making it available to *S. aureus*.

Haemoproteins are the most abundant source of iron in the host with 70% of total iron stores found associated with haemoglobin. Skaar *et al.* demonstrated that *S. aureus* preferentially utilises haem rather than transferrin as an iron source (Skaar *et al.*, 2004). *S. aureus* captures haem using two haem-iron uptake systems. The haem transport system (*htsABC*) encodes an ABC transporter permease made up of HtsB and HtsC. A Fur box is located upstream of *htsA* indicating that the haem transport system is likely to be under the control of Fur. Mutations in *htsC* and *htsB* caused decreased virulence of *S. aureus* in intravenously infected mice (Skaar *et al.*, 2004).

The iron surface determinants (Isd) of *S. aureus* are also involved in capture of haem from host proteins and the subsequent transport of haem across the bacterial envelope (section 1.3.2.6). It has been suggested that haem-iron is predominantly utilised early in systemic infection. At later stages bacteria found in tissues devoid of haem-iron may utilise siderophores to aquire iron (Skaar *et al.*, 2004).

The accumulation of haem in the cytoplasm is potentially toxic and acts as a signal to stress-sensing systems of *S. aureus*. As a result expression of HrtA and HrtB

proteins is induced. These proteins are involved in export of haem from the cytoplasm. The transcription of certain virulence factors is also decreased and host tissue damage is minimised. *S. aureus hrtA* or *hrtB* mutants express increased levels of immunomodulatory proteins such as SSL5, Map-W, Efb, FLIPr and Eap and have enhanced virulence (Torres *et al.*, 2007).

1.6 Bacteria, platelets and cardiovascular disease

Following vascular damage opportunistic pathogens can gain entry to the human circulatory system which leads to the development of bacteraemia. Complications of bacteraemia include the development of serious cardiovascular infections such as infective endocarditis (IE), disseminated intravascular coagulation (DIC), thrombocytopenia, atherosclerosis and myocardial infarction (Fitzgerald *et al.*, 2006a).

Infective endocarditis (IE) is characterised by the build up of vegetative bodies on heart valve surfaces which contain clusters of bacteria, fibrin and aggregated platelets (Mylonakis & Calderwood, 2001). Complications of IE include heart failure due to valve damage and septic embolism. Prior valve damage is a risk factor for IE and in the past chronic rheumatic heart disease was often associated with IE. However, *S. aureus* is able to cause endocarditis in patients with undamaged heart valves. Increased use of intravascular devices and invasive procedures in hospital settings has lead to an increase in bacteraemic infections and a new at-risk population group for IE. Enterococci and streptococci can cause IE but *S. aureus* is now acknowledged to be the leading cause. High mortality rates are associated with nosocomial IE and emergence of multi-drug resistant strains has complicated treatment of this condition (Moreillon & Que, 2004).

The ability of *S. aureus* and other pathogens to cause platelet aggregation is thought to contribute to the development of IE. Inhibitors of platelet aggregation lower bacterial titres in thrombi and result in smaller vegetations in experimental endocarditis (Kupferwasser *et al.*, 1999; Nicolau *et al.*, 1993). Strains of *S. aureus* that are defective in platelet binding and subsequent activation are also less virulent in enodcarditis models. *S. aureus* is thought to have a high affinity for pre-existing lesions on heart valve surfaces (Niemann *et al.*, 2004). Bacteria can colonize sterile developing thrombi on damaged heart valves and subsequently enhance thrombi by capturing and activating circulating platelets. In patients with no prior heat damage

IE is believed to develop when small circulating thrombi consisting of bacteria and platelets are deposited on the heart valve surface.

1.6.1 Biology and function of platelets

Platelets are anucleate cells which play a crucial role in haemostasis and contribute to inflammation and anti-microbial defence (Ni & Freedman, 2003). They are the most numerous cells found in the bloodstream. Platelet activation leads to formation of a thrombus at sites of vascular damage which acts as a hemostatic plug. Granules within platelets contain ADP, serotonin, von Willebrand Factor (vWF), fibrinogen and calcium. Release of granule contents upon activation can regulate thrombus formation and its subsequent growth (Fitzgerald *et al.*, 2006a).

Thrombus formation is initiated by platelet adhesion to extracellular matrix (ECM) proteins that become exposed at sites of vascular damage. Platelets have a number of receptors on their cell surface which recognise ECM components. The most abundant receptor on the platelet surface is the $\alpha_{IIb}\beta_3$ integrin also known as glycoprotein (GP) IIb/IIIa which recognises fibrinogen, fibronectin and vWF. Other integrin receptors found on platelets interact with collagen ($\alpha_2\beta_1$), fibronectin ($\alpha_5\beta_1$), vitronectin ($\alpha_V\beta_3$) and laminin ($\alpha_6\beta_1$). The GPIb/V/IX complex interacts with vWF and is crucial in regulating haemostasis in high shear conditions. Receptors GPVI and FcγRIIa belong to the immunoglobulin receptor superfamily and interact with collagen and the Fc region of antibodies respectively. Other receptors on platelets recognise factors such as thrombin (receptors PAR-1 and PAR-4) and ADP (receptors $P2Y_1$ and $P2Y_{12}$) (Calvete, 1999; Fitzgerald *et al.*, 2006a).

The initial adhesion of platelets generally occurs through the interaction of GPIb/IX/V and GPVI with vWF and collagen. Circulating vWF binds to subendothelial ECM components exposed at sites of vascular damage. The vWF molecule undergoes a conformational change which exposes the high affinity binding site for GP1b on platelets, resulting in platelet capture. Subsequent activation and spreading of platelets is accompanied by secretion of prothrombotic factors such as ADP and serotonin, rapid elevation of cytosolic calcium and cytoskeletal rearrangements. The GPIIb/IIIa receptor is a heterodimer consisting of α and β subunits which exists in a low-affinity state on resting platelets. Each subunit has a large extracellular domain, a transmembrane domain, and a short cytoplasmic tail.

Upon activation of platelets intracellular signals are transmitted to the β3 cytoplasmic tail in a process termed inside-out signaling. This results in conformational changes in the extracellular domain of GPIIb/IIIa converting it to its activated form in which it has a high affinity for fibrinogen and vWF (Xiao *et al.*, 2004). Cross-linking of adjacent platelets via fibrinogen or vWF into aggregates leads to thrombus formation (Gibbins, 2004). Subsequent integrin clustering is triggered and stimulation of protein tyrosine kinase activity (outside-in signaling). The cytoplasmic tails of GPIIb/IIIa are further stimulated and ligand binding and platelet activation become irreversible (Ni & Freedman, 2003; Shattil & Newman, 2004; Xiao *et al.*, 2004).

Shear stress in flowing blood is an important factor in vascular cell adhesion. Under low shear conditions found in large arteries and veins, platelet adhesion is mediated through interactions of platelet receptors with collagen ($\alpha_2\beta_1$ and GPVI), fibronectin ($\alpha_5\beta_1$) and laminin ($\alpha_6\beta_1$). Under high shear conditions present in small arteries and arterioles, platelet adhesion is dependent on the GPIb/V/IX complex binding to subendothelial bound vWF. Under these conditions aggregate formation occurs by cross-linking of activated platelets via vWF, with fibrinogen and fibrin playing stabilizing roles (Jackson *et al.*, 2003).

Upon stimulation by agonists platelets release platelet-microbicidal-proteins, (PMPs; (Yeaman *et al.*, 1997). These are small cationic peptides that disrupt the cytoplasmic membrane of bacteria resulting in permeabilization. Thromin-induced PMPs can also inhibit synthesis of macromolecules such as DNA and proteins leading to bacterial cell death (Xiong *et al.*, 2002). *S. aureus* endocarditis isolates are more resistant to PMPs than isolates from soft-tissue infections (Bayer *et al.*, 1998; Yeaman *et al.*, 1992). *S. aureus* strains resistant to PMPs are more virulent than sensitive strains in experimental endocarditis (Dhawan *et al.*, 1998; Mercier *et al.*, 2000).

1.6.2 Bacterial interaction with platelets

Many bacteria can adhere to and activate platelets. This is believed to be an important property of endovascular pathogens that cause endocarditis such as *S. aureus* and the oral streptococci. Adhesion of bacteria to resting platelets is believed to trigger intracellular signalling and platelet activation. Conformation changes in GPIIb/IIIa result in fibrinogen binding and cross-linking of adjacent platelets into platelet aggregates (Figure 1.12). *In vitro* bacteria-mediated platelet aggregation is

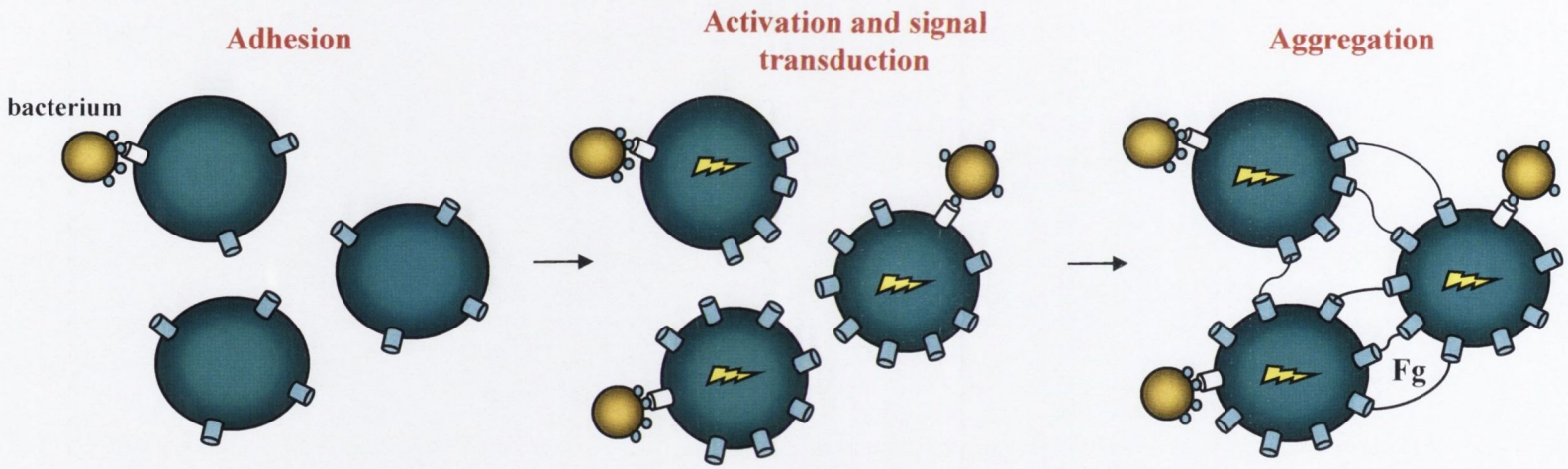


Figure 1.12. Platelet activation and aggregation mediated by bacteria

Bacteria bind to a specific receptor on resting platelets via a surface exposed protein. Binding to receptors can also be mediated via a bridging molecule such as fibrinogen (not shown here). Adhesion to platelets triggers 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.

preceded by a lag phase which reflects the time taken for aggregation to occur after bacteria and platelets come into contact. Lag times depend on the bacterial species, the cell density and the expression levels of bacterial surface components. Many studies have focused on platelets interaction with *S. aureus* and the viridans group Streptococci. Other bacterial species which can trigger platelet activation include *P. gingivalis, H. pylori, Borrelia burgdorferi and Borrelia hermsii* (Fitzgerald *et al.*, 2006a).

1.6.2.1 The interaction of *S. aureus* with platelets

In vitro studies have demonstrated that *S. aureus* can interact with platelets in a number of ways. Direct adhesion of *S. aureus* to platelets does not require plasma components (Nguyen *et al.*, 2000; Siboo *et al.*, 2005; Sullam *et al.*, 1996). Other mechanisms of adhesion require plasma proteins such as fibrinogen to act as bridges between *S. aureus* and platelets.

Several surface associated proteins of *S. aureus* can mediate the initial adhesion to platelets. *S. aureus srtA* mutants that can not anchor cell wall proteins were less virulent in experimental endoarditis models (Weiss *et al.*, 2004). The fibrinogen-binding surface protein ClfA is an important factor in promoting platelet adhesion and aggregation *in vitro* and in the pathogenesis of experimental endocarditis (Loughman *et al.*, 2005; Moreillon *et al.*, 1995; O'Brien *et al.*, 2002a; Que *et al.*, 2005). Cells expressing high levels of ClfA cause platelet aggregation with short lag times (1-2 min) (Loughman *et al.*, 2005).

Loughman *et al.* determined that ClfA-expressing cells mediate adhesion and aggregation to platelets in a fibrinogen-dependent manner. ClfA binds avidly to fibrinogen which is present in plasma. Bound fibrinogen acts as a bridge and crosslinks bacteria to platelets via GPIIb/IIIa. Low affinity GPIIb/IIIa is able to bind fibrinogen coating the bacterium. Both ClfA and GPIIb/IIIa bind to the C terminal γ-chain in a flexible region that protrudes from either end of the bivalent fibrinogen molecule. However, adhesion bacteria to platelets via fibrinogen is not sufficient to stimulate platelet activation. ClfA-specific antibodies are also required to interact with the platelet FcγRIIa receptor which clusters to trigger activation and intracellular signalling (Figure 1.13A). For platelet activation to occur a sufficient level of ClfA protein is required on the bacterial surface. This is referred to as the "threshold for platelet activation" (Loughman *et al.*, 2005). There is also evidence that ClfA can

also bind directly to an unknown 118 kDa platelet membrane protein. It is not known whether this interaction can lead to subsequent activation of platelets (Siboo *et al.*, 2001).

A non-fibrinogen binding mutant of ClfA (ClfA PY) was still able to stimulate platelet aggregation but with an extended lag time (8-10 min). Complement proteins were required stimulate platelet aggregation by cells expressing the ClfA-PY variant. Complement proteins deposited on the bacterial surface are presumed to interact with a complement receptor on platelets. Specific anti-ClfA antibodies were also required to trigger activation (Figure 1.13.B; Loughman *et al.*, 2005). A similar mechanism of platelet activation has been identified for *S. sanguis* strain NCTC 7863 (Ford *et al.*, 1996). Complement-dependent platelet activation may be common amongst IE-causing pathogens. Potentially any bacterium could cause aggregation by expressing a surface protein to which antibodies are present in the host.

With bacteria from the exponential growth phase rapid activation of platelets is mediated by the FnBPA and FnBPB proteins. The fibrinogen-binding A domain of FnBPA behaves in a similar fashion to ClfA. A fibrinogen bridge is formed between *S. aureus* expressing FnBPA and GPIIb/IIIa while specific IgG forms a bridge to FcγRIIa. The fibronectin-binding region BCD of FnBPA can also activate platelets by binding to fibronectin N-terminal type I domains by the tandem β-zipper mechanism and forming a bridge to GPIIb/IIIa, which also binds fibronection (Fitzgerald *et al.*, 2006b).

Studies have shown that protein A (Spa) contributes to platelet activation under high shear conditions. Bacterial cells expressing Spa are thought to adhere to platelet receptor GP1b via a vWF bridge under shear stress (Hartleib *et al.*, 2000; Pawar *et al.*, 2004). Monoclonal antibodies to vWF and GP1b inhibited platelet activation under these conditions. Spa has been shown to mediate adherence of staphylococci to immobilized collagen and vWF under flow (Mascari & Ross, 2003). ClfA also contributes to platelet activation at high shear rates through interaction with GPIIb/IIIa (Pawar *et al.*, 2004). Spa also binds directly to a platelet complement receptor gC1qR/p33 that is found only on the surface of activated platelets (Nguyen *et al.*, 2000; Peerschke & Ghebrehiwet, 2001; Peerschke *et al.*, 2003). This interaction may contribute to colonisation of sterile thrombi by *S. aureus* through binding to activated platelets within developing thrombi.

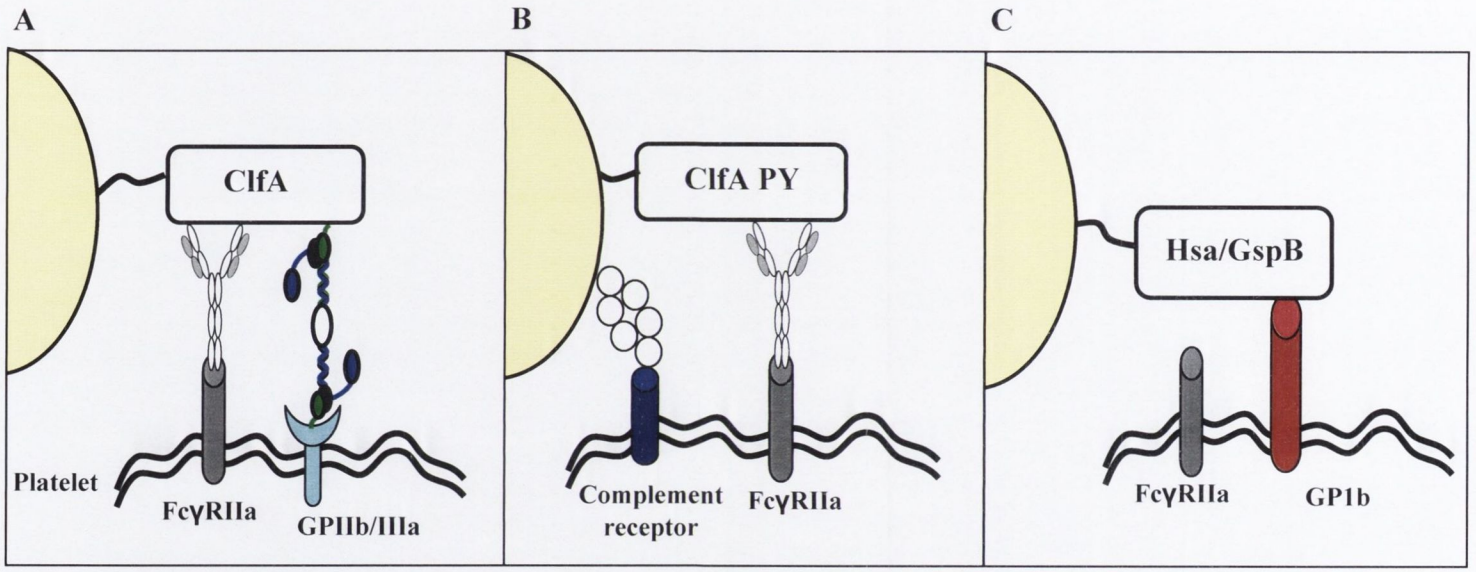


Figure 1.13. Mechanisms of bacterial interactions with platelets

(A) Fibrinogen mediated platelet activation. Bacteria expressing fibrinogen binding proteins become cross-linked to platelets via surface bound fibrinogen. Activation also requires specific antibodies to crosslink bacteria to the platelet Fc recpetor. (B) Complement mediated platelet activation. Platelet activation can be mediated by the deposition of complement proteins on the bacterial surface which cross-links bacteria to a complement receptor on platelets. Specific antibodies are required to engage platelet receptor FcγRIIa. (C) Direct platelet activation. *S. gordonii* strains expressing GspB or Hsa promote direct binding to sialic acid moieties on platelet receptor GPIb. Receptor clustering and signaling events lead to activation of GPIIb/IIIa and aggregation of platelets.

In *S. gordonii* surface proteins GspB and Hsa mediate direct interactions with platelet receptors. A homolog of these proteins known as SasA or SraP (serine-rich adhesin) can be found in *S. aureus* (Roche *et al.*, 2003a). Expression of SraP by *S. aureus* promoted direct platelet binding *in vitro* and enhanced virulence in experimental endocarditis models. Unlike GspB and Hsa, SraP does not bind to platelet receptor GP1b and it is not clear whether SraP-mediated platelet binding by *S. aureus* plays a role in activation (Siboo *et al.*, 2005).

Other *S. aureus* surface associated proteins implicated in platelet interactions include ClfB and SdrE. When expressed by *L. lactis* these proteins were able to cause platelet activation, however lag times to aggregation were extended (O'Brien *et al.*, 2002a). ClfB has been shown to contribute to the pathogenesis of experimental endocarditis and patients recovering from IE have elevated titres of anti-ClfB antibodies (Entenza *et al.*, 2000; Rindi *et al.*, 2006).

Rapid aggregation of human platelets can also be mediated by the secreted toxin α–hemolysin. Aggregation is a result of pore formation on platelets by α–hemolysin. This causes calcium influx across the platelet membrane triggering activation and prothrombinase formation (Arvand *et al.*, 1990; Bhakdi *et al.*, 1988).

S. aureus produces a secreted fibrinogen-binding protein, Efb which can interact with platelets and inhibit their activation (Shannon & Flock, 2004). The fibrinogen binding domain of Efb shares homology with the C-terminal repeats of staphylococcal coagulase (Boden & Flock, 1994). Efb recognises the α-chain of fibrinogen bound to ADP-stimulated platelets (Palma *et al.*, 2001). It can also bind directly to an unidentified receptor on activated platelets. *S. aureus efb* mutants were less pathogenic than wild-type strains in a rat wound-infection model. It was postulated that production of Efb can delay wound healing by inhibiting platelet function (Palma *et al.*, 1996).

S. aureus has numerous mechanisms for adhering to and activating platelets which suggests this interaction is an important aspect of *S. aureus* pathogenesis. Platelet activation is believed to be a factor in the establishment of endocardial infections and a greater understanding of the mechanisms involved is required to develop novel therapeutics.

1.6.2.2 The interaction of streptococci with platelets

Streptococci are the second leading cause of IE (Moreillon & Que, 2004). The viridans group of streptococci are commensals of the human oral cavity. Periodontal disease and poor dental hygiene practices are risk factors associated with the development of streptococcal IE.

S. gordonii strain M99 expresses a serine-rich glycoprotein, GspB on its cell surface that promotes direct binding to and activation of human platelets (Bensing & Sullam, 2002). Hsa is a homologue of GspB expressed by *S. gordonii* strain DL1 (Takahashi *et al.*, 2002). Basic regions located between the two serine-rich-repeat domains of Hsa and GspB are believed to mediate binding to sialic acid moieties on platelet receptor GPIb (Figure 1.13; Bensing *et al.*, 2004; Takamatsu *et al.*, 2005).

Strains of *S. sanguis* that adhere to and activate platelets are associated with increased severity of disease in experimental endocarditis models. *S. sanguis* isolates exhibit three phenotypes based on their ability to interact with platelets (Kerrigan *et al.*, 2002). Type I strains (133-79 and SK36) bind directly to platelets via the serine-rich surface glycoprotein SrpA (Kerrigan *et al.*, 2002; Plummer *et al.*, 2005). SrpA shares structural similarities with GspB and Hsa of *S. gordonii* and is believed to mediate bacterial adhesion by interacting with sialic-acid moieties on GPIb. Bacterium specific IgG is also required to interact with FcγRIIa on platelets and trigger activation (Herzberg *et al.*, 1983; Kerrigan *et al.*, 2002).

Type II strains of *S. sanguis* are defective in platelet binding and are thought to mediate aggregation in a complement-dependent manner. In the absence of direct binding to platelets, assembly of complement and IgG on the bacterial cell was required for platelet activation. A model was proposed in which platelet activation was stimulated by cross-linking of bacteria to platelet receptors via surface deposited complement components and IgG-Fc receptor interactions. The identity of the specific complement components and platelet receptors involved were not elucidated, although a role for C5b-9 was suggested (Ford *et al.*, 1996; Ford *et al.*, 1997). Lag times to platelet aggregation were longer for these strains than SrpA-expressing strains (approximately 16 minutes; Kerrigan *et al.*, 2002). The extended lag times are believed to reflect the time taken for complement assembly to occur on the bacterial cell surface (Ford *et al.*, 1996). The platelet-aggregation-associated protein (PAAP) of *S. sanguis* also induces platelet aggregation although it is not involved in initial adhesion to platelets (Herzberg *et al.*, 2005). PAAP is a rhamnose-rich glycoprotein

which contains a collagen-like platelet-interactive domain (Erickson & Herzberg, 1993). This protein is reported to interact with platelet membrane proteins of 175 kDa and 230 kDa to mediate platelet aggregation (Gong *et al.*, 1995). Type III strains of *S. sanguis* do not adhere to or aggregate platelets (Kerrigan *et al.*, 2002).

S. pyogenes causes rapid aggregation of platelets in plasma (Kurpiewski *et al.*, 1983). The cell-surface fibrinogen-binding protein M is required to induce platelet thrombus formation under physiological shear. This process is fibrinogen-dependent and requires IgG specific to M proteins. Bound fibrinogen is believed to promote bacterial adhesion to resting platelets via GPIIb/IIIa and streptococcal-bound IgG is required to interact with FcγRIIa on platelets (Sjobring *et al.*, 2002) .

The ability of *S. agalactiae* to stimulate platelet aggregation is also dependent on the expression of a fibrinogen-binding surface protein, FbsA. Aggregation of platelets is dependent on GPIIb/IIIa, FcγRIIa, and antibody to FbsA similar to aggregation mediated by *S. pyogenes* (Pietrocola *et al.*, 2005; Sjobring *et al.*, 2002).

1.7 Rationale for this study

S. aureus is now acknowledged to be the leading cause of infective endocarditis. The ability of *S. aureus* to interact with platelets is thought to be a crucial virulence determinant in the pathogenesis of this disease (Sullam *et al.*, 1996). Greater understanding of the mechanisms involved in *S. aureus*-platelet interactions is required to identify novel targets for therapeutics. ClfA and FnBPA mediate rapid aggregation of platelets through interaction with the fibrinogen γ-chain (Fitzgerald *et al.*, 2006b; Loughman *et al.*, 2005). ClfB binds to the α-chain of fibrinogen and to the tail region of cytokeratin 10. Although ClfB is known to cause platelet aggregation and contributes to the pathogenesis of experimental endocarditis in rats, little is known about the mechanism by which platelet activation occurs (O'Brien et al., 2002a; Entenza *et al.*, 2000).

The recently identified Isd proteins of *S. aureus* are involved in iron acquisition from haem-containing proteins. *In vivo* bacteria are exposed to conditions in which iron is scarce and expression of Isd proteins is induced (Skaar & Schneewind, 2004). Multiple roles have been identified for the Isd proteins in immune evasion and adhesion to host proteins. IsdA in particular has been shown to mediate adhesion to

multiple ligands including fibrinogen and fibronectin (Clarke *et al.*, 2004). This raises the possibility that Isd proteins may interact with platelets.

1.7.1 Aims and objectives

- Isolation of non-fibrinogen binding mutants of ClfB based on previous mutagenesis studies carried out with ClfA and FnBPA.
- Determination of the ClfB binding site in the fibrinogen α-chain.
- Assessment of the ability of non-fibrinogen binding mutants of ClfB to interact with cytokeratin 10. This will indicate if the same sites in ClfB are required for fibrinogen and keratin binding.
- Comparison of the ability of *L. lactis* expressing ClfB and a non-fibrinogen-binding ClfB mutant to cause activation of platelets. This will establish if the fibrinogen-binding activity of ClfB is necessary for platelet activation.
- Study of platelet aggregation mediated by *S. aureus* strains grown in iron-limiting conditions. This will indicate if the Isd proteins can contribute to platelet adhesion and aggregation. Strains of *S. aureus* defective in individual proteins will also be constructed to determine which can mediate platelet aggregation.

Chapter 2

Materials and Methods

2.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 2.1. *L. lactis* was grown statically at 28° C in M17 (Difco) agar or broth incorporating 0.5% (w/v) glucose (GM17). *S. aureus* and *S. epidermidis* were grown on tryptone soy agar (TSA) or broth (TSB) at 37° C with shaking (200 r.p.m.) for liquid cultures. *E. coli* strains were grown in L broth or agar (Difco) at 37° C with shaking (200 r.p.m.). Strains in L broth, TSB or GM17 were snap frozen and maintained at -70° C. The following antibiotics (Sigma) were added to the media as required: chloramphenicol (Cm) at 10 μg/ml, erythromycin (Em) at 10 μg/ml and tetracycline (Tc) at 2 μg/ml.

2.1.1 Growth of bacteria in iron and nutrient limiting conditions

To reflect growth conditions *in vivo*, bacterial strains were grown in RPMI 1640 (Sigma). RPMI medium is an iron deficient medium which was originally designed for the cell culture of human leukocytes (Moore *et al.*, 1967). Starter cultures were diluted in 10 ml of RPMI to an OD_{600} 0.05 and grown at 37°C with shaking (200rpm). Glassware was acid treated to eliminate iron by incubation with 0.1M HCl, followed by thorough washing with dH_2O and sterilization by autoclaving.

2.1.2 Growth of bacteria in iron limiting conditions

Bacterial strains were also grown in TSB from which iron was depleted. TSB (Oxoid) was incubated with 20 g/l of Chelex 100 resin (sodium form, Sigma), a polyvalent metal ion chelator for 16 h at 4 °C. TSB was then filtered to remove the resin and autoclaved. Iron was further depleted from Chelex-treated TSB by the addition of 2,2'-dipyridyl (Sigma) a high-affinity chelator of Fe^{2+} which also binds Fe^{3+}.

2.1.3 Growth and induction of *L. lactis* strains carrying pNZ8037 derivatives

Nisin is an antimicrobial peptide produced by certain *L. lactis* strains. The gene that encodes nisin, *nisA* is found on the conjugative transposon Tn5276. Nisin is an autoregulator and through interaction with the two-component regulatory system NisKR can activate transcription of *nisA* (Kuipers *et al.*, 1995). *L. lactis* strain NZ9800 is defective in nisin production so expression of genes cloned in-frame with the *nisA* start codon can be induced by addition of extracellular nisin (Figure 2.1; de Ruyter *et al.*, 1996). The nisin-controlled expression system (NICE) of *L. lactis* can

therefore be utilised to control levels of protein expression and also to study *S. aureus* proteins in isolation. *L. lactis* strains carrying pNZ8037 derivatives were grown and induced with nisin as follows. Starter cultures of *L. lactis* strain NZ9800 (pNZ8037) were diluted $^{1}/_{100}$ into 5 ml of fresh GM17 medium containing 10 µg/ml Cm and grown to OD_{600} of 0.5. Nisin (Sigma) was added to exponential phase cultures at final concentrations that ranged between 0.025 and 3.2 ng/ml. Induced cultures were grown at 28 °C for 16 h.

2.2 Plasmids

The plasmids used in this study are listed in Table 2.2.

2.3 DNA manipulations

Standard methods were used in DNA manipulations (Sambrook *et al.*, 1989). Restriction endonucleases were purchased from New England Biolabs and Roche and were used according to the manufacturer's instructions. DNA ligase and shrimp alkaline phosphatase were purchased from Roche. Confirmatory DNA sequencing was carried out by Lark Technologies or GATC-Biotech.

2.3.1 Preparation of plasmid and genomic DNA

Plasmid DNA was extracted from bacteria using WizardPlus™ Minipreps kit (Promega). Genomic DNA was prepared using Bacterial Genomic DNA purification kit (Edge BioSystems). Plasmid and genomic extracts from *S. aureus* and *S. epidermidis* required the addition of 200 µg/ml of lysostaphin to digest cell wall peptidoglycan. Lysozyme (1mg/ml) and mutanolysin (1000U/ml) were used to digest the cell wall peptidoglycan of *L. lactis* prior to DNA preparation.

2.3.2 Polymerase chain reaction (PCR)

PCR reactions were typically carried out in 50 µl volumes using 1 U Phusion™ Hot Start DNA polymerase in Phusion HF buffer (Finnzymes). 10ng of plasmid DNA or 20ng of genomic DNA were used as templates for PCR. Primers (Sigma-Genosys) and dNTPs (Bioline) were used at final concentrations of 0.2 mM and 200µM, respectively. Primer sequences are listed in Table 2.3. Initial denaturation was carried out at 98 °C (30 sec) followed by 30 cycles of denaturation (10 sec) at 98 °C, 20 sec annealing (temperature dependent on primer used) and extension at 72 °C.

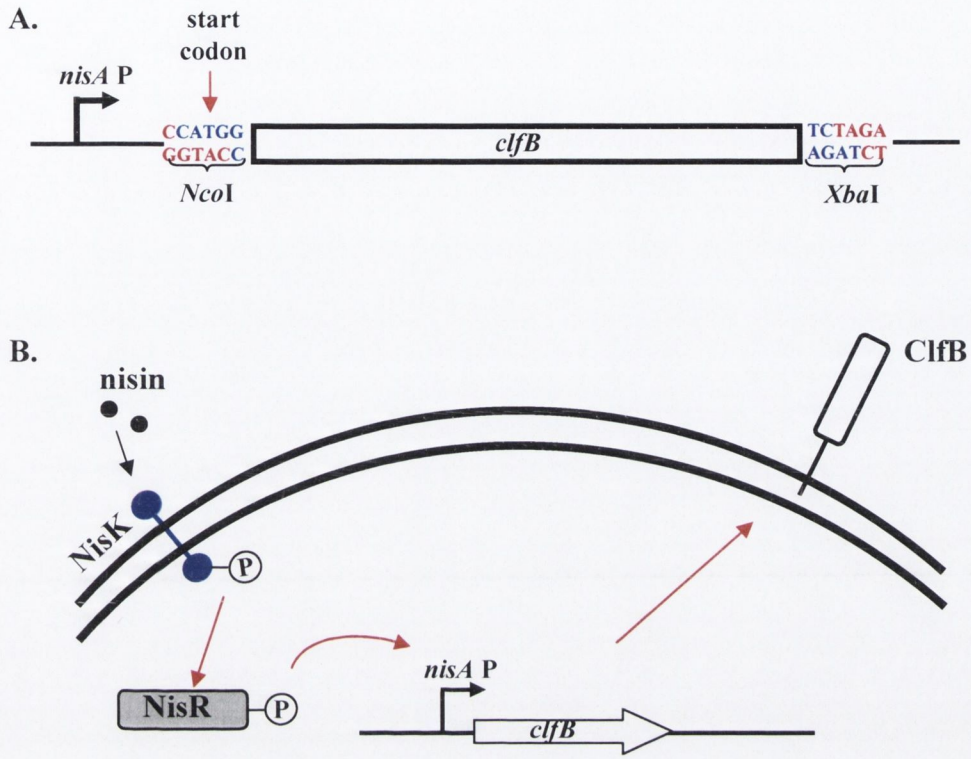


Figure 2.1. Nisin-inducible expression of ClfB from vector pNZ8037

A. Fusion of target genes to *nisA* promoter in pNZ8037. The ATG start codon of *clfB* was fused with the *nisA* start codon. Nucleotides shown in red are derived from the pNZ8037 vector and those shown in blue are derived from the PCR-amplified gene.

B. Expression of target genes from *nisA* P in pNZ8037. Nisin binds to the sensor-histidine kinase NisK resulting in phosphorlylation of the NisR response regulator. NisR then binds to the *nisA* promoter (*nisA* P) sequence and activates transcription of translationally fused *clfB.* ClfB is sorted to the cell wall and is displayed on the cell surface.

Table 2.1 Bacterial strains

Strain	Relevant Characteristics	Source/ Reference
E. coli		
XL-1 Blue	Cloning host	Stratagene
Topp3	Expression host for recombinant proteins. Protease deficient strain.	Stratagene
S. aureus		
8325-4	NCTC 8325 strain cured of prophages	Novick, 1967
RN4220	Restriction-deficient derivative of 8325-4	Keriswirth *et al.*, 1983
8325-4 *clfB*	8325-4 *clfB::lacZ* Em^R	McAleese *et al.*, 2001
SH1000	8325-4 with repaired defect in *rsbU*	Horsburgh *et al.*, 2002
Newman	NCTC 8178.	Duthie and Lorenz, 1952
Newman *clfA*	Newman *clfA5*. Frameshift mutation in *clfA*	Fitzgerald *et al.*, 2006
Newman *clfA clfB*	Newman *clfA5*, *clfB::lacZ* Em^R	Fitzgerald *et al.*, 2006
Newman *coa*	Newman *coa::*Tc^R	McDevitt *et al*, 1992
Newman *coa clfA*	Newman *clfA5*, *coa::*Tc^R	This study
Newman *coa clfA clfB*	Newman *clfA5*, *clfB::lacZ* Em^R, *coa*::Tc^R	This study
Newman *isdA*	Newman frameshift mutation in *isdA*	Clarke *et al.*, 2007
Newman *isdB*	Newman frameshift mutation in *isdB*	This study

Table 2.1 Bacterial strains, continued

Strain	Relavant Characteristics	Source/ Reference
Newman *isdH*	Newman *isdH::*Em^R	N. Yanagisawa
Newman *isdA isdB*	Newman frameshift mutation in *isdA*, frameshift mutation in *isdB*	This study
Newman *clfA clfB isdA*	Newman frameshift mutation in *isdA*, *clfA5*, *clfB::lacZ* Em^R	This study
Newman *clfA clfB isdB*	Newman *clfA5*, *clfB::lacZ* Em^R, frameshift in *isdB*	This study
Newman *clfA clfB isdA isdB*	Newman *clfA5*, *clfB::lacZ* Em^R, frameshift mutation in *isdA*, frameshift mutation in *isdB*	This study
Newman *clfA clfB isdH*	Newman *clfA5*, *clfB::*Tc^R, *isdH::*Em^R	This study
S. epidermidis		
TU3298	Strain capable of accepting and maintaining, recombinant plasmids	Augustin *et al.*, 1990
L. lactis		
MG1363	Derivative of NCD 0712, plasmid free	Gasson, 1983
NZ9800	Derivative of MG1363 containing nisin-sucrose conjugative transposon *Tn5276*, Δ*nisA*	Kuipers *et al.*, 1993

Table 2.2 Plasmids

Plasmid	Relevant Characteristics	Source/ Reference
pQE30	*E. coli* expression vector, polyhistidine-tagged Amp^R	Quiagen
pQE30 *clfB*	pQE30encoding domain N123 of ClfB Amp^R	E. Walsh
pQE30 *clfB* PF	pQE30*clfB* with point mutations P326A and F328A Amp^R	This study
pQE30 *clfB* R331A	pQE30*clfB* with point mutation at residue R331 Amp^R	This study
pQE30 Aα-Fg	pQE30 encoding Aα-chain of fibrnogen 1-625 Amp^R	E. Walsh
pQE30 Aα-Fg T322P	pQE30 Aα1-625 with point mutation T322P Amp^R	This study
pQE30 Aα-Fg S317P	pQE30 Aα1-625 with point mutation S317P Amp^R	This study
pNZ8037	*L. lactis* expression vector, *nisA* nisin inducibe promoter Cm^R	De Ruyter *et al.*, 1996
pNZ8037*clfA*	pNZ8037 containing full length *clfA* gene Cm^R	R. Dower
pNZ8037*clfB*	pNZ8037 containing full length *clfB* gene Cm^R	T. Loughman
pNZ8037*clfB* Q235A	pNZ8037*clfB* with point mutation at Q235A Cm^R	This study
pNZ8037*clfB* N526A	pNZ8037*clfB* with point mutation at N526A Cm^R	This study
pJH1	Temperature sensitive plasmid containing frameshift *clfA*5 Em^R	Fitzgerald *et al.*, 2006
pBluescript II SK	*E. coli* cloning vector Amp^R	Stratagene
pBlueΔ*isdB*	pBluescript II SK containing *isdB* DNA incorporating a frameshift mutation Amp^R	This study
pTS*ermC*	*S. aureus* plasmid containing temperature sensitive-replicon Em^R	J. Higgins

Table 2.2 Plasmids, continued

Plasmid	Relevant Characteristics	Source/ Reference
pHM1	Shuttle plasmid containing pBlueΔ*isdB* and pTS*ermC* Amp^R/Em^R	This study
pCU1	Derivative of pC194 and pUC19. Shuttle vector, Amp^R in *E. coli* and Cm^R in *S. aureus*	Augustin *et al.*, 1992
pCU1 *isdB*	pCU1 containing full length *isdB* gene Amp^R/Cm^R	This study
pCU1 *isdA isdB*	pCU1 containing full length *isdA* and *isdB* genes Amp^R/Cm^R	This study

Table 2.3 Primers

Primer	Sequence (5′-3′)[a,b]
PF F	GGACAATTTTCATTA**GCT**TTA**GCT**ACAGACCGAGCAAAG
PF R	CTTTGCTCGGTCTGT**AGC**TAA**AGC**TAATGAAAATTGTCC
R331A F	CCTTTATTTACAGAC**GC**AGCAAAGGCACCTAAATCA
R331A R	CCTGATTTAGGTGCCTTTGCT**GC**GTCTGTAAATAAAGG
Aα-S317P F	GCTGGAAC**C**CTGGGAGCTCTGGAAC
Aα-S317P R	GTTCCAGAGCTCCCAG**G**GTTCCAGC
Aα-T322P F	TGGGAGCTCTGGA**C**CTGGAAGTACTGGAAAC
Aα-T322P R	GTTTCCAGTACTTCCAG**G**TCCAGAGCTCCCA
N526A F	GTTGGAATAATGAG**GCT**GTTGTACGTTATGGTGG
N526A R	CCACCATAACGTACAAC**AGC**CTCATTATTCCAAC
Q235A F	CATTTGACCCTAAT**GCA**AGTGGTAACACATTTATGG
Q235A R	CCATAAATGTGTTACCACT**TGC**ATTAGGGTCAAATG
D270A F	GTTTAACTGGTAATGGAG**C**CGTGGATTATTCTAATTC
D270A R	GAATTAGAATAATCCACG**G**CTCCATTACCAGTTAAAC
W522A F	GACTACAGTATTTTCGGT**GC**GAATAATGAGAATGTTGTACG
W522A R	CGTACAACATTCTCATTATTC**GC**ACCGAAAATACTGTAGTC
Y446A F	CTAAATTATCAGATAGC**GC**CTATGCAGATCCAAATGAC
Y446A R	GTCATTTGGATCTGCATAG**GC**GCTATCTGATAATTTAG
FP*isdB*	GTCCTGCAGATTCTTACATTAGCTGACGCA
RB*isdB*	GGCGGATCCTTTGTTCATGTTGTAGAAACA
FB*isdB*	GAAGGATCCAAAAGAATTTAAATCATTTTA
RX*isdB*	CGCTCTAGAATCTTGAATTTTATTTAATTC

Table 2.3 Primers, continued

Primer	Sequence (5′-3′)[a,b]
pC*isdB* F	CAGCTGCAGCCTATGTCATAGATATTTCATAATC
pC*isdB* R	CGCGTCTAGACCTATGTCATAGATATTTCATAATC
pC*isdA* F	CAGCTGCAGACATAATCCTCCTTTTTATGATTG

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

When amplifying plasmid DNA, a 15 sec extension time per 1kb DNA was used. For high complexity genomic DNA a longer extension time was used (30 sec per kb). A final extension step was carried out at 72 °C for 5min. PCR products were purified using Wizard SV gel and PCR clean-up system (Promega).

2.3.3 Site-directed mutagenesis

Site-directed mutagenesis of plasmids pQE30 *clfB* and pQE30 Aα-Fg was carried out using the Quickchange protocol (Stratagene). Complementary forward and reverse primers incorporating the desired mutation were extended by thermal cycling to produce a mutated plasmid with staggered DNA nicks. PCR products were digested with *Dpn*I for 1 h at 37°C with to eliminate methylated, parental DNA and then transformed into *E. coli* strain XL-1 Blue. The nisin- inducible plasmid pNZ8037 *clfB* was subjected to site-directed mutagenesis as described above. *Dpn*I digested PCR products were transformed into *L. lactis* strain NZ9800. Transformants were screened by restriction mapping and DNA sequencing.

2.4 Transformation

Plasmids were transformed into *E. coli* TOPP3 and XL1-Blue cells made competent by $CaCl_2$ treatment. *E. coli* transformants were screened for the presence of recombinant plasmids using the rapid colony screening procedure developed by Le Gouill & Dery, 1991.

2.4.1 Preparation and electroporation of *S. aureus* RN4220

Plasmids were introduced into *S. aureus* by electroporation into strain RN4220 (Augustin & Gotz, 1990). Electrocompetent RN4220 cells were prepared by growing RN4220 to mid-exponential phase in TSB. Cells were washed in 0.5 M sucrose and harvested by centrifugation at 4 °C. Electrocompetent RN4220 (200 µl) were mixed with (0.1 µg) plasmid DNA in a 0.2 mm electrode gap cuvette. Electroporation was carried out at 200 Ω resistance, 25 µF capacitance and 25 V. Cells were incubated for 1 hour at 37 °C with shaking (200 rpm) in TSB prior to plating on antibiotic containing media. Transformants were screened by PCR, restriction mapping and Western immunoblotting.

2.4.2 Preparation and electroporation of *S. epidermidis*

Electrocompetent *S. epidemidis* cells were prepared by an adapted protocol based on the method described by Löfblom *et al* (2007). *S. epidermidis* strain TU3298 grown in Basic Medium (1% casein hydrolysate, 2.5% yeast extract, 0.5% glucose, 0.5% NaCl and 0.1% K_2HPO_4, pH 7.4), was diluted 1/100 in fresh BM. Bacterial cells were grown at 37 °C with shaking to an OD_{600} of 0.5-0.6. Cells were harvested by centrifugation at 3000 g for 10 min and washed in ice cold 10% glycerol. A further two washes were carried out before final resuspension in 2 ml of ice cold glycerol. Electrocompetent cells were aliquoted, snap frozen and stored at -70 °C.

Thawed electrocompetent cells (90 µl) were incubated with 2-4 µg of plasmid DNA for 20 min at room temperature. Electroporation was carried out in 0.1 cm electrode gap cuvettes (Flowgen) which were pulsed at 2 kV, 25 µF capacitance and 100 Ω resistance. Cells were immediately added to 1 ml of Basic Medium and incubated at 37 °C for 1 h with shaking. Following recovery, cells were plated on TSA with selective antibiotic.

2.4.3 Preparation and electroporation of *L. lactis*

Electrocompetent *L. lactis* NZ9800 and MG1363 cells were prepared according to the protocol outlined by Wells *et al.* (1993). An overnight culture of *L. lactis* was diluted 1/100 in in GM17 medium containing 2.5 % (w/v) glycine. Cells were grown statically at 28°C to a $0D_{600}$ nm of 0.6 and then chilled on ice for 10 min. Cells were washed twice in ice cold storage buffer (0.5 M sucrose, 10% glycerol) and harvested by centrifugation at 5000g for 10 min. Electrocompetent cells were resuspended in 1 ml of storage buffer, snap frozen and stored at -70 °C.

Electroporation was carried out using 50 µl of electrocompetent cells. 1-2 µl of ethanol precipitated, plasmid DNA was added to electrocompetent cells in a 0.1 cm electrode gap cuvette (Flowgen). Cells were pulsed at 2.5 kV, 25 µF capacitance and 200 Ω resistance. The cells were immediately added to 0.96 ml of recovery medium (GM17, 0.5 M sucrose, 20 mM $MgCl_2$, 2 mM $CaCl_2$) and incubated on ice for 10 min. Cells were incubated for a further 2 hours at 30°C before plating on GM17 agar containing antibiotic.

2.5 Phage transduction

Phage 85 was used to transfer chromosomal mutations and plasmids marked with antibiotic resistance between *S. aureus* strains. Plasmids were introduced into strain

RN4220 by electroporation prior to preparation of phage stocks. Bacterial cultures of donor strains were diluted in phage broth (20g/l Nutrient broth No. 2, Oxoid supplemented with 10mM $CaCl_2$) and grown at 37°C for 4h with shaking. The donor strains were incubated with phage 85 stock for 30 min at room temperature. Molten top agar (20g/l Nutrient broth No. 2, 3.5g/l Agar No. 1, Oxoid supplemented with 10mM $CaCl_2$) was added to the bacteria and phage and poured over 2 phage base plates (20g/l Nutrient broth No. 2, 7g/l Agar No. 1, Oxoid supplemented with 10mM $CaCl_2$). Following overnight incubation at 37°C the top agar was removed to an Oakridge tube and centrifuged for 10 min at 15 000 rpm. The supernatant containing the phage and the desired plasmid or mutation was filtered and stored at 4°C.

Plasmids and chromosomal mutations were introduced into recipient strains by infection with page 85 carrying the desired plasmid or mutation. A 10 ml overnight culture of the recipient strain was harvested and incubated for 40 min at 37 °C with phage stock (containing desired antibiotic marker) in L-broth supplemented with $CaCl_2$. Control transductions contained no phage. Sodium citrate (0.02 M) was added at this point to eliminate contaminating phage particles and cells were harvested by centrifugation. Following resuspension in sodium citrate (0.02 M), bacterial cells were incubated on ice for 2h. The recipient strains were plated on TSA containing 0.05% (w/v) sodium citrate and the appropriate antibiotic.

2.6 Allelic replacement mutagenesis

S. aureus strains containing pHM1 or pJH1 were grown at 28°C in TSB broth containing erythromycin. Serial dilutions were plated in duplicate on TSA agar containing erythromycin. One set of plates was incubated at 44°C and the other at 28°C. Plates growing at 44°C had 10^3–fold less colonies growing on them than those incubated at the lower temperature. Putative integrants were single colony purified prior to inoculation into drug free TSB broth. Cultures were incubated at 28°C overnight to encourage plasmid replication and excision. Cultures were then diluted 1/100 in fresh broth and grown for 8 hours at 44°C to promote loss of the plasmid. Three rounds of temperature shifting were carried out. Cultures were finally diluted and plated on TSA agar. Colonies were screened for sensitivity to erythromycin. PCR was carried out to detect a novel restriction sites present in the amplified fragments of strains containing the desired mutations. Expression of proteins was analysed by Western immunoblotting.

2.7 Preparation of solubilised cell wall proteins

Cell-wall associated proteins of *S. aureus* were isolated as described previously (Hartford *et al.*, 2001). Overnight cultures of *S. aureus* were washed twice in PBS. An OD_{600} nm of 10 was resuspended in 250 µl of 20mM Tris (pH 8), 10mM $MgCl_2$ containing 30 % raffinose. Complete EDTA-free protease inhibitor cocktail (10 µl of a 10x stock) and lysostaphin (200 µg/ml) were added to the cells and incubated at 37°C for 10 min. When isolating cell wall proteins of *L. lactis*, mutanolysin (500 U/ml) and lysozyme (200 µg/ml) were used instead of lysostaphin to break down cell wall peptidoglycan. Cell wall proteins released by the murolytic enzymes were harvested by centrifugation at 5000 rpm for 10 min. The supernatant containing the cell wall associated proteins was removed and used in SDS-PAGE or stored at -20°C.

2.8 SDS-PAGE

Protein samples were diluted in 2 fold final sample buffer (125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and 0.002% (w/v) bromophenol blue) and boiled for 5 min. 20 µl volumes of the diluted protein samples were loaded onto acrylamide gels (4.5% stacking and 7.5% separating gel) and separated by SDS-PAGE (Laemlli, 1970). Electrophoresis was carried out at 120 V after which proteins were visualised by Coomassie blue staining or electroblotted onto PVDF membranes (Roche) for Western immunoblotting.

2.9 Western immunoblotting

Proteins were electroblotted onto PVDF membranes (Roche) for 1h at 100 V using a wet transfer cell (Bio Rad). Membranes were incubated for 1 h at 4°C in TS buffer (10mM Tris-HCl, pH 7.4, 150Mm NaCl) containing 10 % (w/v) skimmed milk (Marvel) to block non-specific interactions. Primary antibodies diluted in 10 % (w/v) Marvel/TS buffer were incubated with the membranes for 1 h at room temperature with shaking. Polyclonal anti-ClfB, anti-ClfA, anti-IsdB and anti-IsdA antibodies were used at a 1:5000 dilution. Polyclonal anti-IsdH antibodies were used at a 1:10 000 dilution. Unbound antibody was removed by three 10 min washes with TS buffer containing 0.01% Tween. Secondary antibodies (HRP conjugated) diluted in 10 % (w/v) Marvel/TS TS buffer were incubated with the membranes for 1 h at room temperature with shaking. Unbound secondary antibody was removed by washing three times with 0.01% Tween/TS buffer. Chemiluminesent substrate LumiGlo (New

England Biolabs) was used as per manufacturers' instructions. Blots were exposed to X-Omat autoradiographic film (Kodac) and visualised using a Kodac X-OMAT 1000 Processor developing machine. The intensity of protein bands resulting from equal loadings of bacterial cell-wall extracts or purified proteins was assessed visually.

2.10 Whole cell immunoblots

Cultures of bacterial strains were washed twice in PBS and adjusted to an OD_{600} nm of 1. Doubling dilutions of washed cells (5 µl) were dotted onto nitrocellulose membrane (Protran). The membrane was blocked for 1 h with 10% (w/v) skimmed milk powder (Marvel) in TS buffer. Primary antibody was diluted in 10 % (w/v) Marvel/TS buffer and incubated with the membrane for 1 h at room temperature with shaking. Three 10 min washes with TS buffer were performed to remove unbound antibody. Secondary (HRP conjugated) antibody was diluted in 10 % (w/v) Marvel/TS buffer and incubated with the membrane for 1 h at room temperature with shaking. Unbound secondary antibody was removed by washing three times with TS buffer. The membrane was developed in the dark using the chemiluminescent substrate LumiGlo (New England BioLabs) as described above.

2.11 Coagulase clumping test

Serial dilutions of bacteria were mixed with equal volumes of human plasma (diluted 1/3 in PBS) in siliconized glass cuvettes (BioData). The mixtures were incubated for at 37°C and the formation of gel clots was monitored over 2 hours.

2.12 Fibrinogen culmping test

Bacterial cells from fresh agar plates were emulsified in 10 µl of saline to which 10 µl of fibrinogen (at 1 mg/ml, Calbiochem) was added. Slides were shaken to mix the bacteria and fibrinogen. Visible clumping was compared to positive and negative controls.

2.13 Docking of GTWNP peptide into the 3D crystal structure of ClfB

The amino acid α-chain fibrinogen peptide PRPGSTGTWNP (residues 352-362) inhibits binding of rClfB to full length fibrinogen. Altering the W residue within this peptide reduced inhibition of fibrinogen binding (E. Walsh, personal communication). Based on this information a five amino acid peptide, GTWNP was selected for

docking into the 3D crystal structure of ClfB using Autodock (carried out by M. Brennan). The Autodock program (http://autodock.scripps.edu/) can be used to predict the interaction between small molecules and receptors. It has been successfully utilized to predict protein-ligand complexes. The docking result with the most favourable energy for binding in the ClfB hydrophobic trench was analysed using Chimera molecular modelling software.

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

Recombinant domains of *S. aureus* surface associated proteins were expressed from pQE30 with an N-terminal hexa-histidine (His_6) affinity tag to allow purification by nickel affinity chromatography. The pQE30 vector contains an IPTG inducible promoter for controlled expression of recombinant proteins. The vector is designed with sequences located 5' to the MCS that encodes for 6 x His residues.

The pQE30 constructs were transformed into the protease-defective *E. coli* TOPP3 strain for large-scale purification. Cultures were grown to OD_{600} nm of 0.5-0.6 and then induced with 1 mM IPTG for 3 h at 37°C. Cells were harvested by centrifugation and resuspended in 30 ml PBS containing EDTA protease inhibitors (Roche) prior to prior to cell rupture in a French pressure cell. The lysate was centrifuged at 17 000 *g* for 20 min. DNAse was added to the supernatant which was 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. The column washed with binding buffer 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). Eluted protein was collected in 3 ml fractions. Elution was monitored by measuring the A_{280} nm of the eluate. Peak fractions were analysed by SDS-PAGE. Ten µl of each fraction was separated on 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.

Recombinant ClfB region A protein was further purified by anion exchange chromatography on a HiTrap Mono Q column (5 ml Amersham Pharmacia) using a

NaCl gradient for elution. This was followed by 16 h dialysis in PBS and a second nickel affinity purification and dialysis. Protein concentrations were determined using the BCA assay kit (Pierce) and by measuring absorbance at 280 nm using a Nanodrop^{TM} 1000 spectrophotometer.

2.15 Adherence of bacteria to immobilized fibrinogen and cytokeratin 10

NUNC microtitre plates were coated with serial dilutions (100 µl) of recombinant murine cytokeratin 10 (rMK10, 454-570) in carbonate buffer (15mM $Na2CO_3$, 35mM $NaHCO_3$, pH 9.6). Serial dilutions of fibrinogen (100 µl, Calbiochem) were added to 96-well flat bottomed plates (Sarstedt). Following 16 h incubation at 4 °C, microtitre wells were washed three times with PBS. 100µl of filtered BSA (5% w/v) in PBS was added to each well and plates were incubated for 2h at 37°C. Wells were washed three times with PBS. Bacterial suspensions were adjusted to an OD_{600} nm of 1 and 100 µl of bacteria was added per well. Plates were incubated for a further 2 h at 37°C. Wells were washed three times with PBS and 100 µl of 25% (v/v) formaldehyde was added for 15 min to fix adherent cells. Wells were washed three times with PBS and the adherent cells stained with 100 µl of 0.5 % (w/v) crystal violet for 1 min. Excess stain was removed by washing with PBS. Cell-bound crystal violet was solubilised with 100 µl of 5 % (v/v) acetic acid per well. Absorbance at 570 nm was determined ELISA plate reader (Labsystems).

2.16 Binding of recombinant proteins to fibrinogen

Fibrinogen (100 µl) at 10 µg/ml was added to 96-well flat bottomed plates (Sarstedt) and incubated 16 h incubation at 4 °C. Wells were washed three times with PBST (0.1 % Tween 20) and incubated at 37 °C for 2 h with 10 % (w/v) Marvel/PBS buffer to block non-specific interactions. Wells were washed three times with PBST and serial dilutions of recombinant region A ClfB, ClfB PF and ClfB R331 were added to wells. Following a 2 h incubation at 37 °C, wells were washed three times with PBST. Rabbit polyclonal anti-ClfB antibodies (1:5000) in 10 % (w/v) to Marvel/PBS buffer were used to detect bound ClfB. Following a 1 h incubation at room temperature, plates were washed with PBST and goat anti-rabbit-horseradish peroxidase (Dako 1:2000) or anti-His-horseradish peroxidase (1:500) in 10 % (w/v) in Marvel/PBS buffer were used to detect bound antibodies. After washing, 100 µl of a chromogenic substrate (1 mg/ml tetramethylbenzidine and 0.006

% H_2O_2 in 0.05 M phosphate citrate buffer pH 5.0) was added to each well and plates were developed for 10-15 min. The reaction was stopped by the addition of 2 M H_2SO_4 (50 µl/well), and the absorbance at 450 nm was read in an ELISA plate reader (Labsystems).

2.17 Preparation of platelet rich plasma

Platelet rich plasma (PRP) was prepared as described previously (Loughman *et al.*, 2005). Briefly, blood was drawn into a syringe containing 3.2% (w/v) sodium citrate. Whole blood was divided into 5 ml volumes and centrifuged at 150g for 10min. The top layer consisting of platelet rich plasma (PRP) was removed and the red blood cells centrifuged again to obtain platelet poor plasma (PPP).

2.17.1 Preparation of washed platelets

Blood was drawn into a syringe containing acid-citrate-dextrose (ACD, 25 mM citric acid, 75 mM Na citrate, 135 mM D-glucose). Following preparation of platelet rich plasma (as described above) the pH of the platelets was adjusted to 6.5 using ACD. Prostaglandin E1 (1µM) was added to prevent activation of platelets during centrifugation. PRP was centrifuged at 720 g for 10min. The supernatant was carefully removed and discarded. One ml of JNL buffer (6 mM dextrose, 130 mM NaCl, 9mM NaCl2, 10 mM Na Citrate, 10 mM Tris, 3mM KCL, 0.8 mM KH2PO4, and 0.9mM MgCl2; pH 7.4) was layered on top of the platelet pellet and removed carefully so as not to disturb the pellet. The platelet pellet was resuspended in 1ml of JNL and then diluted to obtain a platelet count of $3x10^8$ platelets/ml. Washed platelets were supplemented with 2mM $CaCl_2$.

2.17.2 Preparation of gel filtered platelets

Washed platelets were prepared as described above without the final dilution step. Instead washed platelets were passed through a 10ml Sepharose 2B column (Sigma) that had been equilibrated in JNL buffer. Gel filtered platelets were collected and diluted to obtain a platelet count of $3x10^8$ platelets/ml. Washed platelets were supplemented with 2mM $CaCl_2$ and 1 mg/ml fibrinogen as required.

2.18 Removal of contaminating IgG from commercial supplies of fibrinogen

Fibrinogen depleted of plasminogen, von Willebrand Factor and fibronectin was purchased from Enzyme Research Groups and solubilised in PBS. To eliminate contaminating IgG the fibrinogen was passaged through a column of protein A coupled to sepharose (1 ml, Amersham Biosciences). The column was washed with 10 ml of PBS. One ml of fibrinogen at 10 mg/ml was loaded onto the column. The column was washed with 10 ml of PBS and the flow-through containing the purified fibrinogen was collected. Purified fibrinogen was concentrated back to 10 mg/ml in a centrifugal filter device (Amicon). Depletion of IgG was confirmed by ELISA as described in section 2.20.1. Bound IgG was eluted from protein A coupled sepharose with 5 ml of 100mM glycine, pH 2.5. The column was washed with H_2O and stored in 20% ethanol at 4 °C.

2.18.1 Removal of IgG from human serum

Human serum was depleted of IgG as described in above. Serum samples were diluted 1:20 and analyzed by SDS-PAGE to confirm loss of IgG.

2.19 Depletion of ClfB-specific antibodies from pooled human IgG

Ten ml cultures of *L. lactis* pNZ8037 and *L. lactis* pNZ8037clfB induced with 3.2 ng/ml of nisin were pelleted and washed twice in PBS. Cells were resuspended in 1 ml of pooled human IgG (Gammmagard, Baxter) at 5% (w/v) and incubated a blood tube rotator (Stuart Scientific) for 1 h at 4°C. Bacterial cells were pelleted and the absorbed IgG was collected and filtered using a 0.45μM filter (Sarstedt). Depletion of anti-ClfB antibodies was confirmed by testing reactivity with recombinant ClfB by ELISA as described in section 2.20.

2.20 Determination of specific anti-ClfB 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 2.5mM (100 μl) recombinant ClfB region A in of 50 mM sodium carbonate, pH 9.5, for 16 h at 4°C. Wells were washed three times with PBS and blocked with 100 μl BSA (5 % w/v) for 2 h at 37°C. Wells were washed three times with PBS and incubated with 5 μg pooled human IgG, anti-*L. lactis* depleted IgG and anti-*L. lactis* ClfB depleted IgG in

100 µl 2 % BSA (w/v). Plates were incubated for 1.5 h at room temperature. Unbound antibody was removed by washing wells three times with PBS. Bound antibody was detected by incubation with protein A-peroxidise (1 h at 37°C). Following three washes with PBS binding was quantified as described in section 2.16.

2.20.1 Determination of contaminating IgG levels in fibrinogen

Serial dilutions of fibrinogen and fibrinogen purified of IgG were coated onto microtitre plates (Sarstedt). Plates were incubated at 4 °C for 16 h. Wells were washed 3 times with PBS and blocked with 100 µl of 10% Marvel in TS buffer. Plates were incubated for 2 h at 37 °C. Wells were washed 3 times with PBS and 100 µl of protein A-peroxidise (1 mg/ml) in 10% Marvel/TS buffer was added to each well. Plates were incubated at room temperature with shaking for 1 h after which they were washed with PBS. Binding was quantified as described in section 2.16.

2.21 Heat inactivation of complement proteins in human serum

Human serum samples were thawed at room temperature. Inactivation of complement proteins in human serum was carried out by heating to 56° C for 30 min. Once cooled, heat inactivated serum was added to GFP at a final concentration of 10 % (v/v).

2.21.1 Adsorption of complement from serum by zymosan treatmen

Complement was removed from serum samples by treatment with the yeast polysaccharide zymosan (Sigma). Five hundred µl of serum sample was thawed and mixed with complement-activating zymosan at 100 mg/ml. This mixture was incubated at 37 °C for 30 min with stirring. The serum/zymosan mixture was centrifuged at 14000 rpm for 1 min to pellet the complement-coated zymosan particles. The supernatant was removed, aliquoted and frozen. Aliquots were thawed at room temperature before addition to GFPs at a final concentration 10 % (v/v).

2.22 Platelet adhesion assay

Cultures of bacterial strains were pelleted by centrifugation, washed in PBS and resuspended to an OD_{600} nm of 1. Wells of NUNC microtitre plates were coated with 100 µl of bacteria in triplicate and plates were incubated for 16 h at 4 °C. Negative controls included 1% BSA and Tris Buffered Saline (TBS, 0.5M Tris Base, 9% NaCl,

pH 7.6). Fibrinogen at 50 µg/ml was included as a positive control. Following incubation microtitre wells were washed 3 times with PBS. 1% BSA (100 µl) was added to wells and plates were incubated for 90 min at 37 °C. Wells were three times with 100 µl of JNL buffer. Washed platelets (50 µl) were prepared as described in section 2.17.1 and added to wells. Plates were incubated at 37 °C for 40 min and subsequently washed three times with JNL buffer containing $CaCl_2$. 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 for 2 h at 37°C. Sodium hydroxide (20 µl of 1M) was added to each well to stop the reaction and absorbance at 405 nm was read in an ELISA plate reader (Labsystems).

2.23 Platelet aggregation

L. lactis cells were induced and grown to stationary phase as described in section 2.1.3. *S. aureus* cells were grown to mid-exponential phase or stationary phase. Cells were washed twice with M17 broth or PBS and resuspended to an OD_{600} nm of 1.6. Bacterial cells (25µl) were added to 225µl of PRP in siliconized flat-bottom glass cuvettes (BioData). PRP and bacteria were incubated with stirring (900 rpm) in an aggregometer (Bio-Data) at 37° C. Light transmission was monitored for 25 min. Aggregation was inhibited by anti-GPIIb/IIIa (Abciximab, Eli Lily) antibodies at a 1/100 dilution and anti-FcγRIIa (IV3 kindly provided by R. Klimkowski, Medarex, USA) antibodies at a 1/50 dilution. The anti-GPIb monoclonal antibody AN51 was purchased from Dako and was used at a 1/50 dilution. Gel filtered platelets were supplemented with purified fibrinogen at a final concentration of 0.5 mg/ml. Pooled IgG and depleted IgG were added to a final concentration of 2 µg/ml. 25µl of human serum was added to a final volume of 225 µl GFP.

2.24 Statistical analysis

The data presented by this study represents the mean of three experiments ± SEM unless otherwise stated. Statistical analysis was performed using GraphPad software. The unpaired t-test was used to determine the significance of differences in platelet adhesion or aggregation between bacterial strains, with significance defined as $p < 0.05$.

Chapter 3

Interaction of clumping factor B of *Staphylococcus aureus* with the α-chain of fibrinogen

3.1 Introduction

S. aureus expresses several cell wall anchored proteins that can bind to fibrinogen. These include ClfA, ClfB and the FnBPA and FnBPB proteins. In addition *S. aureus* can produce a number of secreted proteins such as Map, Efb and Emp which also bind fibrinogen. The ability to interact with human extracellular matrix components is crucial in the establishment of *S. aureus* infections. Adherence of *S. aureus* to fibrinogen contributes to the ability of this organism to colonise implanted medical devices, to interact with platelets and to evade phagocytosis by macrophages (Arrecubieta *et al.*, 2006; Fitzgerald *et al.*, 2006; Higgins *et al.*, 2006).

The fibrinogen binding capacity of ClfA, ClfB and the FnBPs is localized to the A domains of these proteins. The A domains consist of sub-domains N1, N2 and N3. Sub-domain N1 is a protease sensitive region which can be cleaved in ClfA by aureolysin and serine protease (SspA) without affecting ligand binding (McDevitt *et al.*, 1994; McDevitt *et al.*, 1997). Cleavage of the N1 domain of ClfB by aureolysin results in loss of fibrinogen binding ability in the post-exponential growth phase (McAleese *et al.*, 2001). The fibrinogen binding N2N3 domains of ClfA, FnBPA and ClfB vary considerably in sequence. Domains N2N3 of ClfA and FnBPA are 20% identical while N2N3 of ClfA and ClfB share 22% sequence identity. Despite sequence variation the fibrinogen binding domains of these proteins share similar structural organisation (Figure 1.5). Crystal structure studies of ClfA revealed that the N2N3 domains are organised into a novel immunoglobulin-type fold which was named the D-variant or DEv-type IgG fold. Sequence alignments and secondary structure predictions indicated that the N2N3 domains of ClfB, FnBPA and FnBPB are also organised into DEv-type IgG folds (Deivanayagam *et al.*, 2002). This was later confirmed for ClfB when the crystal structure of the N2N3 domains was solved (M. Hook, unpublished data).

The DEv-type IgG fold is also found in SdrG, a cell wall-anchored protein of *S. epidermidis*. The solution of the crystal structure of N2N3 SdrG in complex with a synthetic fibrinogen peptide provided valuable insight into the structural changes that occur upon ligand binding. A common mechanism of ligand binding termed the "dock, lock and latch" model was proposed to occur in the structurally related fibrinogen-binding proteins. In this model ligands are docked into the hydrophobic trench located at the interface between the N2 and N3 domains. The C-terminal of the N3 domain constitutes a flexible linker known as the "latching peptide". During

ligand binding the latching peptide undergoes conformational changes in which it crosses over the binding trench. The ligand is locked in place by this event and secured by hydrogen bonds that form between the ligand and latching peptide. Finally, β-strand complementation creates a new β-sheet in the N2 domain where the C-terminal β-strand of the N3 domain inserts between β-sheets of the N2 domain (Figure 1.5; Ponnuraj *et al.*, 2003).

ClfA, FnBPA and FnBPB bind to a flexible unfolded peptide at the C terminal of the γ-chain which protrudes from each of the two D domains of fibrinogen (Figure 3.1). In-silico models of a γ-chain peptide docked into the crystal structure of ClfA and FnBPA identified residues of these two proteins that interact with fibrinogen. In the most energetically favourable conformations the γ-chain peptide was found in the hydrophobic pocket between domains N2 and N3 of ClfA and FnBPA. Residues P336 and Y338 of ClfA and residues N304 and F306 of FnBPA occur in equivalent positions within domain N2. Introducing alanine substitutions at these positions caused a complete loss in fibrinogen-binding in both ClfA and FnBPA. These residues appear to be crucial for ClfA and FnBPA to interact with fibrinogen (Deivanayagam *et al.*, 2002; Hartford *et al.*, 2001).

Recent work carried out in this laboratory has conclusively shown that ClfB binds to the α-chain of fibrinogen. The binding site was localized using α-chain mutants and truncated recombinant proteins. Recombinant ClfB A-domain and the full length protein expressed from bacteria bound specifically to the truncated C-terminal domain of the α-chain. This domain of fibrinogen corresponds to the αC-domain and connector region which span residues 221-610 (Figure 3.1).

The αC regions of fibrinogen are highly susceptible to proteolysis and can be cleaved into smaller fragments by plasmin and other proteases (Collet *et al.*, 2005). The 3D structure of this domain has not yet been established although recent studies have shed some light on the domain organisation (Burton *et al.*, 2006; Tsurupa *et al.*, 2002). A compact globular C terminal structure named the αC-domain is connected to the rest of the molecule by a flexible non-globular connector (αC-connector). The αC-connector spans residues 221-391 (Figure 3.1). In human fibrinogen this region consists of a 43 residue segment followed by ten 13 residue tandem repeats (Figure 3.2). The globular αC-domain consists of residues 392-610. It has been shown that the αC-domains can interact with each other and the central E domain via

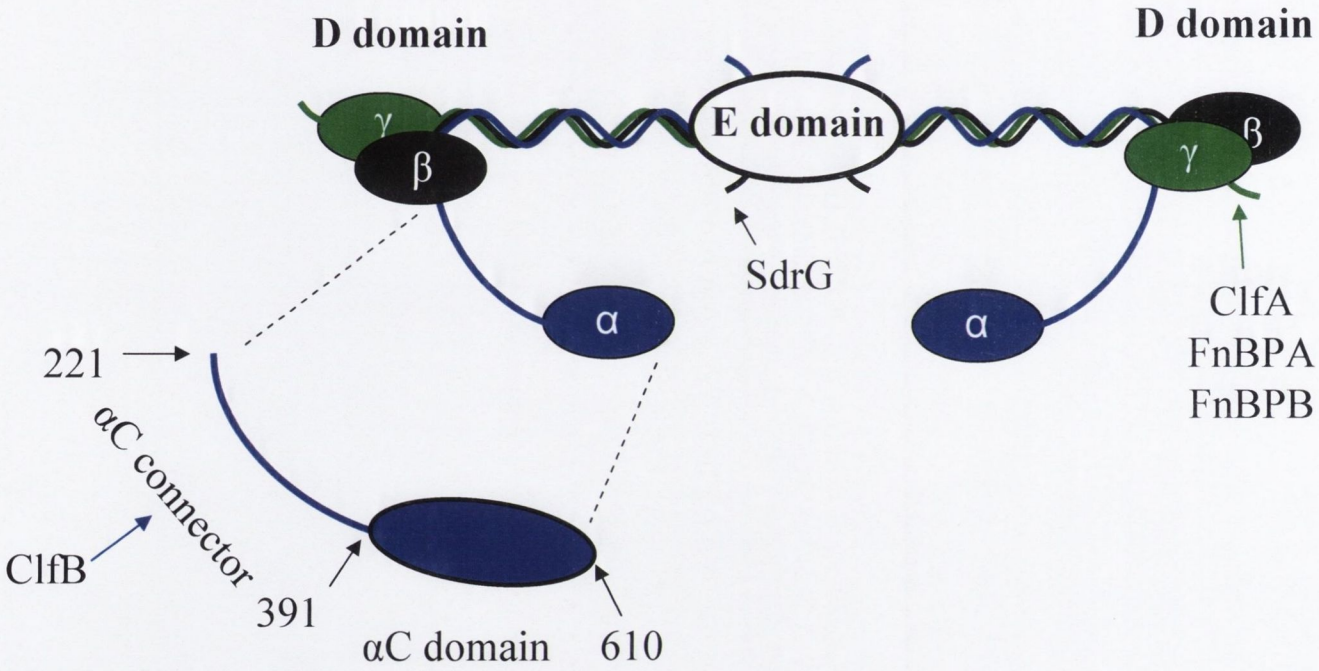


Figure 3.1. MSCRAMM binding sites in fibrinogen

ClfA, FnBPA and FnBPB all bind to a flexible, unfolded extension of the C-terminus of the 2 γ-chains shown in green. The binding sites of SdrG are the N terminal residues of the 2 Bβ-chains known as fibrinopeptides A which project from the E domain. ClfB binds to the αC-connector, a non-globular region spanning residues 221-391 of the two αC regions.

fibrinopeptide B (Litvinov *et al.*, 2007). Following fibrinopeptide B cleavage, the αC-domains participate in fibrin assembly (Medved *et al.*, 1985; Weisel & Medved, 2001). The ClfB binding site within fibrinogen was identified as the αC-connector. Tandem repeat 5 of the connector (residues 316-328) appeared to be necessary for ClfB binding (Figure 3.2). The work described in this chapter identified residues within tandem repeat 5 crucial for ClfB binding and eliminated the possibility of other binding sites within the homologous, tandem repeats of the αC-connector.

ClfB is also able to bind cytokeratin 10 (Walsh *et al.*, 2004). Cytokeratins belong to the family of proteins that form intermediate filaments (IF) which provide mechanical strength to vertebrate epithelial cells. Keratin filaments are composed of heterodimers of type I and type II keratin. Cytokeratin 10 is a type I keratin and is associated with cytokeratin 1. Other combinations of keratin filaments are found in basal proliferating cells but cytokeratin 1 and 10 are found in the differentiated layer of the human epidermis (Steinert & Marekov, 1995). Confocal microscopy and flow cytometry have been used to show that cytokeratin 10 is present on the surface of desquamated epithelial cells found in the anterior nares (O'Brien *et al.*, 2002b). The ability of ClfB to bind cytokeratin 10 is believed to be an important factor in colonisation of the desquamated nasal epithelium (Schaffer *et al.*, 2006).

Cytokeratin 10 is composed of a central rod domain which has a highly conserved secondary structure forming α-helical coiled coils. The rod domain is flanked by the N-terminal head domain and the C-terminal tail domain which vary considerably in length and sequence. Sub-domains V1 of the head domain and V2 of the tail domain are rich in glycine and serine repeats (Figure 3.3; Zhou *et al.*, 1988). ClfB binds specifically to the V2 domain of the tail region of cytokeratin 10 (Walsh *et al.*, 2004). This domain is involved in intermediate filament organisation and epithelial barrier function. The repeats within the V2 domain are believed to form structures known as Ω loops with a typical sequence of Tyr-(Gly/Ser)$_n$. Ω loops are continuous segments of protein sequence which adopt a loop shape in three dimensional space, with small distances between the segment termini (Fetrow, 1995; Leszczynski & Rose, 1986; Pal & Dasgupta, 2003). Repeat 5 (NSGSSGTGSTTGNQ) of the αC-connector of fibrinogen resembles the Ω loops of the tail region of cytokeratin 10. The similarity in the binding regions of ClfB's two ligands raises the possibility that a common binding site in ClfB for fibrinogen and cytokeratin 10 may exist.

This study aimed to isolate non-fibrinogen binding mutants of ClfB. If a common binding site exists within ClfB for fibrinogen and cytokeratin 10 alterations that affect binding of one ligand might also affect binding to the other. Site-directed mutagenesis was used to alter residues in the hydrophobic trench of ClfB. Evidence from previous studies shows common structural organisation of the A regions of ClfB and the other cell wall-anchored fibrinogen binding proteins of *S. aureus*. These proteins all adhere to flexible, non-globular regions of fibrinogen and a common model for ligand binding the "dock, lock and latch" mechanism has been proposed. Based on these observations, residues in the hydrophobic trench of ClfB corresponding to residues of ClfA and FnBPA which eliminate fibrinogen binding were initially selected for change. Other residues with side chains pointing into the trench which may interact with a bound ligand were also considered for mutagenesis. A non-fibrinogen binding mutant was also required to study of the interaction between ClfB and platelets as described in Chapter 4. In order to study the effect of ClfB on platelet activation it was preferable to express this protein from the heterologous host *L. lactis*. Expression of proteins from the nisin-controlled expression system (NICE) in *L. lactis* allows them to be studied in isolation of other *S. aureus* proteins and also provides a means of controlling the level of protein on the bacterial cell surface (Figure 2.1).

3.2 Results

3.2.1 Site directed mutagenesis of DNA encoding residues in the putative binding trench of ClfB

Chimera molecular modelling software was used to visualise the 3D crystal structure of ClfB (M. Hook, unpublished data). Residues in the putative hydrophobic trench were considered for alteration as follows: (i) Residue R331 was selected since it has a large side chain pointing into the trench which may come in contact with potential ligands. (ii) P336 and F338 were chosen because they are located in equivalent positions to residues P336 and Y338 of ClfA and N304 and F306 of FnBPA which are known to be important in fibrinogen binding (Deivanayagam *et al.*, 2002; Hartford *et al.*, 2001). It was possible to alter residues P336 and F338 in the same experiment because they are located close enough to each other for altered codons to be incorporated in the same mutagenic primer. The residues involved are shown in Figure 3.4. The Quickchange method described in section 2.3.3 was used to

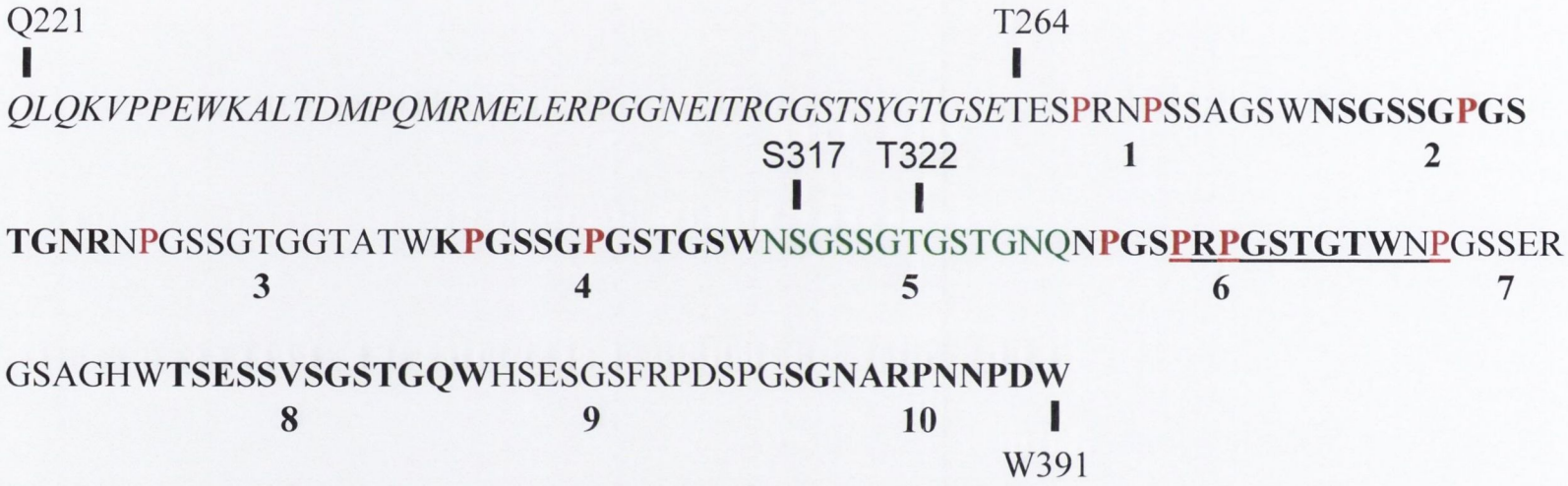


Figure 3.2. Amino acid sequence of the N-terminal section of the fibrinogen αC-connector

The ten tandem repeats of the αC-connector begin at residue T264 and are shown in alternating normal and bold face type. The ClfB binding site, tandem repeat 5 is highlighted in green. The S317 and T332 residues that were substituted with proline residues within tandem repeat 5 are indicated. Proline residues are shown in red. The peptide PRPGSTGTWNP, which inhibits binding of recombinant ClfB to full length fibrinogen is underlined.

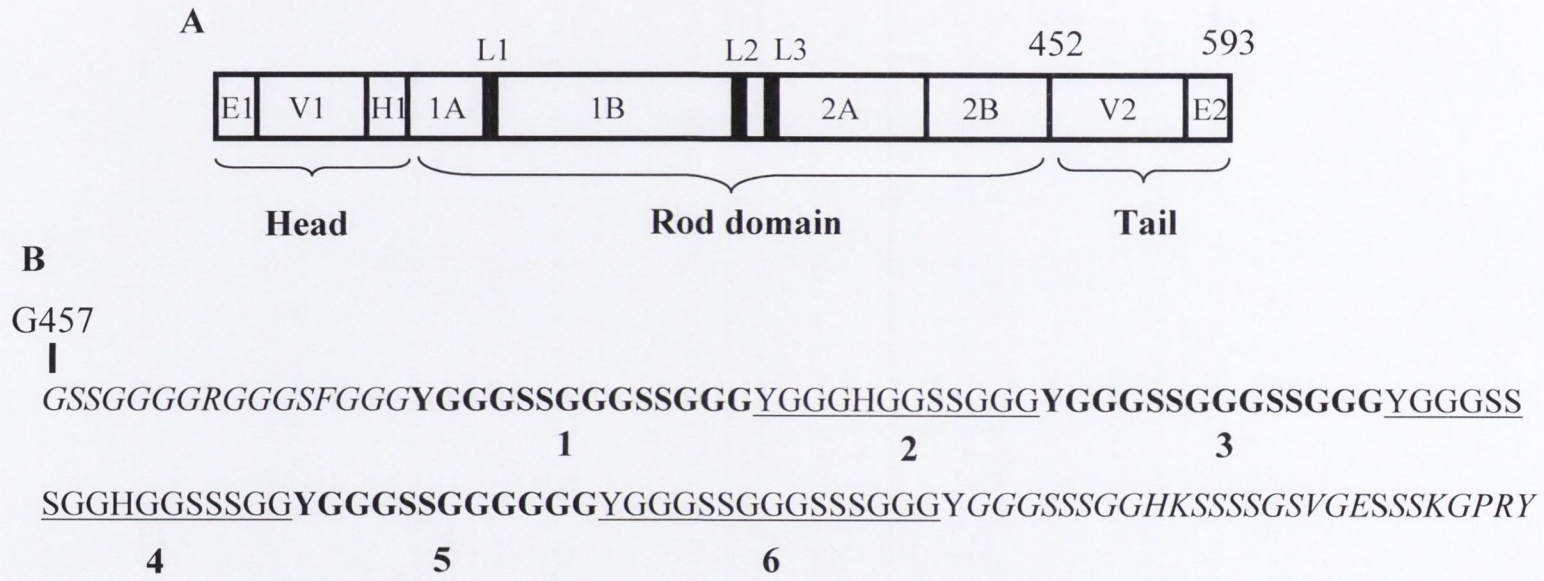


Figure 3.3. Structural organisation of cytokeratin 10

A. Cytokeratin 10 is composed of a central rod domain flanked by a head and tail domain. The central rod domain has α-helical sub-domains 1A, 1B, 2A and 2B which are separated by linkers L1, L2 and L3. The V1 and V2 sub-domains of the head and tail domains are rich in glycine and serine repeats. ClfB binds to the V2 sub-domain of the tail domain.

B. Sequence of the tail region of human cytokeratin 10. The 6 Tyr-$(Gly/Ser)_n$ repeats of the V2 tail domain are shown in alternating normal and bold face type.

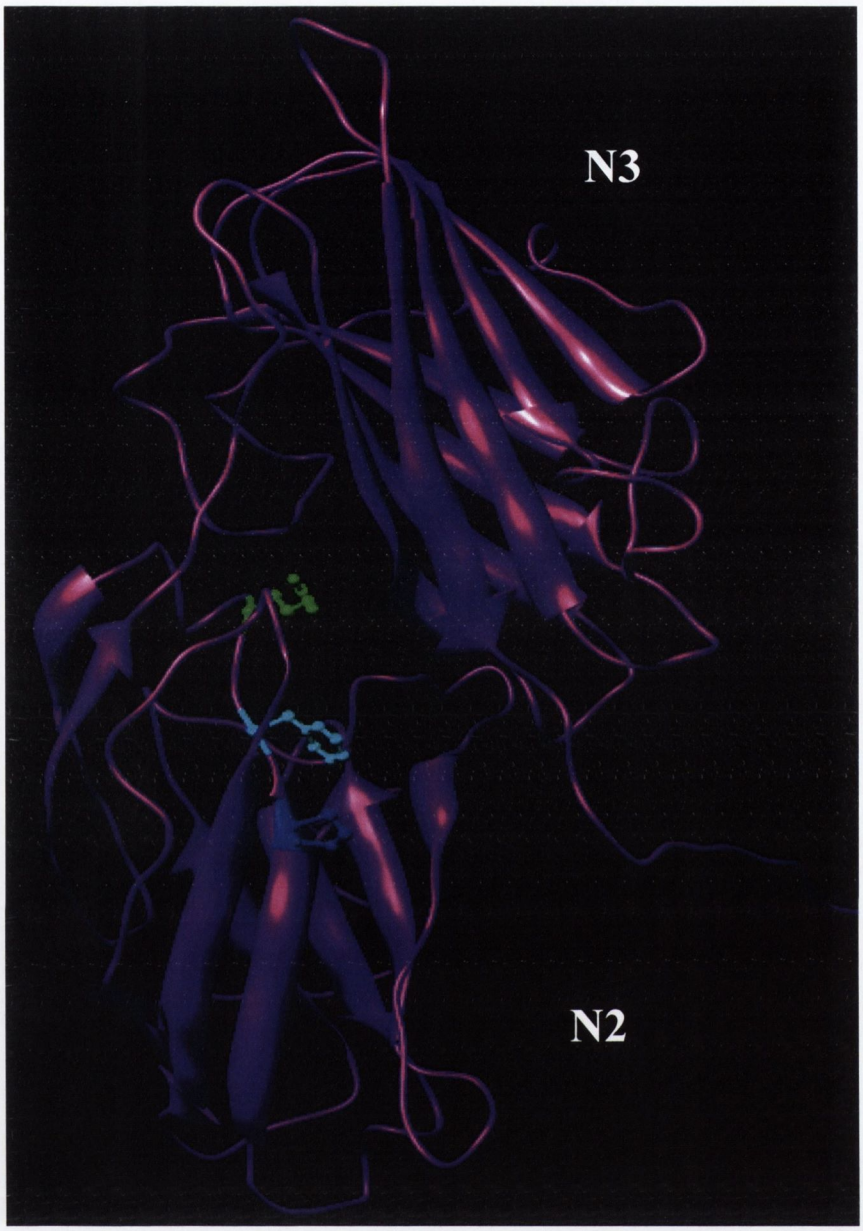


Figure 3.4. 3D crystal structure of domains N2 and N3 of ClfB

Structural model of N2 and N3 subdomains of ClfB. Side chains of alanine substituted residues are shown in the ball and stick format. Residues P326 and F328 are shown in navy and light blue respectively. Residue R331 is shown in green.

introduce alanine codons at the positions described. The template for mutagenic PCR was plasmid pQE30 containing the A domain (45-542) of ClfB (pQE30 *clfB*). PCR products were digested with *Dpn*I to remove wild-type parental DNA and transformed into *E. coli* XL-1 Blue cells. Plasmids were isolated from transformants and the presence of mutations was confirmed by DNA sequencing.

3.2.2 Fibrinogen binding by alanine substituted variants of ClfB

Plasmids pQE30 *clfB*, pQE30 *clfB*PF and pQE30 *clfB*R331A were transformed into protease-defective *E.coli* strain TOPP3. The 6-His tagged A domains of ClfB, ClfB P336A F338A (ClfB PF) and ClfB R331A were purified by nickel chetate affinity chromatography as described in section 2.14. Solid phase ELISA-type assays were carried out to compare the fibrinogen binding ability of the wild-type ClfB A domain and the A domains of ClfB PF and ClfB R331A. Microtitre plates were coated with 10 μg/ml of fibrinogen and subsequently blocked with BSA. Dilutions of recombinant ClfB, ClfB PF and ClfB R331A were added to the fibrinogen coated wells.

Binding of wild-type ClfB and the mutants was detected using rabbit polyclonal anti-ClfB antibody. Bound antibody was detected with goat anti-rabbit antibody conjugated to peroxidise. Plates were developed as described in section 2.16. Altering residues P336 and F338 did not have a noticeable effect on fibrinogen binding by ClfB. ClfB and ClfB PF bound to fibrinogen in a dose-dependent and saturable manner with almost identical half-maximum binding values of 0.071 μM and 0.072 μM, respectively (Figure 3.5).

Binding of recombinant ClfB and ClfB R331A to immobilised fibrinogen was detected using anti-His tag antibodies. ClfB R331A bound to fibrinogen in a dose-dependent and saturable manner, virtually indistinguishable from the wild-type protein, demonstrating that residue R331 is not important for ClfB to bind fibrinogen (Figure 3.6).

3.2.3 Docking of fibrinogen peptide GTWNP into the 3D crystal structure of ClfB

Another approach was adopted in order to narrow down the number of sites within *clfB* to target for mutagenesis. The fibrinogen αC-connector peptide PRPGSTGTWNP (residues 352-362) inhibited binding of recombinant ClfB to full

length fibrinogen. Altering the tryptophan residue within this peptide reduced inhibition of fibrinogen binding (E. Walsh, personal communication). Taking this into account GTWNP was selected as a peptide for docking into the crystal structure of ClfB using the Autodock computer modelling program (carried out by M. Brennan, Royal College of Surgeons). The docking result with the most favourable energy was further analysed using Chimera software. Residues P326 and F328 were not in close proximity to the GTWNP peptide and no interaction was predicted. Residue R331 was found within 4 Å of the peptide. Other residues of the ClfB N2 and N3 sub-domains within 3-4 Å of the peptide were then considered for mutagenesis. Of these residues Y446 within 4 Å of the peptide and residues D270, Q235, W522 and N526 within 3 Å of the peptide were selected. Residue Q235A was predicted to form a hydrogen bond with the GTWNP peptide. The positions of these residues are shown in Figure 3.7.

3.2.4 Site-directed mutagesesis of pNZ8037 *clfB* and screening of potential ClfB mutants

Quickchange mutagenesis was carried out on the pNZ8037 vector encoding full length ClfB. Complementary primers were used to introduce alanine codons at codons for Y446, D270, Q235, W522 and N526 as described in section 2.3.3. PCR products were digested with *Dpn*1 and transformed into *L. lactis* strain NZ9800. This strain is defective in nisin production so expression of genes cloned in-frame with the *nisA* start codon can be induced by addition of extracellular nisin. Since ClfB and its alanine substituted variants could be expressed directly from *L. lactis* the need for time- consuming protein purification was eliminated.

L. lactis (pNZ8037 *clfB*) and transformants obtained from Quickchange mutagenesis were grown and induced with 3.2 ng/ml of nisin as described in section 2.3.3. Bacterial cells were washed and the $OD_{600\ nm}$ was equalised in PBS. Cells were added to microtitre plates coated with 10 µg/ml of fibrinogen. Adherence of *L. lactis* expressing ClfB and alanine substituted mutants was assessed by crystal violet staining. Of 17 clones obtained from Quickchange mutagenesis to introduce an alanine at residue N526, 6 clones (35%) were deficient in fibrinogen binding (Figure 3.8.A). Fifteen transformants were obtained from Quickchange mutagenesis to introduce an alanine substitution at residue Q235 of fibrinogen. Only 1 of these was deficient in fibrinogen binding (Fig 3.8.B). Fibrinogen binding mutants were

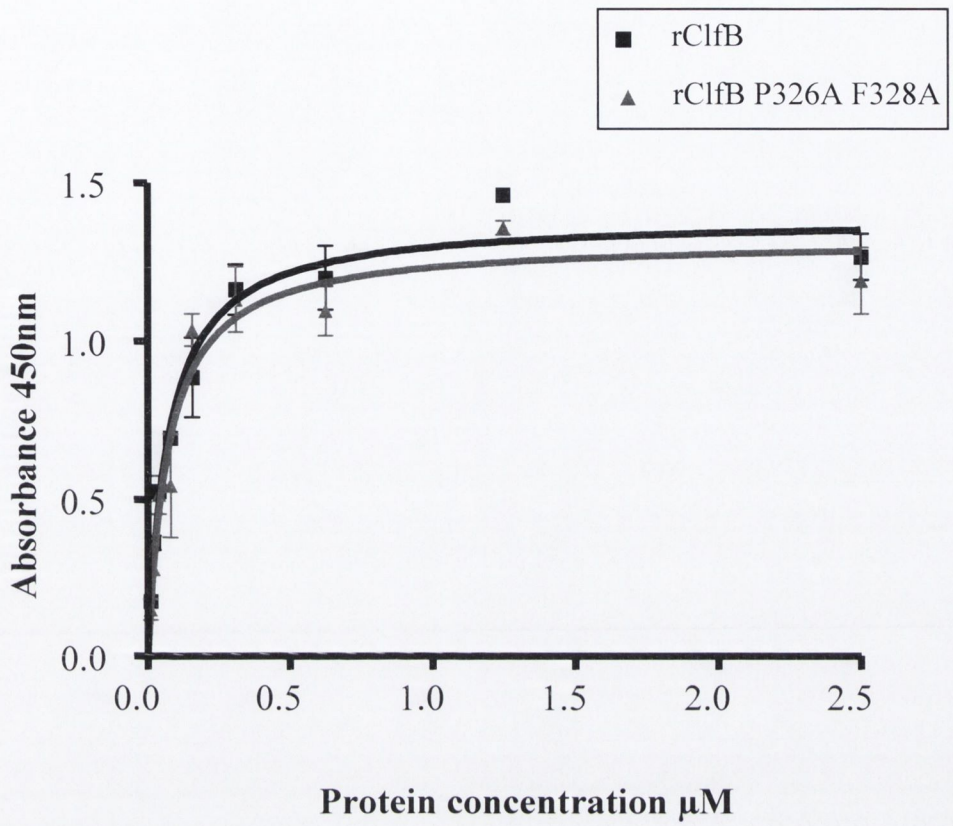


Figure 3.5. Binding of the recombinant A domain of ClfB and ClfB P326A F328A to immobilized fibrinogen

Serial dilutions of recombinant ClfB and ClfB PF were added to microtitre plates coated with fibrinogen. After incubation and washing bound ClfB was detected with polyclonal rabbit anti-ClfB antibody. Bound antibodies were detected using a goat-anti-human antibody conjugated to peroxidase and developed by incubation with a chromogenic substrate. The absorbance at 450 nm was determined. Values represent the means ± standard deviation of triplicate wells. The experiment was performed three times with similar results.

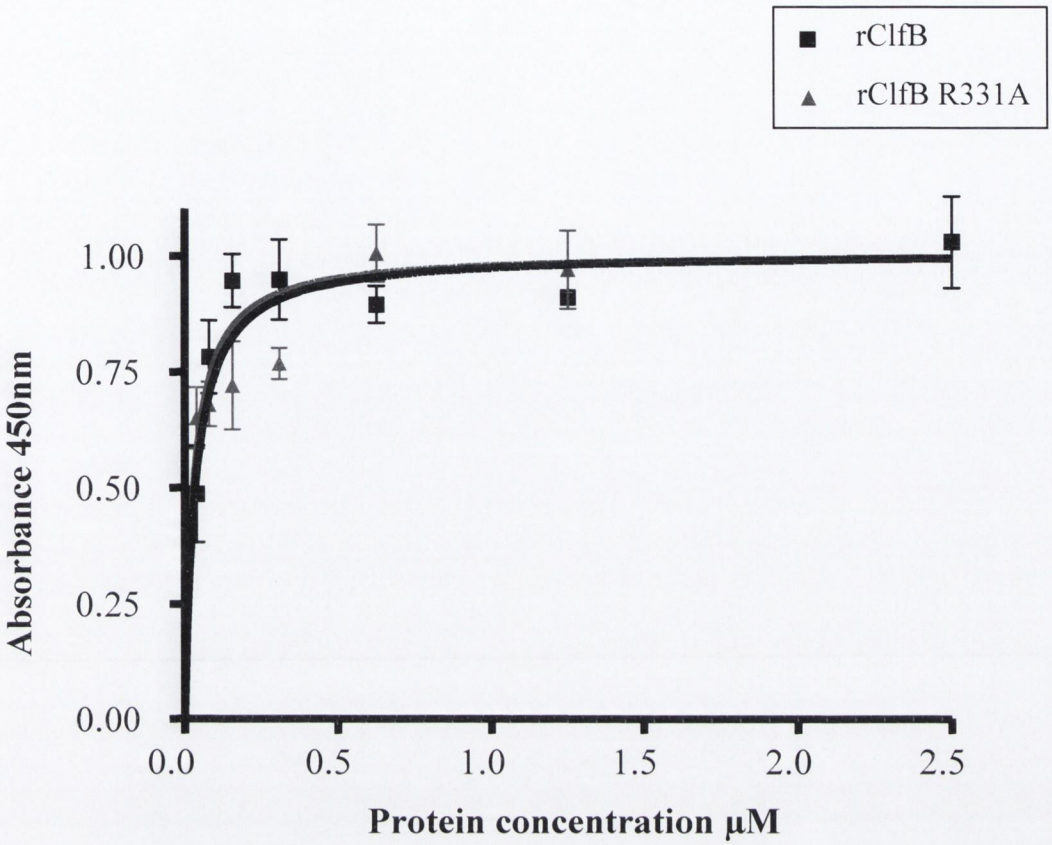


Figure 3.6. Binding of recombinant A domain of ClfB and ClfB R331A to immobilized fibrinogen

Serial dilutions of recombinant ClfB and ClfB R331A were added to microtitre plates coated in fibrinogen. Following incubation and washing bound ClfB was detected with anti-His tag antibody conjugated to peroxidase and developed by incubation with a chromogenic substrate. The absorbance at 450 nm was determined. Values represent the means ± standard deviation of triplicate wells. The experiment was performed three times with similar results.

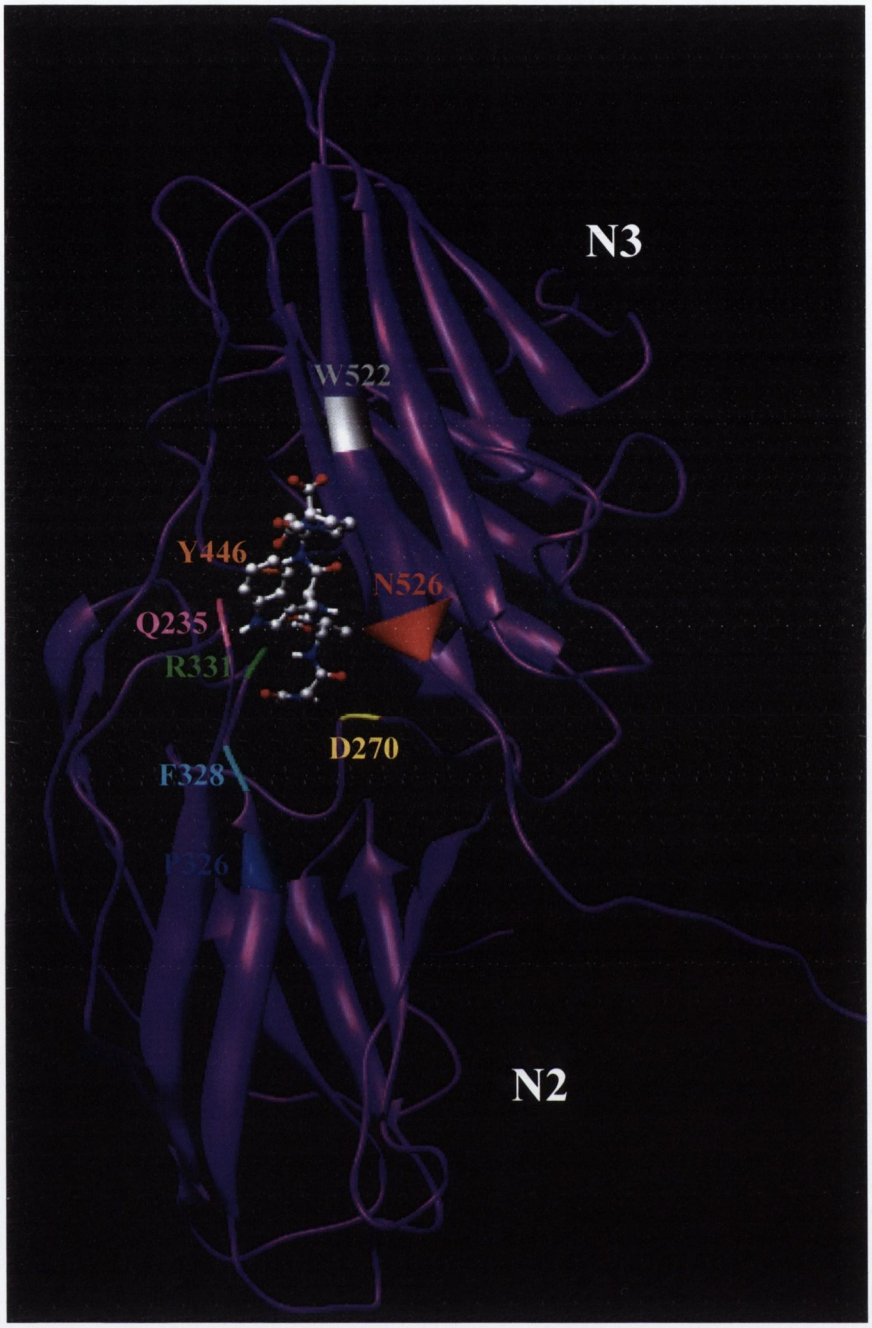


Figure 3.7. 3D crystal structure of N2N3 of ClfB with docked GTWNP peptide

Structural model of ClfB with the fibrinogen peptide GTWNP shown (in ball and stick format) docked in the hydrophobic interface between domains N2 and N3. Residues of ClfB within 3-4 Å of the peptide were selected for mutagenesis and the backbones of these residues are highlighted in colours.

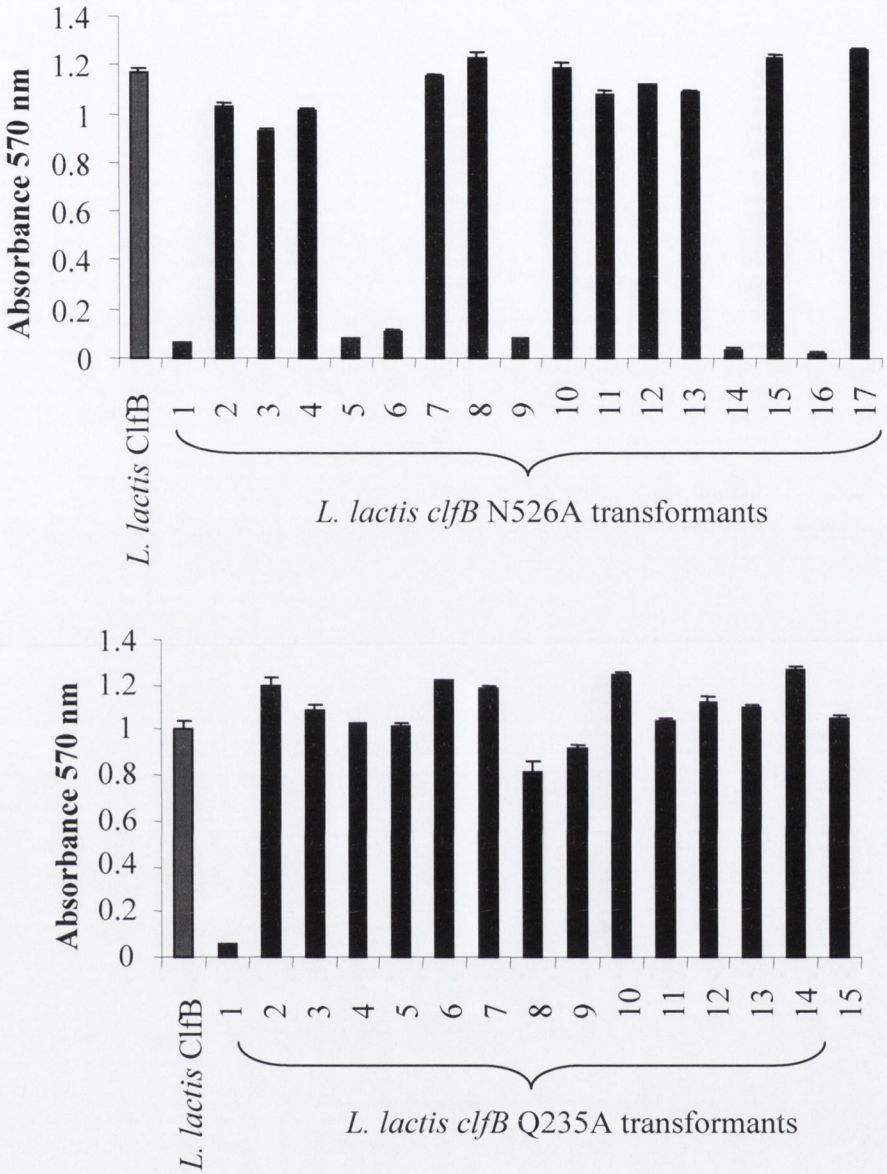


Figure 3.8. Screening of putative ClfB mutants Q235A and N526A

A. *L. Lactis* transformants obtained from site-directed mutagenesis of the codon for residue N526 were grown and induced with 3.2 ng/ml nisin. Washed bacterial cells with OD_{600} of 1 were added to plates coated with fibrinogen. Adherent cells were detected by crystal violet staining.

B. Fibrinogen binding of *L. Lactis* transformants obtained from site directed mutagenesis of residue Q235A.

sequenced and all 6 putative N526A non-adhering clones had an alanine substitution at residue 526 of ClfB. Clone 1 was selected for use and is referred to as pNZ8037 *clfB*N526A. The 1 non-fibrinogen binding clone obtained from mutagenesis of codon Q235 was also sequenced and found to contain the correct mutation. The positions of these two residues in the ClfB structure are shown in Figure 3.9.

All of the transformants obtained from attempted alteration of residues W522, D270 and Y446 were able to adhere to fibrinogen. Sequencing carried on a selection of these transformants revealed wild-type sequence. It appears Quickchange mutagenesis was less successful in *L. lactis* than in *E. coli* where the majority of clones obtained are correct.

3.2.5 Expression of ClfB mutants N526A and Q235A by *L. lactis*

Whole cell immunoblots were carried out to compare expression of wild type ClfB to ClfB Q235A and ClfB N526A on the surface of *L. lactis*. *L. lactis* strains containing plasmids pNZ8037 *clfB*, pNZ8037 *clfB* Q235A and pNZ8037 *clfB* N526A were grown with concentrations of nisin ranging from 0.025 ng/ml to 3.2 ng/ml. Serial dilutions of washed, induced bacterial cells were applied to nitrocellulose membranes and subsequently probed with polyclonal rabbit anti-ClfB antibody and developed as described in section 2.10. The level of expression of wild type ClfB, ClfB Q235A and ClfB N526A by *L. lactis* was directly proportional to the amount of nisin added to the growth medium. There was no noticeable difference in the levels of expression of the wild type and non-fibrinogen binding ClfB proteins detected with rabbit anti-ClfB antibody. Expression was increased approximately 64 fold in fully induced compared to un-induced cells (Fig 3.10).

Western immunobloting was performed on proteins solubilised from the *L. lactis* cell wall to show that ClfB Q235A and ClfB N526A proteins expressed from *L. lactis* were intact. Cell wall extracts of *L. lactis* expressing wild type and mutant ClfB proteins were separated by SDS-PAGE and transferred on to PVDF membranes. Membranes were probed with polyclonal anti-ClfB antibody and developed as described in section 2.9. The ClfB, ClfB Q235A and N526A proteins each migrated as bands of 150 kDa (Figure 3.11). The previously reported migration of ClfB solubilised from the cell wall of *S. aureus* was 150 kDa. A breakdown product due to cleavage of N1 at the SLAVA motif resulted in loss of ability to bind fibrinogen

(McAleese *et al.*, 2001). These results show that the two non-fibrinogen binding mutants were intact and were expressed at the same levels as wild type ClfB.

3.2.6 Adherence of *L. lactis* expressing ClfB Q235A or ClfB N526A to fibrinogen and cytokeratin 10

The ability of *L. lactis* expressing ClfB wild-type, ClfB Q235A and ClfB N526A to adhere to immobilised fibrinogen and to murine recombinant cytokeratin 10 was compared at various nisin inducer concentrations. *L. lactis* strains containing plasmids pNZ8037, pNZ8037 *clfB*, pNZ8037 *clfB* Q235A and pNZ8037 *clfB* N526A were grown with concentrations of nisin ranging from 0.025 ng/ml to 3.2 ng/ml. Washed bacterial cells were normalised to an OD_{600} of 1 and added to microtitre plates coated with either 10 µg/ml of fibrinogen or 10µg/ml of murine cytokeratin 10. Following incubation adherent cells were fixed with formaldehyde and stained with crystal violet as described in section 2.15.

L. lactis containing vector pNZ8037 did not adhere detectably to fibrinogen. *L. lactis* expressing wild-type ClfB adhered to fibrinogen in a dose-dependent and saturable manner. Adherence of *L. lactis* ClfB N526A and *L. lactis* ClfB Q235A to fibrinogen was dramatically reduced (Figure 3.12). Of the two mutants, ClfB Q235A appeared to have a lower affinity for fibrinogen. Therefore *L. lactis* expressing ClfB Q235A was selected for use in future platelet studies.

L. lactis (pNZ8037) could not adhere detectably to the tail region of murine cytokeratin 10. *L. lactis* expressing wild-type ClfB adhered to cytokeratin 10 in a dose dependent and saturable manner. ClfB mutants N526A and Q235A expressed by *L. lactis* had a dramatically reduced ability to bind cytokeratin 10 (Figure 3.13). These results indicate that residues N526 and Q235 located in the hydrophobic trench of ClfB are crucial for binding to both fibrinogen and cytokeratin 10.

3.2.7 Mutagenesis of codons in tandem repeat 5 of the fibrinogen αC-connector

Studies carried out with truncated and deleted variants of the αC-connector of the fibrinogen Aα-chain showed that tandem repeat 5 is crucial for ClfB binding. This 13 residue repeat is very similar in sequence to other repeats in the αC-connector with the exception that proline residues are absent (Figure 3.2). We hypothesised that the presence of proline residues in the sequences of repeats 1-3 may interfere ClfB binding. In order to test this and rule out the possibility of further ClfB binding sites

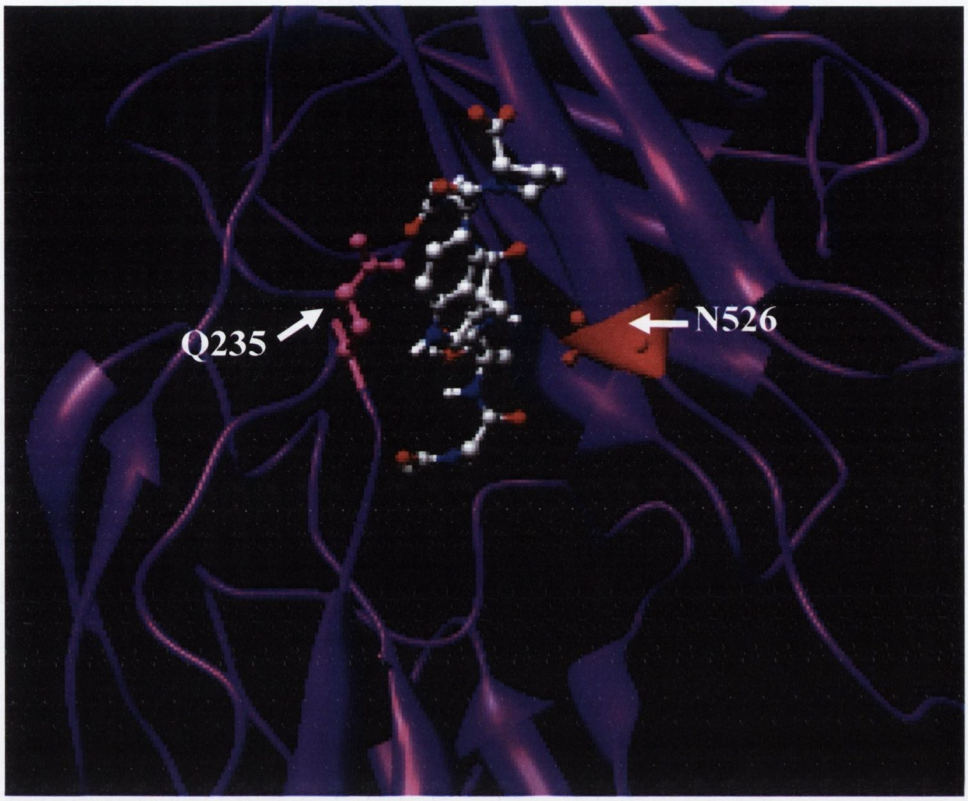


Figure 3.9. Position of Q235 and N526 residues in ClfB

Enlarged section of the crystal structure of N2 and N3 of ClfB with docked GTWNP peptide. Residue Q235 is shown in pink in the ball and stick format. Residue N526A is shown in red in the ball and stick format.

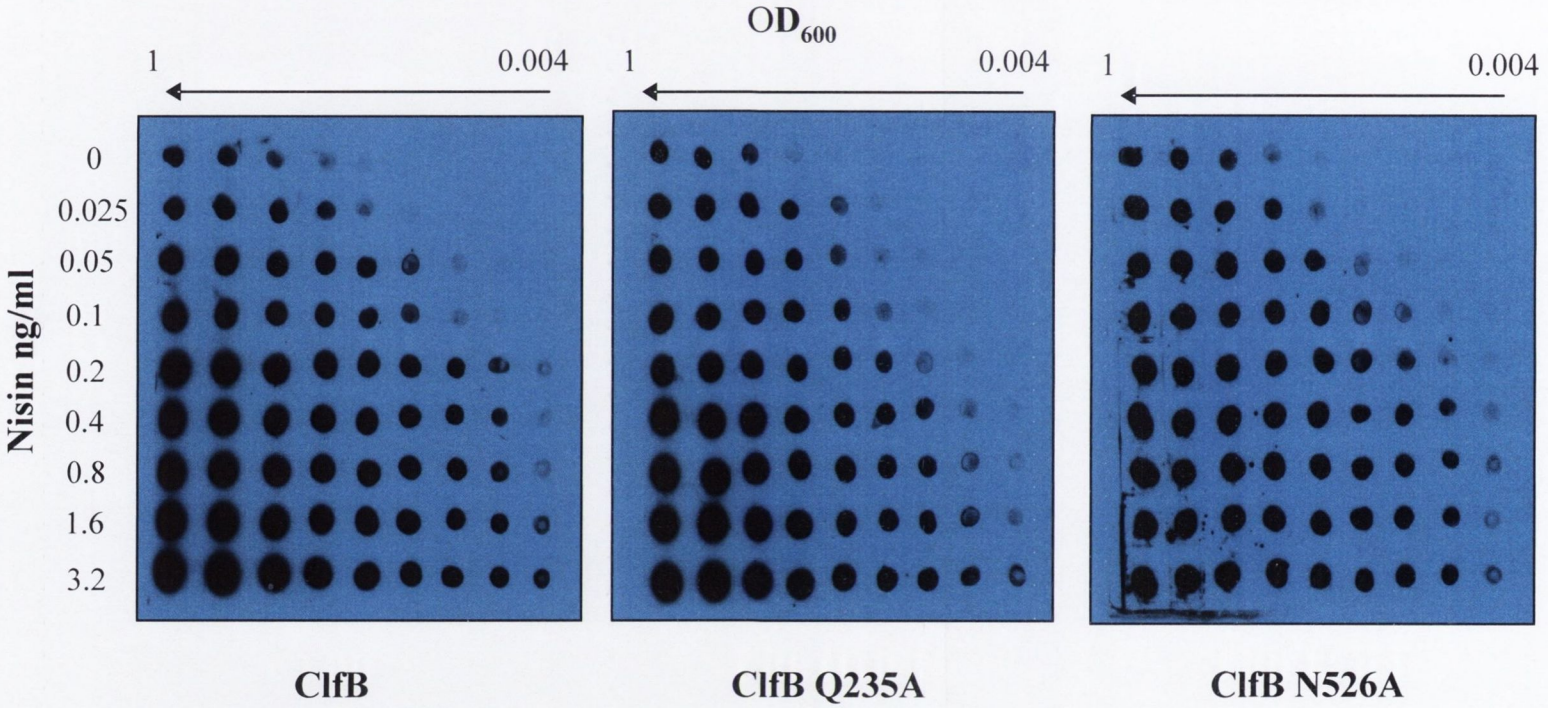


Figure 3.10. Nisin-controlled expression of ClfB, ClfB Q235A and ClfB N526A by *L. lactis*

*L. lactis (*pNZ8037*clfB)*, *L. lactis (*pNZ8037*clfB* Q235A) and *L. lactis (*pNZ8037*clfB* N526A) were grown with increasing concentrations of nisin as indicated. Cells were washed in PBS and resuspended to an OD_{600} of 1.0. Serial dilutions were applied to nitrocellulose membranes which were blocked in 10 % skimmed milk solution for 16 h. Membranes were incubated with rabbit-anti-ClfB antibodies. Bound antibodies were detected with goat-anti-rabbit antibodies conjugated to peroxidase. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

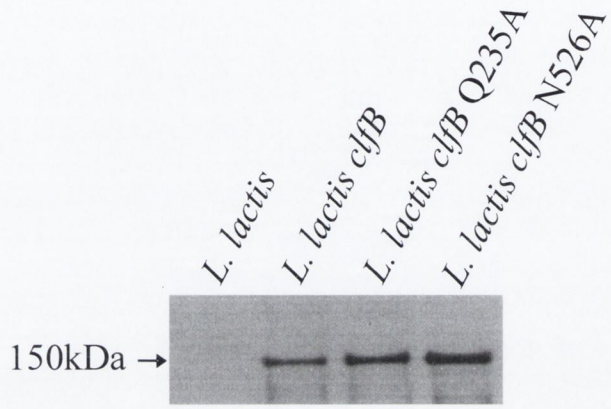


Figure 3.11. Expression of ClfB by *L. lactis*.

*L. lactis (*pNZ8037*)*, *L. lactis (*pNZ8037*clfB)*, *L. lactis (*pNZ8037*clfB* Q235A) and *L. lactis (*pNZ8037*clfB* N526A) were induced with nisin and grown to stationary phase. Cell wall proteins were solubilised with mutanolysin and lysozyme and separated by SDS-PAGE. Cell wall extracts were subsequently electroblotted onto PVDF membranes. Membranes were probed with rabbit anti-ClfB antibody and bound antibody was detected with protein A-peroxidase. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

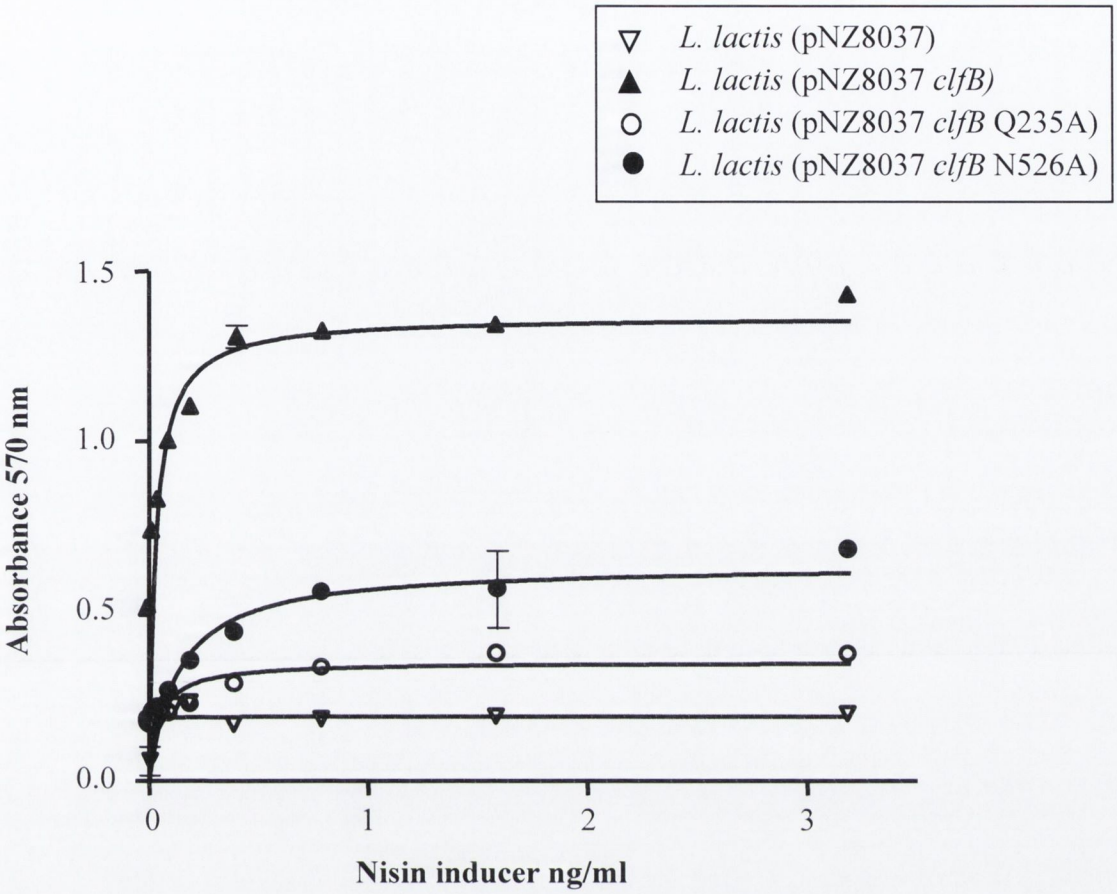


Figure 3.12. Fibrinogen binding by ClfB, ClfB Q235A and ClfB N526A expressed by *L. lactis*

L. lactis (pNZ8037), *L. lactis* (pNZ8037*clfB*), *L. lactis* (pNZ8037*clfB* Q235A) and *L. lactis* (pNZ8037*clfB* N526A) were induced with nisin and grown to stationary phase. Adherence of washed cultures to ELISA plates coated with fibrinogen was assessed by crystal violet staining. Absorbance at 570 nm was determined. Values represent the means ± standard deviation of triplicate wells. The experiment was performed three times with similar results.

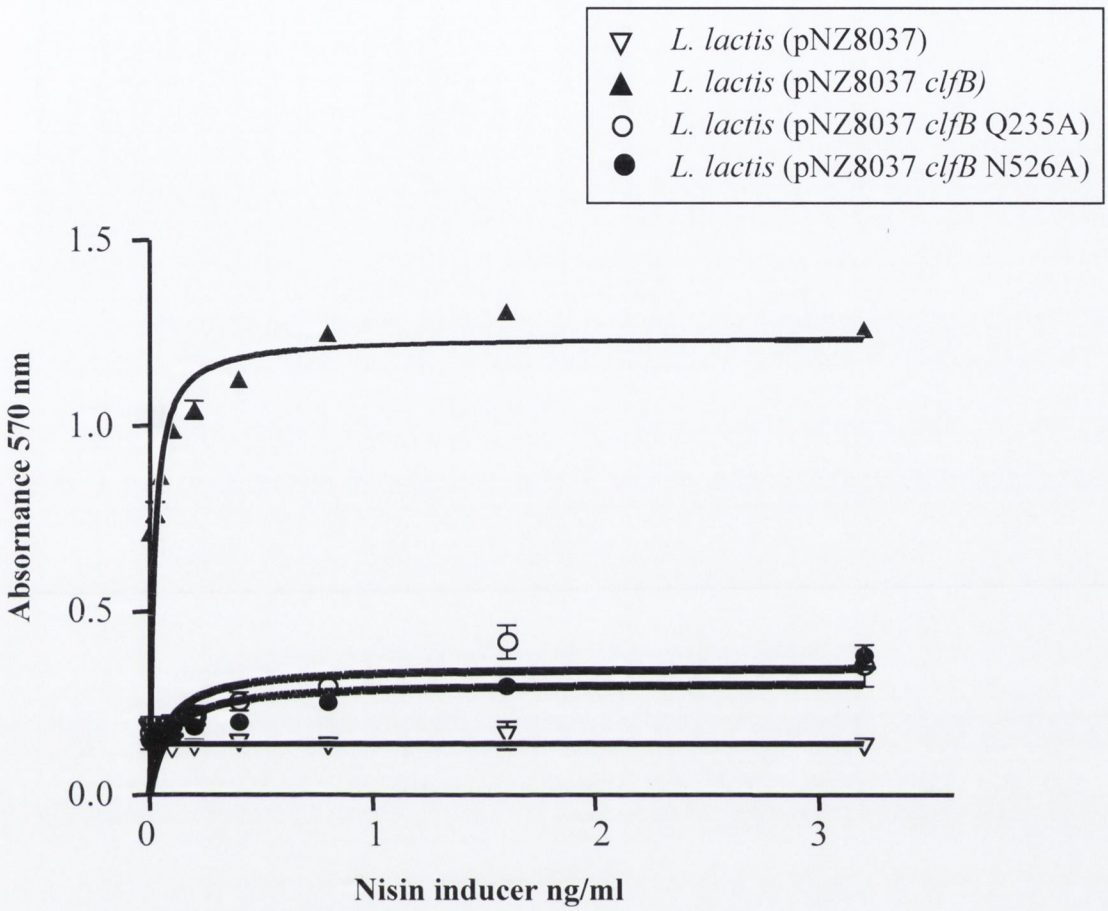


Figure 3.13. Cytokeratin binding by ClfB, ClfB Q235A and ClfB N526A expressed by *L. lactis*

*L. lactis (*pNZ8037*)*, *L. lactis (*pNZ8037*clfB)*, *L. lactis (*pNZ8037*clfB* Q235A) and *L. lactis (*pNZ8037*clfB* N526A) were induced with nisin and grown to stationary phase. Adherence of washed cultures to ELISA plates coated with cytokeratin 10 was assessed by crystal violet staining. Absorbance at 570 nm was determined. Values represent the means ± standard deviation of triplicate wells. The experiment was performed three times with similar results.

in repeats 1-3, proline residues were introduced into repeat 5 to make it more closely resemble other repeats in the αC connector.

Codons for residues S317 and T322 of repeat 5 were chosen for site-directed mutagenesis by the Quickchange protocol. Residue S317 was changed to proline to resemble more closely tandem repeat 3. Residue T322 was changed to proline making it more similar to repeat 2. Complimentary mutagenic primers were used to amplify the pQE30 vector encoding full length Aα-chain (residues 1-625). PCR products were digested with *Dpn*1 to eliminate wild-type DNA and transformed into *E. coli* strain XL-1 Blue. Plasmids were isolated from resulting clones and sequenced. A high proportion of clones obtained had deletions of repeats or contained additions. A total of 8 clones were sequenced for the S317P substitution, only one of which was correct. Of 14 clones sequenced to obtain the T322P substitution only 1 was correct. These were named pQE30 Aα-FgS317P and pQE30 Aα-FgT322P.

3.2.8 Expression of fibrinogen αC-connector

Plasmids pQE30 Aα-Fg, pQE30 Aα-S317P and pQE30 Aα-T322P were transformed into protease defective *E.coli* strain TOPP3. The 6-His tagged Aα-chain proteins were purified by nickel chetate affinity chromatography as described in section 2.14 with the addition of 6 M urea to the bacterial pellet prior to cell rupture by French press and to washing and elution buffers. Recombinant proteins were separated by SDS-PAGE and visualised by Coomassie staining as described in section 2.8. Mutant proteins Aα-S317P and Aα-T322P migrated as bands of approximately 70 kDa indistinguishable from the wild type Aα-chain (Figure 3.14.A).

3.2.9 ClfB-promoted bacterial adherence to Aα-S317P and Aα-T322P

Purifed recombinant Aα-1-625 chain and two mutants (S317P and T322P) were tested for their ability to support adherence of *S. aureus* cells expressing ClfB. In order to analyse ClfB-mediated fibrinogen binding only, a *S. aureus* strain defective in ClfA expression was utilised (Newman *clfA*). ELISA assays were carried out to check that the Aα-S317P and Aα-T322P proteins were able to coat microtitre plates to the same extent as the wild type Aα-chain (Figure 3.14.B). Serial dilutions of the wild-type and mutant proteins were coated onto 96 well plates. Coating of plates was

measured with anti-6xHis antibody. Both proline-substituted mutants coated microtitre plates indistinguishably from the wild-type Aα-chain.

Strain Newman *clfA* was grown to early exponential phase (OD_{600} 0.45-0.5) during which ClfB is known to be maximally expressed. Washed bacterial cells at an OD_{600} of 1 were added to microtitre plates coated with either wild-type Aα-chain or the Aα-S317P or Aα-S322P mutant proteins. Adherence was detected by crystal violet staining as described in section 2.15. Newman *clfA* adhered to the wild type Aα-chain in a dose-dependent and saturable manner but was not able to adhere to the Aα-T322P mutant. Adherence to the Aα-S317P mutant was reduced but not completely abolished (Figure 3.15). The inability of Aα-T322P to support binding to ClfB suggests that there is a single binding site for ClfB in the Aα-chain of fibrinogen located in repeat 5.

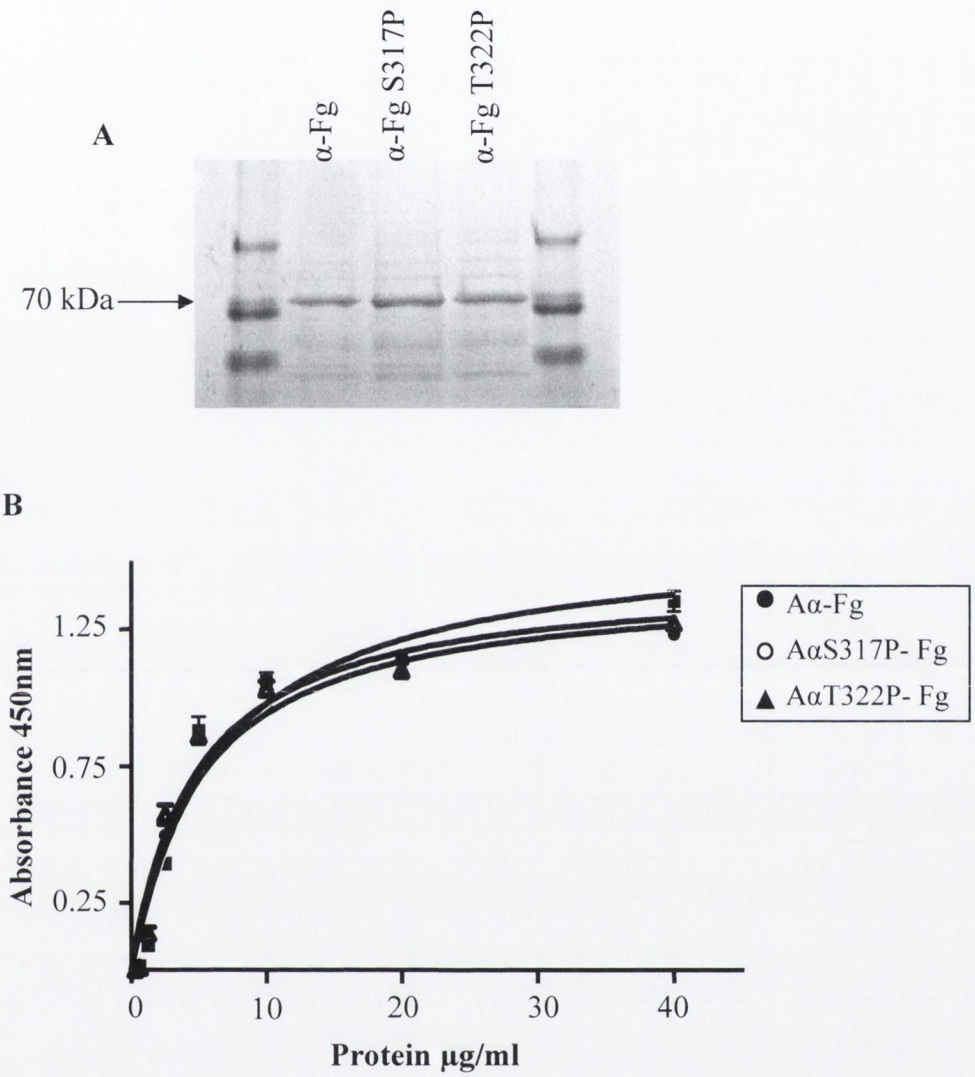


Figure 3.14. Mutants of the Aα-chain of fibrinogen

(A). Purified recombinant α-fibrinogen (Fg), α-Fg S317P and α-Fg T322P proteins were separated on a 7 % SDS-PAGE gel and visualised by Coomassie staining. (B). Increasing concentrations of recombinant Aα-chain proteins were added to ELISA plates. Coating of wells was detected using anti-His tag antibody conjugated to peroxidase and developed by incubation with a chromogenic substrate. Absorbance at 450 nm was determined. Values represent the means ± standard deviation of triplicate wells. The experiment was performed three times with similar results.

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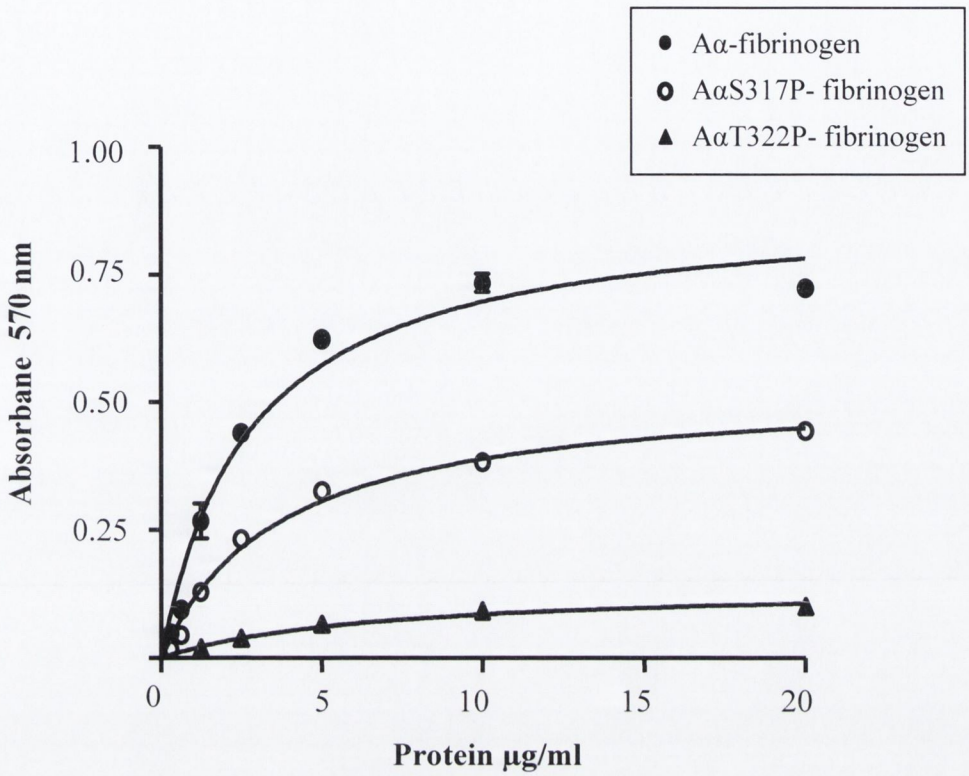


Figure. 3.15. Adherence of Newman *clfA* to immobilised recombinant fibrinogen Aα-, AαS317P- and AαT322P-chains.

Increasing concentrations of recombinant Aα-chain proteins were immobilised on ELISA plates. Exponential phase Newman *clfA* cells were added to the wells and incubated. Adherent bacteria were detected by crystal violet staining. Absorbance at 570 nm was determined. Values represent the means ± standard deviation of triplicate wells. The experiment was performed three times with similar results.

3.3 Discussion

Clumping factor B is a multi-functional surface protein of *S. aureus* which can interact with fibrinogen and cytokeratin 10 (Ni Eidhin *et al.*, 1998; O'Brien *et al.*, 2002b). It plays an important role in nasal colonisation and also contributes to the ability of *S. auerus* to interact with platelets (O'Brien *et al.*, 2002a; Schaffer *et al.*, 2006). The ligand binding domains of ClfB share structural similarities with other fibrinogen binding proteins of *S. aureus*. Non-fibrinogen binding mutants have been isolated for ClfA and FnBPA. This study aimed to isolate non-fibrinogen binding mutants of ClfB and assess their effects on cytokeratin 10 binding. The binding site of ClfB in fibrinogen was also targeted by site-directed mutagenesis to eliminate conclusively other potential binding sites in the Aα-chain.

Structural similarities occur in the fibrinogen binding domains N2N3 of ClfA, FnBPA and ClfB. The sequence of the N2N3 domains of ClfA from strain 8325 NCTC is 20 % identical to the sequence of the same domains in FnBPA. Introduction of alanine substitutions at residues P336 and Y338 of ClfA and equivalent residues in FnBPA eliminated fibrinogen binding by these two proteins (Deivanayagam *et al.*, 2002; Keane *et al.*, 2007). The ClfB N2N3 domains are 22% identical to the same domains of ClfA in strain 8325 NCTC. Alanine substitutions were introduced at residues P336 and F338 of ClfB which are in equivalent positions to P336 and Y338 of ClfA. The recombinant ClfB PF mutant protein was able to bind fibrinogen in a dose-dependent and saturable manner with no apparent decrease in the affinity for fibrinogen compared to the wild-type protein. The half-maximum binding value obtained by ELISA for ClfB wild-type and ClfB PF was approximately 0.07μM. This is close to the half maximum binding value of 0.1μM obtained previously using the same technique (Figure 3.5).

The Autodock modelling program was used to identify residues of ClfB which could interact with a short peptide of the αC-connector. Previous studies carried out in this lab found that αC-connector peptide PRPGSTGTWNP inhibited binding of recombinant ClfB to full length fibrinogen (E. Walsh personal communication). The tryptophan residue at position 341 seemed particularly important and a change to this residue eliminated inhibition of ClfB binding to fibrinogen. The GTWNP peptide which includes Trp 341 and two residues on either side was selected for in-silico docking into the 3D structure of N2N3 domains of ClfB. The most energetically favourable docked result was analysed using Chimera modelling software. Residues

P336 and F338 were too far away from the peptide for any interaction to occur. Since GTWNP is only a small section of the fibrinogen αC-connector it is impossible to know if the full length protein would be in close proximity to residues P336 and F338. Despite its proximity to the GTWNP peptide (4 Å) introducing alanine substitutions at residue R331 had no effect on fibrinogen binding. Based on their proximity to the peptide residues Q235, W522, D270, Y446 and N526 were selected for alteration. Quickchange mutagenesis seemed much more effective in *E. coli* than in *L. lactis* where the majority of transformants were unchanged. This has been observed before in this laboratory. Clones obtained from the attempted alteration of residues D270, Y446 and W522 all bound fibrinogen. It was decided not to sequence all of the clones obtained so it is not possible to determine whether or not any of these clones have the desired mutations which may not affect fibrinogen binding. Of the 6 clones that were sequenced all were found to be wild-type. One way to improve screening for point mutations is to incorporate an adjacent mutation that creates a new restriction site. In this way PCR based screening for mutant clones can be used.

Substitution of residues Q235 and N526 located in the N2 and N3 domains, respectively resulted in clones defective in fibrinogen binding. These were sequenced and found to contain the desired alanine substitutions. Residues Q235 and N526 were predicted to be within 3Å of the GTWNP peptide and residue Q235A was predicted to form a hydrogen bond with the peptide. The same substitutions also eliminated binding to cytokeratin 10 (Figures 3.12 and 3.13). Since the same residues of ClfB are important in the interaction with fibrinogen and cytokeratin 10 these two ligands most likely occupy overlapping binding sites in ClfB. Further evidence for this conclusion was seen in a previous study where binding of a Ω loop peptide of cytokeratin 10 caused quenching of tryptophan fluorescence of ClfB (Walsh *et al.*, 2004). ClfB contains two tryptophan residues, one of which (W522) is positioned in the binding trench. In order to cause tryptophan quenching the cytokeratin 10 peptide must lie in close proximity to this residue in ClfB.

The MSCRAMMs ClfA, FnBPA and FnBPB all bind to the short flexible C-terminal γ-chain peptide that protrudes from domain D of fibrinogen (Deivanayagam *et al.*, 2002). ClfB differs from these proteins in that it binds to the C-terminal regions of the two Aα-chains of fibrinogen. These regions consist of a C-terminal globular domain connected to the rest of the Aα-chain by a flexible αC-connector. ClfB binds

specifically to the αC-connector. *In silico* models of a γ-chain peptide docked into ClfA found residues P336 and Y338 to be in close proximity to the fibrinogen γ-chain C-terminal residues A408-G-D-V411 (Deivanayagam *et al.*, 2002). This region of fibrinogen bears little similarity to the glycine and serine rich repeats which make up the binding site for ClfB in the αC-region. This may explain why altering residues P336 and F338 of ClfB had no effect on fibrinogen binding while changes to equivalent residues in ClfA and FnBPA eliminated binding. These residues may be crucial in interacting with the γ-chain but not the αC-regions of fibrinogen. Nevertheless a common mechanism of ligand binding is still seems probable. Residue N526 of ClfB is in an equivalent position to residues V527 of ClfA and L498 of FnBPA. Alterations to these residues in ClfA and FnBPA caused a reduction in fibrinogen binding (Deivanayagam *et al.*, 2002; Keane *et al.*, 2007). Residues V527, L498 and N526 are positioned at the C-terminal of the N3 domain at the top of the latching peptide. Altering these residues may affect function of the latching peptide. Earlier studies noted that antibodies raised against the C-terminal of ClfA inhibited adherence of bacteria expressing ClfA to immobilised fibrinogen (Hartford *et al.*, 2001). In SdrG residue I581 located at the start of the latching peptide is thought to be important in repositioning of this flexible structure during ligand binding. Structural rearrangements in the latching peptide begin with a deviation in strand direction at residue I581 (Ponnuraj *et al.*, 2003). Equivalent residues in the *S. aureus* fibrinogen binding proteins might act in a similar manner (Figure 3.16). Residues in ClfA and FnBPA equivalent to the Q235 residue in ClfB have never been altered so no direct comparisons can be made. However, residues in adjacent positions were altered in ClfA and caused reductions in fibrinogen binding (Hartford *et al.*, 2001). These residues are believed to lie at the "mouth" of the binding pocket.

Previous studies found the αC-connector peptide PRPGSTGTWNP (residues 333 to 343) could inhibit binding of recombinant ClfB to fibrinogen (E. Walsh personal communication). These residues encompass most of repeat 6 and 2 residues of repeat 7. Alteration of W341 in this peptide eliminated inhibition. Later studies found that deletions of tandem repeats 6 and 7 of the αC-connector did not affect binding of ClfB. Only deletions affecting tandem repeat 5 located between residues 316-328 eliminated ClfB binding. This may seem to be contradictory, however the inhibitory peptide PRPGSTGTWNP bears striking similarity to residues 307-317 which comprise the C-terminal 9 residues of repeat 4 and 2 residues of repeat 5. Alignments

of these two sequences show 63% identity (Figure 3.17.A). The GTWNP peptide itself is 60% identical to residues GSWNS. Therefore peptide PRPGSTGTWNP may inhibit binding of ClfB to full length fibrinogen by mimicking the sequence overlapping repeat 5. In particular the presence of the Trp residue seems to be important.

In human fibrinogen the tandem repeats in the αC-connectors are composed of 13 amino acids. The majority of the residues in the repeats are glycine or serine (Burton *et al.*, 2006; Weisel & Medved, 2001). Omega loops present in the tail region of cytokeratin 10 to which ClfB also binds have a similar composition, Tyr-(Gly/Ser)$_n$. The Y-Y loop peptide of cytokeratin 10 which was previously shown to support ClfB binding is typical of the Ω loops of the V2 tail region (Walsh *et al.*, 2004). Aligning the sequence of the Y-Y loop peptide with tandem repeat 5 of the αC-connector reveals 46% shared sequence identity (Figure 3.17.B). Also tandem repeat 5 has many features of a Ω loop. Typical Ω loops are 6-16 residues in length and have few hydrophobic residues within the putative loop. However tryptophan is commonly found at the beginnings and ends of loops (Pal & Dasgupta, 2003). It seems likely that an Ω loop is also present in the αC-connector of fibrinogen (Figures 3.17.C and D). The docking of the GTWNP peptide was carried out prior to the identification of tandem repeat 5 of the αC-connector as the ClfB binding site. In retrospect, a fibrinogen peptide containing the sequence GSSGTGS which is homologous to the Ω loops of cytokeratin 10 seems to be a better choice.

Repeats of the αC-connector that do not support ClfB binding contain proline residues at positions 2 or 7 or at both sites in their 13 residue sequence. Proline residues are often found in Ω-loops in other proteins and are generally found at turn motifs along with glycine residues (Pal & Dasgupta, 2003). However, no proline residues are found in the Ω-loops of cytokeratin 10 or repeat 5 of the αC-connector of fibrinogen. Proline substitutions were introduced in repeat 5. A serine residue at 317 of repeat 5 was changed to a proline residue to make it more closely resemble repeat 3 (Figure 3.18.A). Another repeat 5 mutant was isolated with a proline substitution at residue T322 which closely mimicked repeat 2 (Figure 3.18.B). The S317P mutant had reduced affinity for ClfB while the T322P mutant was unable to bind ClfB. The fact that the full length Aα-chain containing a mutation T322P was unable to support ClfB binding suggests that repeat 5 is the only site in the Fg α-chain that ClfB binds

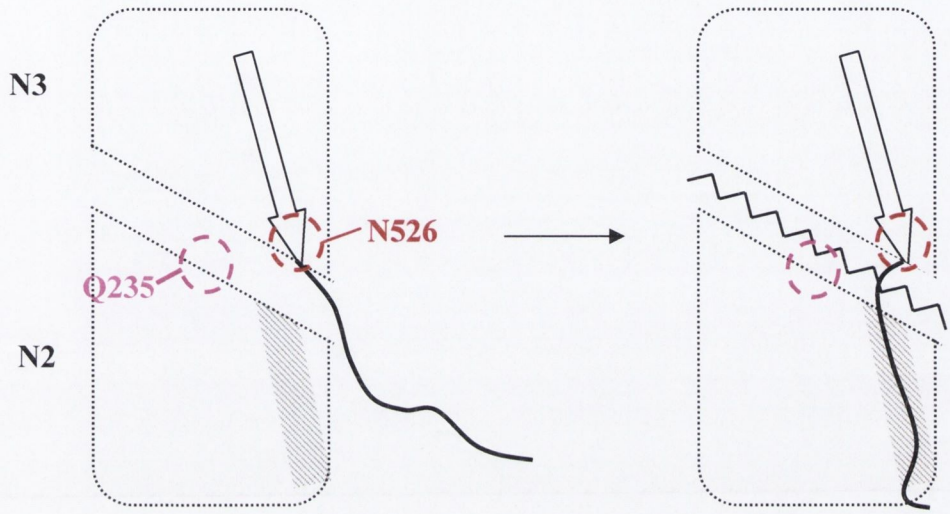


Figure 3.16. Proposed model of ligand binding by ClfB

Upon docking of the fibrinogen αC-connector in the hydrophobic trench between domains N2 and N3, a conformational change occurs at the C terminus of N3 in which the latching peptide crosses over the trench and inserts between β-sheets of N2. Structural rearrangements in the latching peptide are thought to begin with a deviation in strand direction at residue N526 (position indicated in red). Residue Q235 (position indicated in pink) is found at the mouth of the trench.

A

PRPGSTGTWNP αC-connector 333-343

SGPGSTGSWNS αC-connector 307-317

. * * * * * : * * .

B

WNSGSSGTGSTGNQN Fg αC-connector 315-329

YGGGSSGGGSSGGGY K10 Y-Y loop peptide

: . . * * * * * * : * .

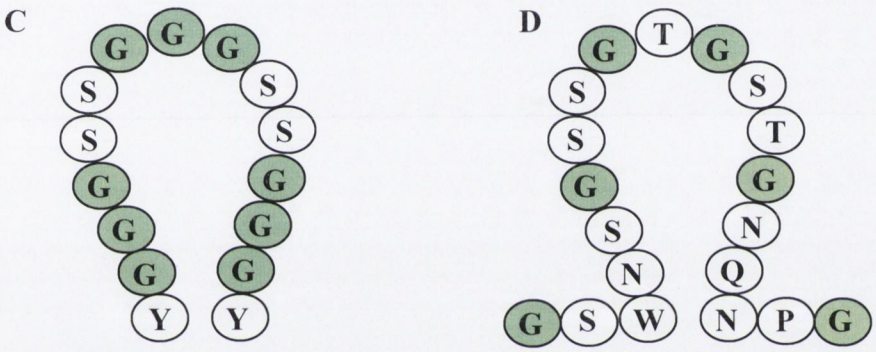


Figure 3.17. Features of the αC-connector of fibrinogen

(A) Alignment of residues 333-343 with residues 307-317 of the αC-connector. GTWNP peptide used in docking study and identical residues in the sequence 313-317 of the αC-connector are highlighted in green. (B) Alignment of Fg αC-connector repeat 5 with Y-Y loop peptide of cytokeratin 10. (C) Ω-loop formed by Y-Y loop peptide of cytokeratin 10. (D) Putative Ω-loop of tandem repeat 5 of the fibrinogen αC-connector. Identical residues (*), conserved residues (:), semi-conserved residues (.)

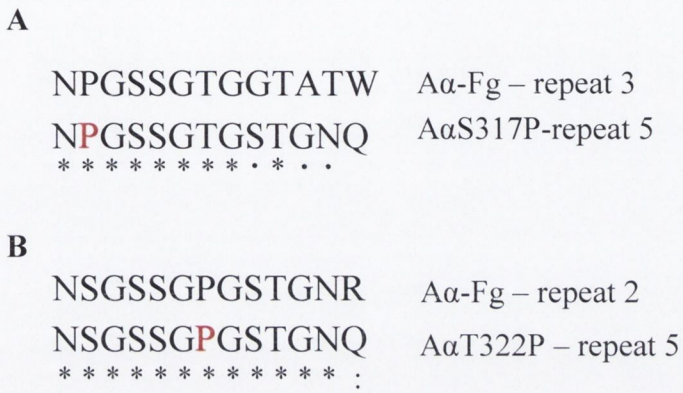


Figure 3.18. Alignment of amino acid sequences of the αC-connector

A. Alignment of αC-connector repeat 3 with repeat 5 of Aα-S317P. Substituted proline residue shown in red.

B. Alignment of αC-connector repeat 2 with repeat 5 of Aα-T322P. Substituted residues are shown in red. Identical residues (*), conserved residues (:), semi-conserved residues (.)

to and that T322 is crucially important for this. The presence of a proline residue in the centre of other tandem repeats and in particular repeat 2, might explain their inability to support binding to ClfB despite an otherwise high degree of sequence similarity. Proline residues are generally thought to introduce rigidity into the structure of proteins (Pal & Dasgupta, 2003). Proteins of the Clf-Sdr family have been established to interact with flexible peptides of fibrinogen (Deivanayagam *et al.*, 2002). Proline substitutions within the ClfB binding site in fibrinogen may decrease flexibility and prevent binding. The S317P substitution creates a sequence in repeat 5 that resembles repeat 3 except for the C-terminal residues (Figure 3.18.A). Presumably these must contribute to the inability of repeat 3 to support ClfB binding in a reduced capacity like the S317 mutant. PCR amplification of the tandem repeat region with mutagenic primers resulted in many clones with deleted or extra repeats. The DNA encoding this region has a high GC content which is believed to interfere with PCR reactions. Other studies have encountered problems when attempting to PCR-amplify regions of high GC content in full-length cytokeratin 1 and 10 (Walsh *et al.*, 2004).

Current studies in the laboratory of Prof. M. Hook, Texas A&M University are attempting to solve the X-Ray crystal structure of ClfB in complex with the tail region of cytokeratin 10. The results of this study will conclusively show if ClfB binds to cytokeratin 10 and by implication, fibrinogen by the dock, lock and latch mechanism. Evidence found in this study supports the dock, lock and latch mechanism for binding. Changes to ClfB that reduced binding to both fibrinogen and cytokeratin 10 are located in the hydrophobic trench between N2 and N3. Substituting a residue predicted to be in a crucial position involved in the function of the latching peptide reduced binding to both ligands. The binding site for ClfB in fibrinogen was conclusively shown to be repeat 5 of the αC-connector which is very similar in sequence to the Y-Y loop peptide of cytokeratin 10. It seems likely that an Ω loop is also present in the fibrinogen Aα-chain and that ClfB can recognise this structure.

Chapter 4

Mechanism of platelet aggregation mediated by clumping factor B of *Staphylococcus aureus*

4.1 Introduction

The interaction of bacteria with platelets leads to the development of cardiovascular infections such as infective endocarditis (IE). The increased use of intravascular devices and invasive procedures in hospital settings has lead to an increase in bacteraemic infections and a new at-risk population group for IE. *S. aureus* is now recognised as the leading cause of IE in the developed world. This condition is characterised by the build-up of vegetative bodies on heart valve surfaces which contain clusters of bacteria, fibrin and aggregated platelets (Moreillon & Que, 2004). The ability of *S. aureus* to cause platelet aggregation is thought to contribute to the development of IE. Binding of *S. aureus* to platelets in sterile vegetations may facilitate colonization and isolate the bacterium from the immune system, since neutrophils do not penetrate thrombi (Fitzgerald *et al.*, 2006a).

Aggregation of platelets by bacteria is the result of a multi-step process. Bacteria that cause platelet aggregation may interact with receptors on the platelet surface directly or indirectly through bridging molecules such as fibrinogen. Initial adhesion of bacteria to platelets triggers platelet activation which is characterised by signalling events and calcium oscillations in the platelet. The major platelet integrin GPIIb/IIIa undergoes conformational changes upon platelet activation. The fibrinogen binding site in GPIIb/IIIa is exposed by these changes and in this high affinity state GPIIb/IIIa can bind avidly to fibrinogen and fibronectin in solution (Calvete, 1999). Aggregation of platelets occurs when adjacent platelets interact with the γ-chain of the bivalent fibrinogen molecule. This results in cross-linking of platelets into aggregates. Aggregation occurs after a variable period of time referred to as the lag time. This time reflects the time taken for activation and aggregation to occur after the bacteria and platelets come into contact. *In vitro* studies using light transmission aggregometry measure either the lag time to aggregation or the overall percentage aggregation.

The interaction between *S. aureus* and platelets is complex and involves multiple factors. Several surface-expressed proteins of *S. aureus* have been shown to stimulate platelet activation and aggregation. These include the fibrinogen binding proteins ClfA and ClfB, and the bi-functional fibronectin-fibrinogen binding proteins FnBPA and FnBPB (Fitzgerald *et al.*, 2006b; Loughman *et al.*, 2005; O'Brien *et al.*, 2002). Since a number of proteins are involved in *S. aureus*-mediated platelet aggregation many studies have utilised the non-aggregating Gram positive strain *L. lactis* to study

platelet aggregation mediated by individual *S. aureus* proteins. When expressed individually on the surface of *L. lactis*, ClfA, FnBPA and FnBPB were all capable of inducing fast platelet aggregation with lag times of 1-2 min. *L. lactis* expressing ClfB caused platelet aggregation with the average lag time of 7 min. SdrE was also able to cause aggregation with extended lag times (13.6 min) when expressed from *L. lactis*. Although protein A was not capable of inducing platelet aggregation when expressed from *L. lactis*, it enhanced aggregation mediated by *S. aureus* (O'Brien *et al.*, 2002). The studies which characterised these interactions were generally carried out under static or low shear conditions. Recent studies have shown that under high shear rates which mimic *in vivo* conditions in arterioles protein A and ClfA are crucial for platelet aggregation (Kerrigan *et al.*, 2008; Pawar *et al.*, 2004).

Bacteria expressing ClfA and FnBPA cause rapid platelet aggregation. ClfA binds avidly to fibrinogen which is present in plasma at concentrations of 2.5 - 3 mg/ml. The adhesion and aggregation of platelets by ClfA-expressing bacteria has been shown to be fibrinogen-dependent. Initial adhesion of ClfA on the bacterial surface and GPIIb/IIIa on platelets occurs via a fibrinogen bridge. Low affinity GPIIb/IIIa is able to bind fibrinogen coating the bacterium. One end of the bivalent fibrinogen molecule is bound at the γ-chain by ClfA while the other γ-chain is free to interact with GPIIb/IIIa. Previous studies have shown that the level of ClfA protein on the bacterial surface is crucial in this process. A "threshold" level of protein expression is required for platelet activation to occur (Loughman *et al.*, 2005). This was also found to be the case for aggregation mediated by bacteria expressing FnBPA (Fitzgerald *et al.*, 2006b). Adhesion of ClfA to platelets via fibrinogen is not sufficient to stimulate platelet activation. ClfA-specific antibodies are also required to interact with platelet FcγRIIa receptors which cluster to trigger activation and intracellular signalling (Loughman *et al.*, 2005).

ClfA is expressed predominantly in the stationary phase of growth and is the main mediator of platelet aggregation for stationary phase cells (Loughman *et al.*, 2005). In the exponential phase of growth rapid platelet activation is caused by the FnBPA and FnBPB proteins. FnBPA causes platelet aggregation in a similar manner to ClfA. Fibrinogen bound by the A domain or fibronectin bound by the BCD domains of the protein can interact with GPIIb/IIIa on the surface of platelets. Specific antibodies to FnBPA are also required to trigger activation and subsequent aggregation (Fitzgerald *et al.*, 2006b). Studies have shown ClfA and FnBPA can

cooperate in valve colonisation and invasion in IE. Fibrinogen binding by ClfA and FnBPA promotes early colonisation of heart valves while fibronectin binding by FnBPA promotes internalisation and spread into the adjacent endothelium (Heying *et al.*, 2007; Que *et al.*, 2005).

Longer lag times to aggregation occur with bacteria lacking the aforementioned potent proaggregatory surface components. Instead, a slower process involving complement assembly coupled with specific antibody binding is required for aggregation. The complement system is part of the host innate immune response. Complement activation results in the assembly of complement proteins on the bacterial surface to form the membrane-attack-complex (MAC). Insertion of the MAC complex into the cell membranes of certain bacteria leads to cell lysis. *S. aureus* is protected from the action of the MAC complex by the peptidoglycan cell wall. Instead complement components deposited on the *S. aureus* cell surface act as opsonins and aid the process of phagocytosis. In complement-dependent platelet activation complement components deposited on the bacterial cell serve to cross-link bacteria to a platelet complement receptor. Activation also requires specific antibodies to cross-link bacteria to the platelet receptor FcγRIIa. Platelet aggregation mediated by certain strains of *Steptococcus sanguis* such as NCTC 7863 takes place by this mechanism. *Strep. sanguis* NCTC 7863 requires a functional complement system and specific antibodies to trigger platelet aggregation with varying lag times of over 7 min (Ford *et al.*, 1996). A non-fibrinogen binding mutant of ClfA (ClfA PY) also stimulates platelet activation by a complement-dependent mechanism. Bacteria expressing ClfA PY caused platelet aggregation with a lag time of 8-10 min (Loughman *et al.*, 2005). The longer lag times to aggregation are believed to correspond to the time taken for complement proteins to assemble on the bacterial surface.

Clumping factor B is known to cause platelet aggregation and has been shown to contribute to the pathogenesis of experimental endocarditis in rats (Entenza *et al.*, 2000; O'Brien *et al.*, 2002). Elevated levels of antibodies to ClfB as well as FnBPA and ClfA have been identified in the sera of IE patients (Rindi *et al.*, 2006). Despite its ability to bind fibrinogen, *L. lactis* expressing ClfB caused aggregation with longer lag times (6-8 min) than ClfA and FnBPA (O'Brien *et al.*, 2002). Unlike ClfA, FnBPA and FnBPB which all bind to the γ-chain of fibrinogen, ClfB binds to a flexible region of the α-chain known as the αC-connector (Walsh *et al.*, 2008).

This study investigated the contribution of ClfB to platelet aggregation mediated by exponential growth phase *S. aureus* cells. The lag times to aggregation were assessed for strain Newman with mutations in *clfA* and *clfB*. A *L. lactis* strain expressing ClfB (*L. lactis* ClfB) from the nisin inducible vector pNZ8037 was also utilised in platelet aggregation studies. The ability to control ClfB expression from this strain was used to determine if a threshold level of ClfB is necessary for platelet activation. Several strategies were utilised to establish the mechanism by which ClfB causes platelet aggregation. The role of fibrinogen in ClfB-mediated aggregation was assessed using a non-fibrinogen binding mutant of ClfB (ClfB Q235A). The involvement of individual plasma proteins was studied using gel filtered platelets and washed platelets. Inhibitors of platelet receptors were also utilised to determine their involvement in ClfB-mediated aggregation.

4.2 Results

4.2.1 Construction of *S. aureus* strains Newman *coa clfA* and Newman *coa clfA clfB*

In the exponential growth phase *S. aureus* can cause rapid platelet activation by expressing the potent pro-aggregatory proteins FnBPA and FnBPB (Fitzgerald *et al.*, 2006b). Strain Newman was selected for this study because it does not have FnBPA and FnBPB attached to its cell surface due to mutations that result in secretion of truncated forms of each protein (Grundmeier *et al.*, 2004). Strain Newman expresses high levels of coagulase and ClfA. Coagulase causes formation of fibrin clots in plasma. ClfA is expressed predominantly in the stationary growth phase. However, it is also weakly expressed from the SigA promoter in the exponential growth phase (Higgins, 2005). To prevent interference from these two proteins in aggregation experiments mutations were introduced into the *coa* and *clfA* genes by allelic replacement mutagenesis. It was therefore possible to study the effect of ClfB in the exponential phase of growth without interference from other pro-aggregatory proteins.

Phage 85 was used to introduce the *coa::*Tc^R mutation into strain Newman *clfA* as described in section 2.5. The resulting strain, Newman *coa clfA* was unable to form fibrin clots in the coagulase tube test described in section 2.11. Phage transduction was used to introduce a *clfB::lacZ* Em^R mutation into strain Newman *coa clfA*.

Western immunblots were carried out to confirm the successful introduction of mutations in *clfA* and *clfB* genes. Cell wall extracts of Newman, Newman *coa*,

Newman *coa clfA* and Newman *coa clfA clfB* from the exponential and stationary growth phases were prepared by lysostaphin digestion. Cell wall extracts were separated by SDS-PAGE and electroblotted onto PVDF membranes. Membranes were probed with polyclonal anti-ClfB or anti-ClfA antibody. Strains Newman, Newman *coa* and Newman *coa clfA* all express ClfB in exponential phase. ClfB is seen as a 150 kDa band in the cell wall extract of each of these strains (Figure 4.1A) which corresponds to the full length form of this protein. ClfB was not detected in the cell wall extract of Newman *coa clfA clfB* indicating that the phage transduction was successful. In the stationary growth phase ClfA was detected as a 170 kDa band from the cell wall extracts of strains Newman and Newman *coa* but not from Newman *coa clfA* and Newman *coa clfA clfB* (Figure 4.1B) as expected.

4.2.2 Contribution of ClfB to strain Newman-mediated platelet aggregation

Strains Newman, Newman *coa*, Newman *coa clfA* and Newman *coa clfA clfB* were grown to an OD_{600} of 0.45-0.5 in the early exponential phase during which ClfB is maximally expressed. Bacterial cells were washed and adjusted to OD_{600} of 1.6. Bacterial cells were added to platelet rich plasma (PRP) and aggregation was measured by light transmission from an aggregometer for 25 min. Results were considered negative if no aggregation occurred after 25 min incubation.

Figure 4.2 shows a typical aggregation trace. Strains Newman and Newman *coa* had the same lag times to aggregation of approximately 3 min. Comparing Newman *coa* and Newman *coa clfA* an increase in lag time was observed, from 3 to 4.67 min. This difference was found to be statistically significant ($p = 0.0123$). This indicates that the low level of ClfA on exponential phase cells contributes to aggregation.

Strain Newman *coa clfA clfB* had a mean lag time of 6.5 min in two of the donors tested. In the third donor no aggregation was detected after 25 min. When the 6.5 min lag time was compared to the lag time caused by strain Newman *coa clfA* the difference was statistically significant ($p = 0.0191$). These results shows that both ClfA and ClfB contribute to the ability *of S. aureus* from the exponential phase of growth to interact with platelets.

4.2.3 Comparisson of fibrinogen binding by ClfB and ClfA expressed from *L. lactis*

Strains *L. lactis* (pNZ8037 *clfA*) and *L. lactis* (pNZ8037 *clfB*) express ClfA and ClfB, respectively, in a nisin inducible manner. Whole cell immunoblots demonstrated that *L. lactis* (pNZ8037 *clfB*) maximally induced with nisin expressed 64-fold more ClfB protein than uninduced cells (section 3.2.5). Previous studies determined that fully induced *L. lactis* (pNZ8037 *clfA*) also expressed 64-fold more ClfA than an un-induced strain (Loughman *et al.*, 2005). The overall increase in ClfA and ClfB protein expression from the pNZ8037 vector appears to be similar.

The ability of *L. lactis* expressing ClfB (*L. lactis* ClfB) to adhere to immobilized fibrinogen was assessed and compared to *L. lactis* expressing ClfA (*L. lactis* ClfA). *L. lactis* ClfB, *L. lactis* ClfA and a strain containing the empty pNZ8037 vector were grown and induced with increasing nisin concentrations ranging from 0.025-3.2 ng/ml. Induced cells were washed and the OD_{600} values were normalised. Cells were added to microtitre plate wells coated with 10 µg/ml of fibrinogen. Adherence was measured by crystal violet staining as described in section 2.15. Increasing the level of ClfB on the cell surface correlated with a sharp increase in fibrinogen binding up to concentrations of nisin of 0.4ng/ml. Increasing the inducer concentration above this level did not increase adherence to fibrinogen. *L. lactis* ClfB adhered to fibrinogen less avidly than *L. lactis* ClfA across the range of inducer concentrations used (Figure 4.3). This could be due to a lower level of ClfB expression compared to ClfA or a lower affinity for fibrinogen. *L. lactis* (pNZ8037) did not bind detectably to fibrinogen (Figure 4.3).

4.2.4 Effect of ClfB-expression levels and bacterial cell density on platelet aggregation in PRP

Previous studies have shown that a minimum level of ClfA and FnBPA must be present on the bacterial cell surface for platelet activation and aggregation to occur. This is known as the "threshold level" of protein expression (Fitzgerald *et al.*, 2006b; Loughman *et al.*, 2005). To test if this was also the case for ClfB, the ability to control expression of this protein from the *L. lactis* pNZ8037 vector was utilised. *L. lactis* pNZ8037*clfB* was induced with increasing concentrations of nisin ranging from 0.025-3.2 ng/ml. Washed cells were adjusted to an OD_{600} of 1.6 and incubated with PRP. The lag time to aggregation and percentage aggregation were measured for 25 min by light transmission from an aggregometer. It was found that 0.2 ng/ml of nisin was sufficient to cause platelet aggregation mediated by ClfB. Below this level of

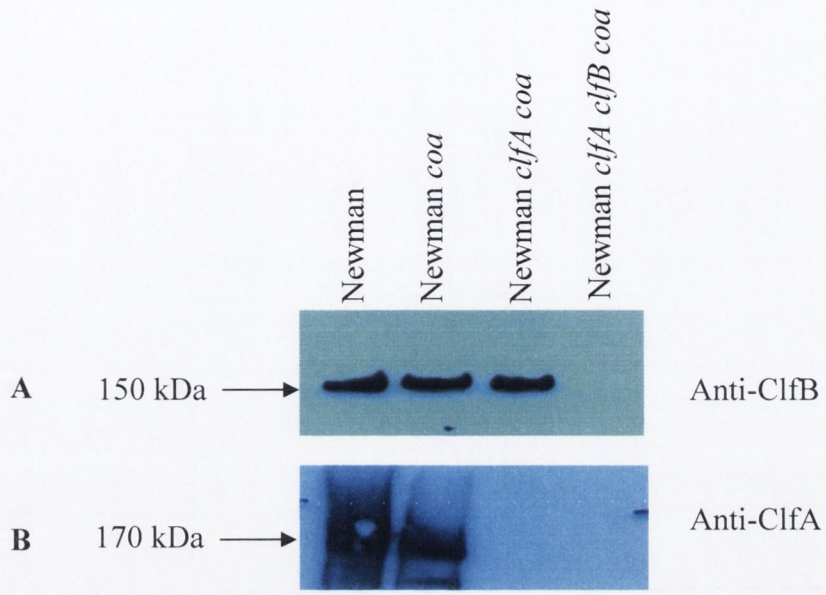


Figure 4.1. Expression of ClfA and ClfB from *S. aureus* strain Newman

S. aureus strains Newman, Newman *coa*, Newman *coa clfA* and Newman *coa clfA clfB* were grown to (A) exponential phase and (B) stationary phase. Cell wall proteins were solubilised by lysostaphin digestion, separated on 7.5% SDS PAGE gels and electroblotted onto PVDF membranes. Membranes were probed with (A) polyclonal rabbit anti-ClfB antibody or (B) polyclonal rabbit anti-ClfA antibody. Bound primary antibodies were detected with HRP conjugated protein A peroxidase. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

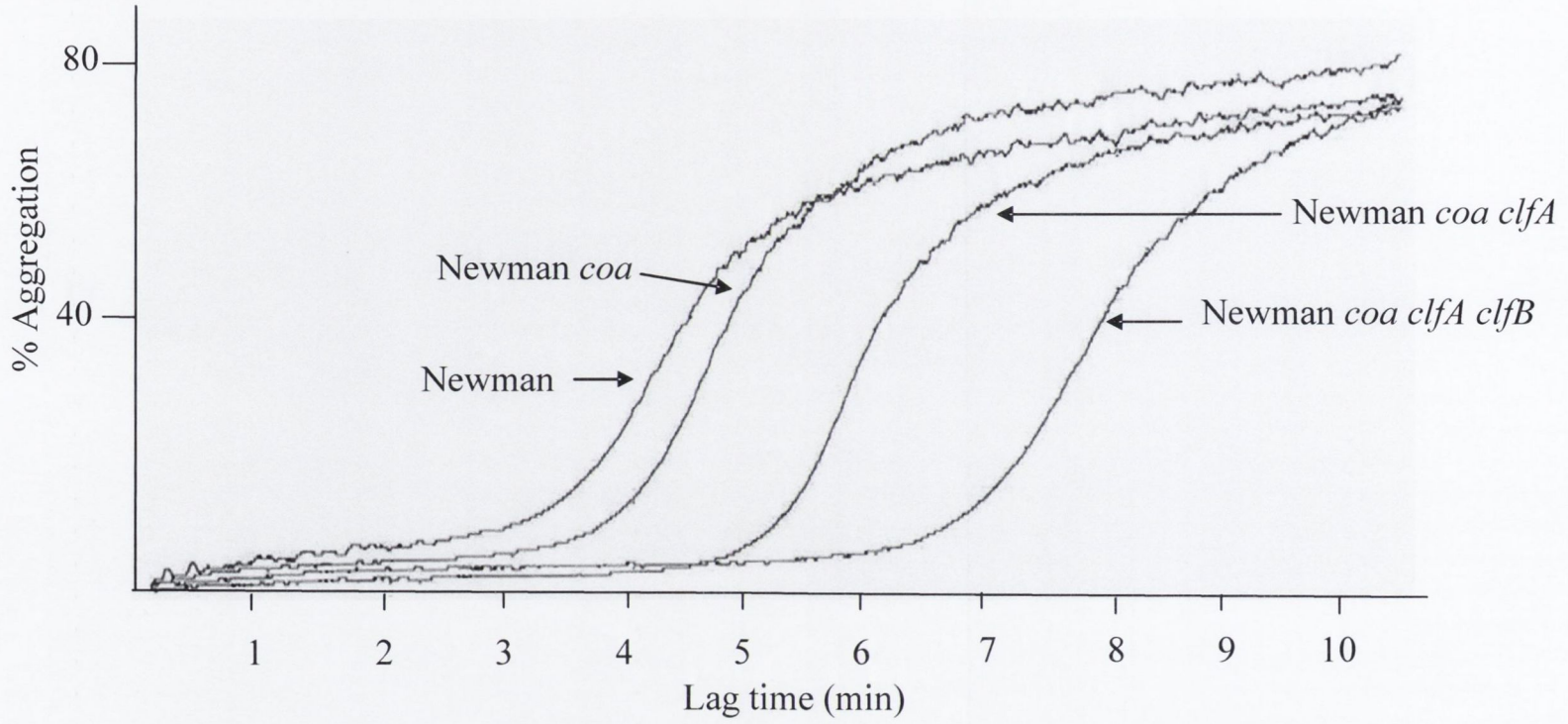


Figure 4.2. Role of ClfB in *S. aureus* mediated platelet aggregation

Strains Newman, Newman *coa*, Newman *coa clfA* and Newman *coa clfA clfB* were grown to exponential phase. Washed bacterial cells were adjusted to an OD_{600} of 1.6 and added to PRP. Light transmission was recorded for 25 min. This experiment was carried out 3 times using 3 different blood donors.

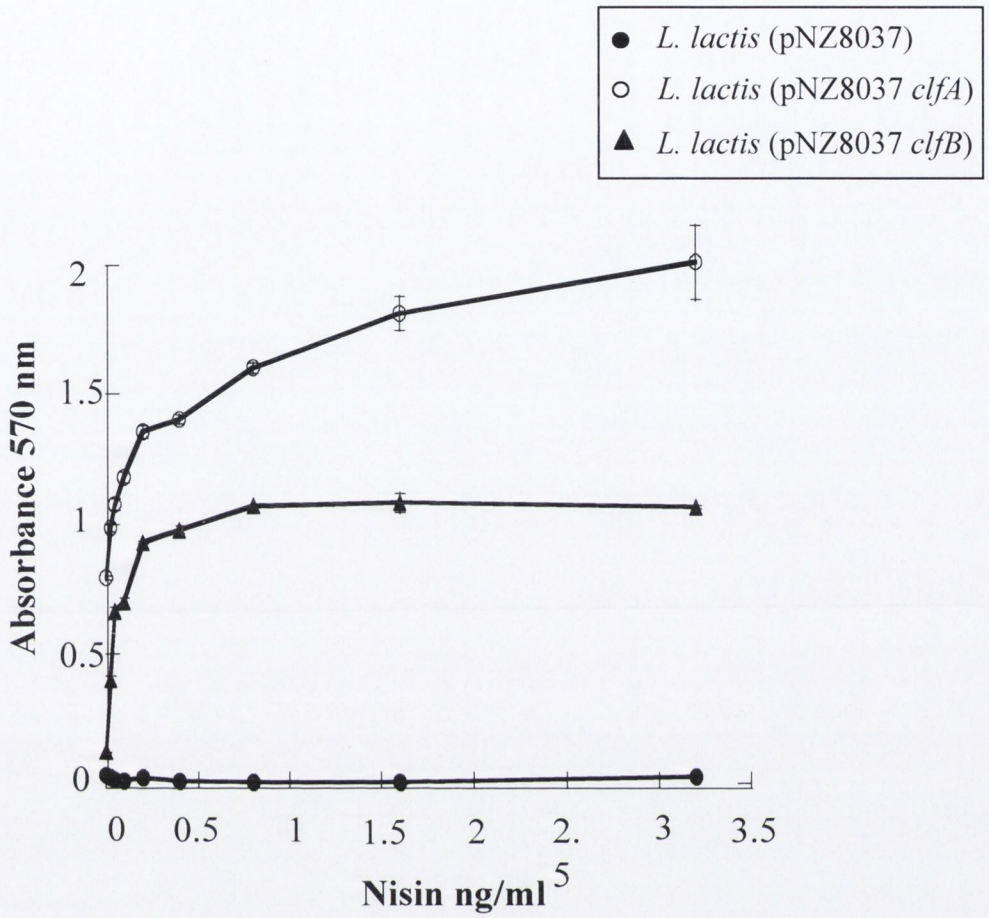


Figure 4.3. Nisin-dependent adherence of *L. lactis* ClfB to immobilized fibrinogen

L. lactis (pNZ8037), *L. lactis* (pNZ8037 *clfA*) and *L. lactis* (pNZ8037 *clfB*) were grown with increasing nisin concentrations. Washed cells were adjusted to OD_{600} of 1.0 in PBS and added to fibrinogen-coated microtitre plates. Cells were allowed to adhere for 2 h at 37°C. Adherent cells were detected by crystal violet staining and the absorbance at 570 nm was determined using an ELISA plate reader.

inducer no aggregation was seen indicating that a certain level of ClfB expression is required to trigger activation (Figure 4.4A). The cells induced with the highest nisin concentrations produced the shortest lag times (6.7 min at 3.2 ng/ml nisin compared to 11.7 min at 0.2 ng/ml nisin $p<0.01$). These results indicate that the lag time to aggregation is dependent on the level of ClfB protein expressed on the bacterial cell surface. There seems to be a decrease in the percentage aggregation caused by cells induced with lower levels of nisin (62 % at 0.2 and 0.4 ng/ml) compared to fully induced cells (78%). This trend was not statistically significant although this would likely change if a larger number of samples were tested.

Experiments were also carried out to determine the threshold number of bacterial cells necessary to cause ClfB-mediated aggregation. Doubling dilutions of fully induced *L. lactis* ClfB with OD_{600} values ranging from 0.1 to 1.6 were added to PRP. It was found that an OD_{600} of 0.8 was necessary to cause platelet aggregation (Figure 4.4B). The lag time to aggregation increased from 8 min at OD_{600} 1.6 to 14 min at OD_{600} 0.8 ($p = 0.0061$). This indicates that bacterial cell density is also important in triggering platelet activation and subsequent aggregation. As a positive control 20 μM ADP was added to platelets. This caused immediate aggregation with no discernible lag time which reached 82% after 25 min (data not shown).

4.2.5 ClfB-mediated platelet adhesion is fibrinogen dependent

Fibrinogen plays an important role in many bacterial interactions with platelets by acting as a bridging ligand between the GPIIb/IIIa receptor on platelets and fibrinogen binding bacterial proteins. A non-fibrinogen binding variant of ClfB with an alanine substitution at residue Q235 was used to asses the importance of fibrinogen in ClfB mediated adhesion to platelets. ClfB Q235A was expressed from *L. lactis*. Whole cell immunoblots and western immunoblots showed that this protein was expressed at the same level as the wild type protein by *L. lactis* and that the expressed protein was intact (Figures 3.10 and 3.11).

Washed platelets (WP) were prepared as described in section 2.17.1. Prostaglandin E1 was added to platelts prior to centrifugation and washing to prevent activation of platelets by physical stress. WP were incubated with microtitre plates coated with *L. lactis*, *L. lactis* ClfB and *L. lactis* ClfB Q235A. Adherence of platelets to bacteria was measured as described in section 2.15. *L. lactis* expressing ClfB and ClfB Q235A bound to WP at 2 fold and 1.6 fold higher levels than *L. lactis* containing

the empty pNZ8037 vector respectively ($p = 0.0046$ and $p = 0.02$, Figure 4.5). Commercial fibrinogen was purified from contaminating IgG by passage over protein A-coupled sepharose as described in section 2. ELISA assays were carried out to confirm the process was successful. Protein A peroxidise did not bind to fibrinogen purified of contaminating IgG in ELISA assays. In contrast protein A-peroxidise bound to the commercial fibrinogen preparation in a dose dependent manner (Figure 4.6). When 1 mg/ml of purified fibrinogen was added to WP adherence of platelets to *L. lactis* expressing ClfB was increased 4 fold ($p < 0.0001$). The small increase seen for *L. lactis* ClfB Q235A adhering to WP when fibrinogen was added was not statistically significant ($p = 0.33$). Addition of fibrinogen to WP did not improve the ability of platelets to adhere to *L. lactis*. These results indicate that platelets can adhere to bacteria expressing ClfB in a fibrinogen-dependent manner.

4.2.6 Inhibition of ClfB-mediated platelet activation and adhesion using antibodies to platelet receptors

The anti-GPIIb/IIIa monoclonal antibody abciximab inhibited aggregation mediated by *L. lactis* expressing ClfB. The antibody was incubated with PRP at 37°C for 5 min prior to the addition of bacterial cells. Anti-GPIIB/IIIa reduced aggregation from 69% to 4% (Figure 4.7A). Abciximab also inhibited adherence of *L. lactis* ClfB to WP substituted with fibrinogen (Figure 4.7B). This indicates that fibrinogen dependent adherence of bacteria expressing ClfB to platelets occurs via GPIIb/IIIa. Aggregation of platelets also requires GPIIb/IIIa for cross-linking of platelets into thrombi following activation.

A function-blocking monoclonal antibody (IV-3) for the FcγRIIa platelet receptor also inhibited platelet activation by *L. lactis* ClfB. Anti-FcγRIIa reduced aggregation from 69% to 2% (Figure 4.7A). The same antibody did not inhibit adhesion of *L. lactis* ClfB to WP substituted with fibrinogen (Figure 4.7B). This demonstrated that FcγRIIa is crucial in platelet activation but not platelet adhesion mediated by ClfB. Immunoglobulin may play a role in triggering activation subsequent to adhesion of bacteria to platelets.

4.2.7 Fibrinogen and ClfB-specific antibodies are required for ClfB-mediated platelet activation and aggregation

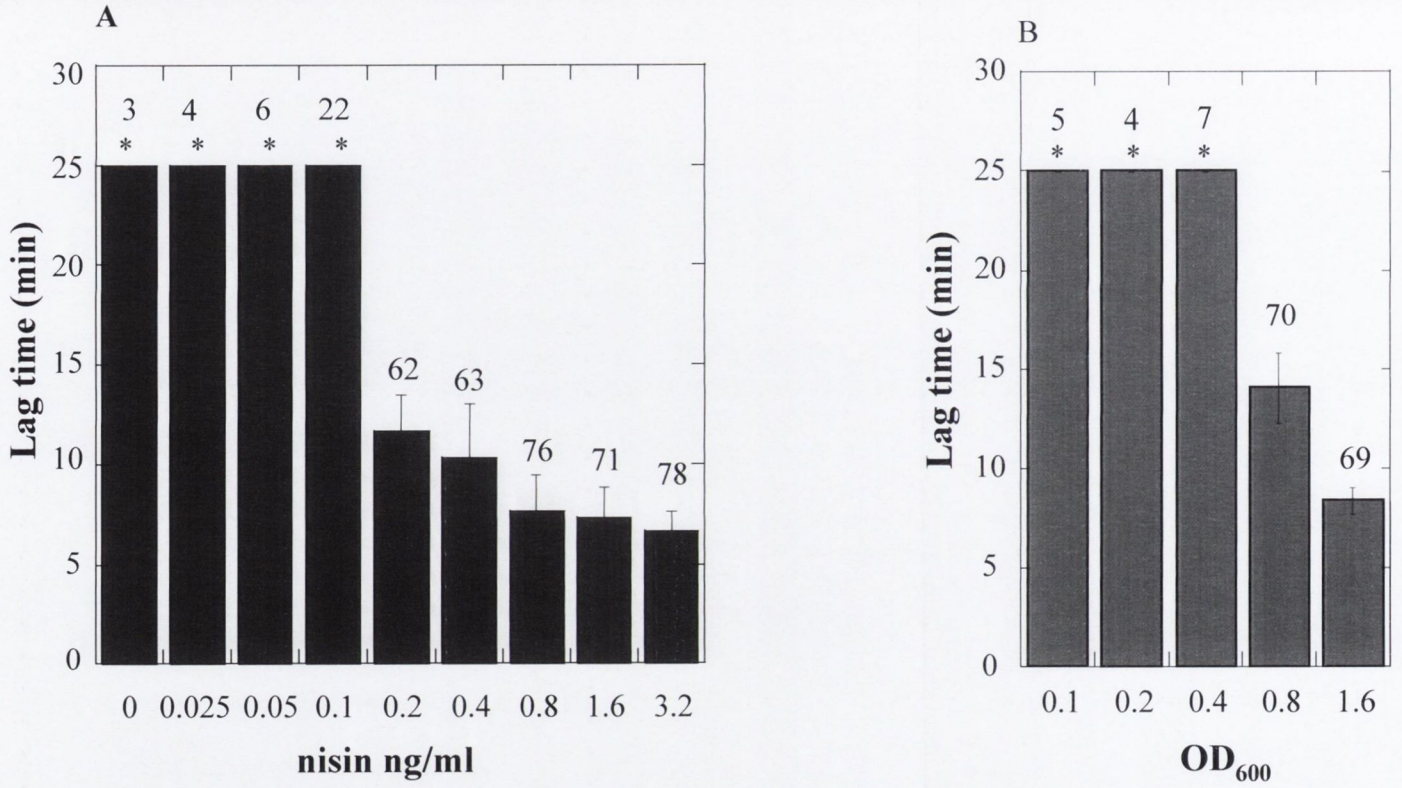


Figure 4.4. Threshold ClfB expression and bacterial cell density necessary for platelet activation

A. *L. lactis* ClfB induced with increasing concentrations of nisin was added to PRP. Light transmission from an aggregometer was recorded over 25 min. * indicates no activation occurred at 25 min. Percentage aggregation is indicated above bars.

B. Doubling dilutions of *L. lactis* ClfB induced with 3.2 ng/ml of nisin were added to PRP. Light transmission was recorded over 25 min. * indicates no activation occurred at 25 min. These experiments were carried out 3 times using 3 different blood donors.

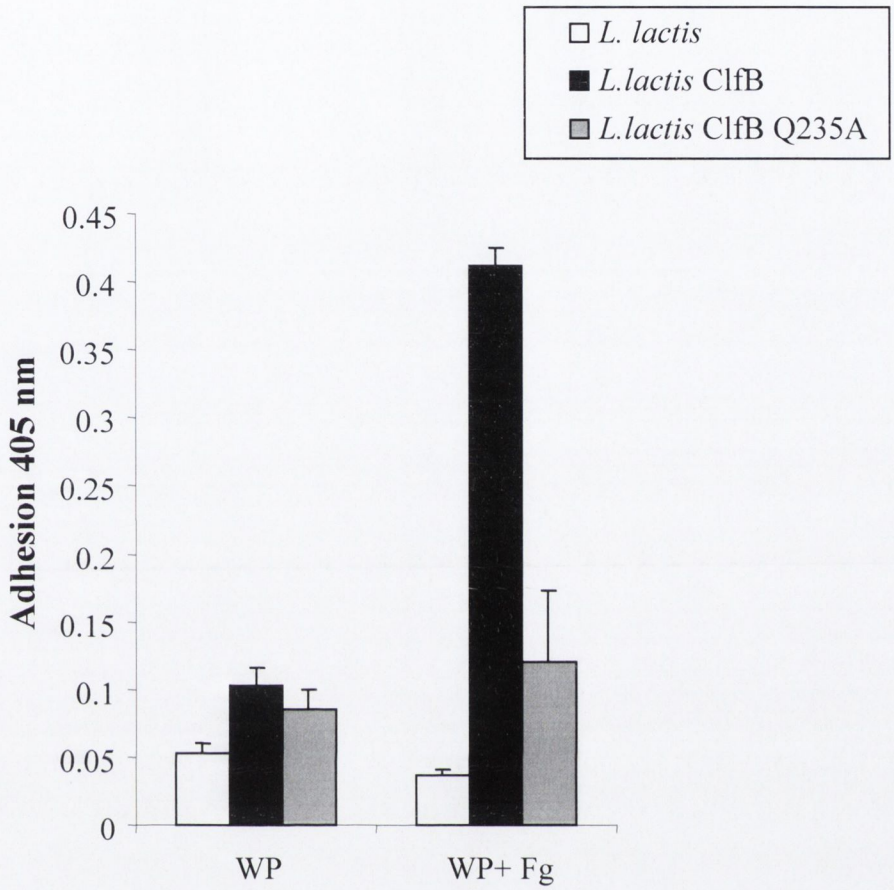


Figure 4.5. Role of fibrinogen in ClfB-promoted platelet adhesion

L. lactis (pNZ8037) and *L.lactis* expressing ClfB and ClfB Q235A were induced with 3.2 ng/ml of nisin, adjusted to an OD_{600} of 1 and incubated on microtitre plates at 4°C for 16 hours. Washed platelets and washed platelets supplemented with purified fibrinogen (Fg; 1 mg/ml) were added to the microtitre plates. Adherent platelets were lysed and a substrate for the intracellular enzyme acid phosphatase was added to each microtitre well. The experiment was carried out 2 times using 2 different blood donors. Results represent the mean ± SD of adherence at 405 nm as measured by an ELISA plate reader.

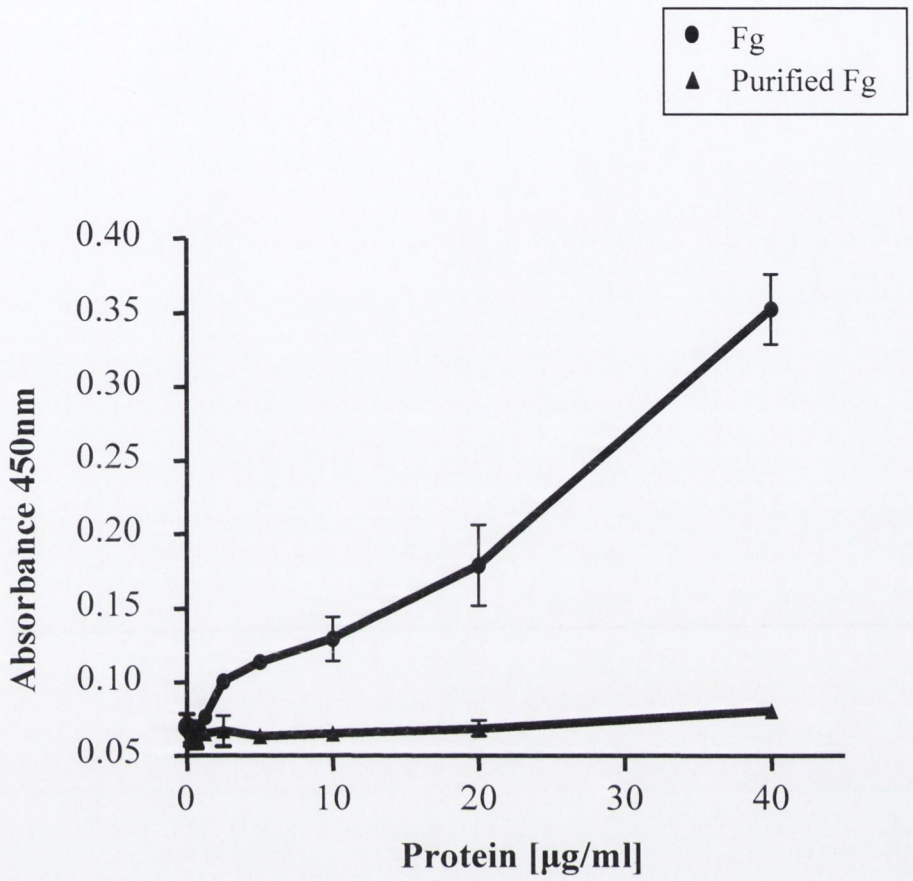


Figure 4.6. Preparation of fibrinogen purified of contaminating IgG for platelet aggregation assays

A commercial fibrinogen preparation was purified by passage through a column of protein A coupled to sepharose. Doubling dilutions of fibrinogen (Fg) and purified fibrinogen were coated onto microtitre plates and protein A-peroxidase was used to detect contaminating IgG. Wells were developed by incubation with a chromogenic substrate and the absorbance at 450 nm was determined. Values represent the means ± standard deviation of triplicate wells.

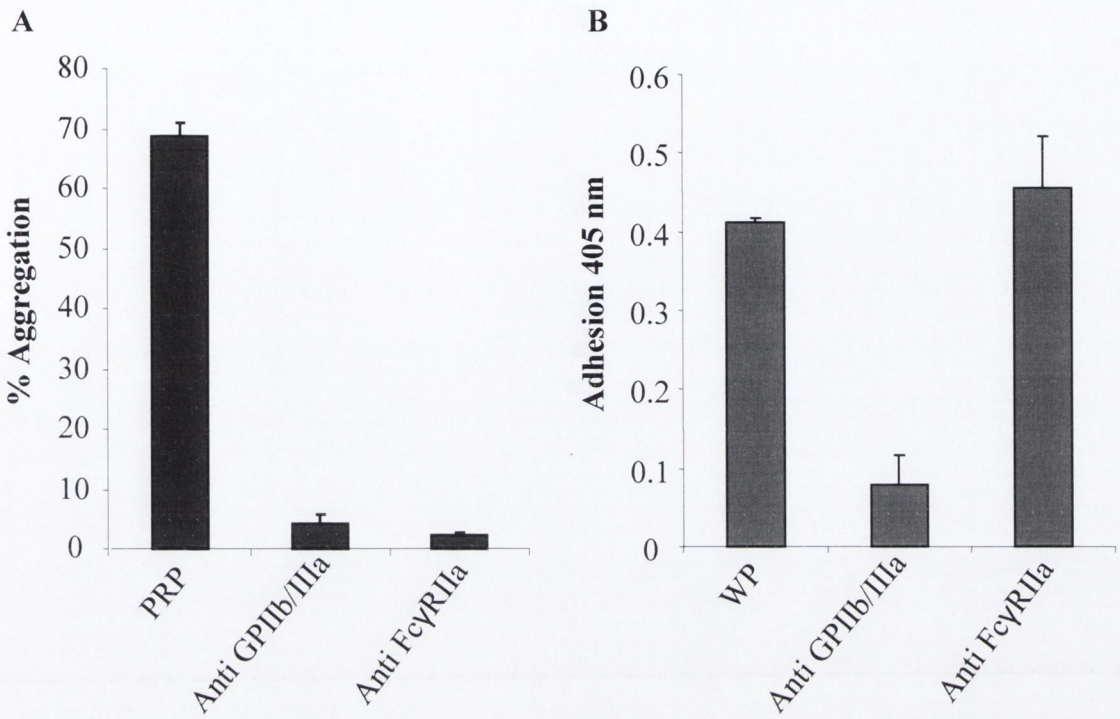


Figure 4.7. Effect of platelet receptor inhibitors on ClfB-mediated platelet activation and adhesion

A. PRP was incubated for 5 min at 37°C with antibodies inhibitory for platelet receptors GPIIb/IIIa or FcγRIIa. Washed *L. lactis* ClfB cells induced with 3.2 ng/ml of nisin were adjusted to an OD_{600} 1.6 and added to PRP. The experiment was carried out 3 times using 3 different blood donors. Results represent the mean ± SD of light transmission (percentage aggregation) observed after 25 min.

B. WP were incubated for 5 min at 37°C with antibodies inhibitory for platelet receptors GPIIb/IIIa or FcγRIIa and added to microtitre plates coated with *L. lactis* ClfB. Adherent platelets were lysed and a substrate for the intracellular enzyme acid phosphatase was added to each microtitre well. The experiment was carried out 2 times using 2 different blood donors. Results represent the mean ± SD of adherence at 405 nm as measured by an ELISA plate reader.

The requirements for ClfB-mediated platelet aggregation were investigated further. *L. lactis* expressing ClfB, ClfB Q235A and a strain containing the empty pNZ8037 vector were induced with 3.2 ng/ml of nisin. Induced cells were washed, adjusted to an OD_{600} of 1.6 and incubated with PRP for 25 min. The lag times and percentage aggregation were very similar for *L. lactis* ClfB and *L. lactis* ClfB Q235A. Bacteria expressing the wild type protein caused aggregation after a lag time of 5.7 min compared to 6 min for ClfB Q235A (Figure 4.8). The percentage aggregation was slightly lower for *L. lactis* ClfB Q235A (70.3% compared to 80.3% for *L. lactis* ClfB wild type) but this was not found to be statistically significant ($p=0.1621$).

To determine which plasma proteins were required for ClfB-mediated platelet activation, platelets were prepared by gel-filtration as described in Section 2.17.2. This process separates platelets from plasma proteins such as complement proteins and immunoglobulin. These factors were then added back to gel filtered platelets (GFP) to assess their roles in platelet aggregation mediated by ClfB. The presence of fibrinogen is necessary for the aggregation of activated platelets by cross-linking activated GPIIb/IIIa receptors. When purified fibrinogen alone was added to GFP neither ClfB nor ClfB Q235A expressed from *L. lactis* was able to cause platelet aggregation (Figure 4.8). The further addition of pooled human antibodies restored the ability of *L. lactis* ClfB to cause platelet aggregation. However fibrinogen and pooled IgG were not able to restore the ability of ClfB Q235A to aggregate platelets, which indicates that additional plasma proteins are required (Figure 4.8). Whole cell immunoblots were carried out to demonstrate that ClfB and ClfB Q235A were expressed equally from *L. lactis* and that both proteins could be recognised by pooled human IgG. No difference was detected in IgG binding between *L. lactis* expressing ClfB and ClfB Q235A (Figure 4.9). This strongly suggests that the proteins were expressed at the same level and that the inability of ClfB Q235A to cause activation of GFP substituted with fibrinogen and IgG was not due to differences in protein expression or due to an inability of pooled human IgG to recognise ClfB Q235A.

In order to determine if antibodies specific for ClfB in human IgG were required for activation, adsorption experiments were carried out to deplete antibodies from pooled human IgG samples (Gammagard). IgG samples were incubated with washed, fully induced *L. lactis* (pNZ8037) or *L. lactis* ClfB cells (section 2.19). Depletion of ClfB specific antibodies was confirmed by ELISA. Gammagard samples adsorbed against *L. lactis* (pNZ8037) bound to recombinant ClfB protein in a dose-dependent

manner similar to the unabsorbed Gammagard sample (Figure 4.10). This demonstrated that pooled human IgG contains ClfB-specific antibodies. Pooled human IgG absorbed against *L. lactis* ClfB did not bind to recombinant ClfB protein, indicating that ClfB-specific antibody was absent from these samples. *L. lactis* ClfB did not cause platelet activation when incubated with GFP substituted with fibrinogen and IgG depleted of specific ClfB antibodies (Figure 4.8). Compared to the experiment in which unabsorbed pooled antibody was added back to GFP and fibrinogen the percentage aggregation was reduced from 83% to 18.7%. IgG absorbed with *L. lactis* (pNZ8037) still supported activation and subsequent aggregation (Figure 4.8).

4.2.8 Complement fixation is required for platelet aggregation mediated by ClfB Q235A

L. lactis ClfB Q235A was unable to cause platelet activation in GFP substituted with fibrinogen and pooled antibody. This indicated that an additional plasma factor was necessary to stimulate activation by ClfB Q235A. Experiments were performed to assess if complement proteins were required for ClfB Q235A-mediated activation. Human serum samples were depleted of complement proteins and tested for their ability to support platelet aggregation by *L. lactis* ClfB and ClfB Q235A. Complement was depleted from serum either by heat inactivation (56 °C for 10 min) or by adsorption with zymosan as described in section 2. Zymosan is a yeast-derived polysaccharide isolated from *Saccharomyces cerevisiae* which activates complement by the alternative pathway. Accumulation of complement components on the polysaccharide surface results in a depletion of complement proteins from serum samples.

L. lactis ClfB Q235A and *L. lactis* ClfB were both able to stimulate platelet aggregation in GFP substituted with human serum and fibrinogen (required for aggregation following activation). The apparent difference in percentage aggregation caused by the mutant and wild type protein (49% compared to 62% respectively) was not statistically significant ($p=0.7325$ Figure 4.11). *L. lactis* ClfB Q235A was not able to stimulate activation when incubated with GFP, fibrinogen and heat inactivated serum. The same strain was also unable to cause activation when GFP were substituted with zymosan-treated serum. In both cases activation by *L. lactis* ClfB

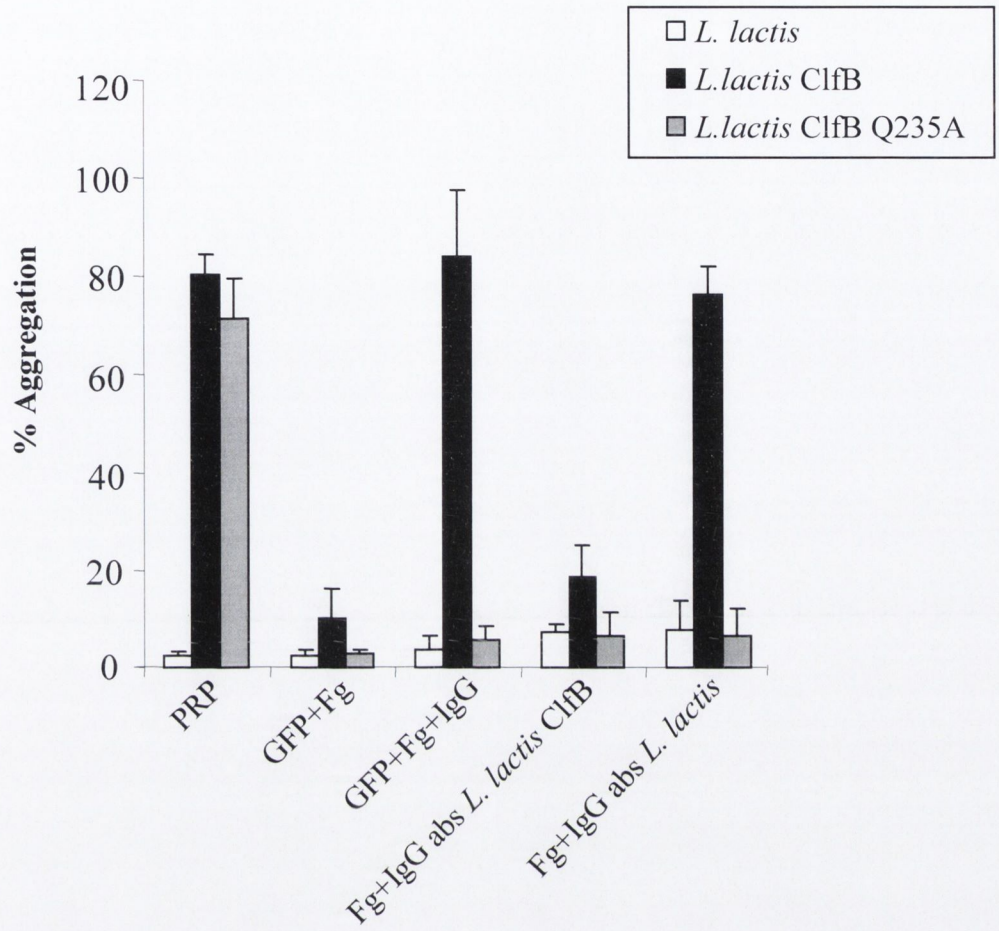


Figure 4.8. Role of ClfB-specific IgG in ClfB-promoted platelet activation

L. lactis (pNZ8037) and *L.lactis* expressing ClfB and ClfB Q235A were induced with 3.2 ng/ml of nisin and adjusted to an OD_{600} 1.6. Washed bacterial cells were added to PRP or GFP. GFP were supplemented with purified fibrinogen (Fg; 1 mg/ml), human IgG samples (2 mg/ml) and IgG samples absorbed (abs) with *L. lactis* pNZ8037 or *L. lactis* ClfB. The experiment was carried out 3 times using 3 different blood donors. Results represent the mean ± SD of light transmission (percentage aggregation) observed after 25 min.

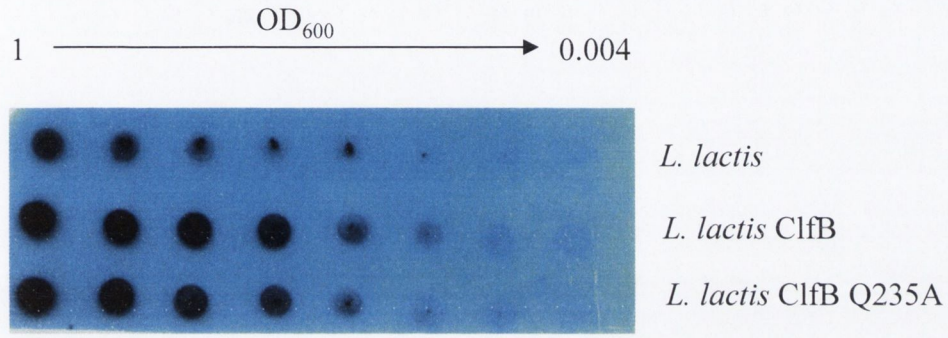


Figure 4.9. Detection of ClfB and ClfB Q235A expressed on the surface of *L. lactis* using pooled human IgG

L. lactis (pNZ8037), *L. lactis* (pNZ8037 *clfB*) and *L. lactis* pNZ8037 *clfB* were induced with 3.2 ng/ml of nisin. Induced cells were washed and doubling dilutions were dotted onto nitrocellulaose membrane. Membranes were probed with pooled human IgG (Gammagard) and HRP-conjugated protein A-peroxidase. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

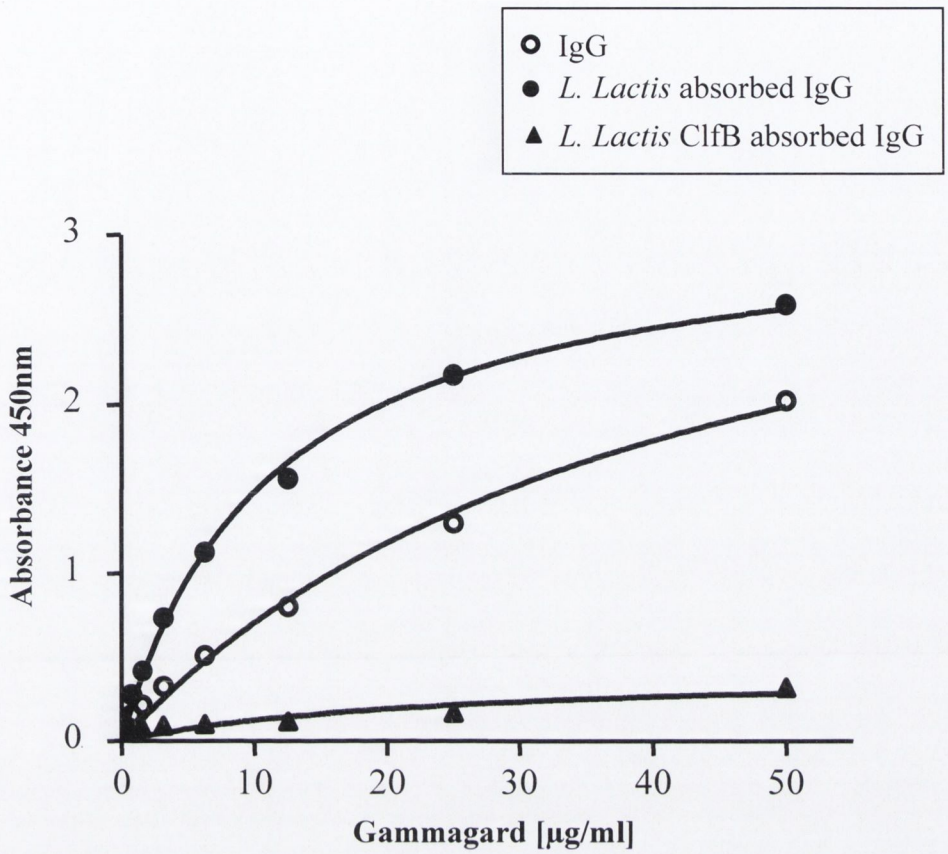


Figure 4.10. Depletion of ClfB-specific antibodies from pooled human IgG

L. lactis (pNZ8037) and *L. lactis* (pNZ8037 *clfB*) induced with 3.2 ng/ml nisin were incubated with pooled human IgG (Gammagard) to absorb ClfB specific antibodies. Depletion of anti-ClfB antibodies was confirmed by ELISA. Recombinant ClfB A domain protein was immobilized in microtitre wells at 2.5 µM. Serial dilutions of IgG samples were added to each well and incubated for 1 h. Antibodies adhering to ClfB were detected using Protein A peroxidase. Wells were developed by incubation with a chromogenic substrate and the absorbance at 450 nm was determined. Values represent the means ± standard deviation of triplicate wells.

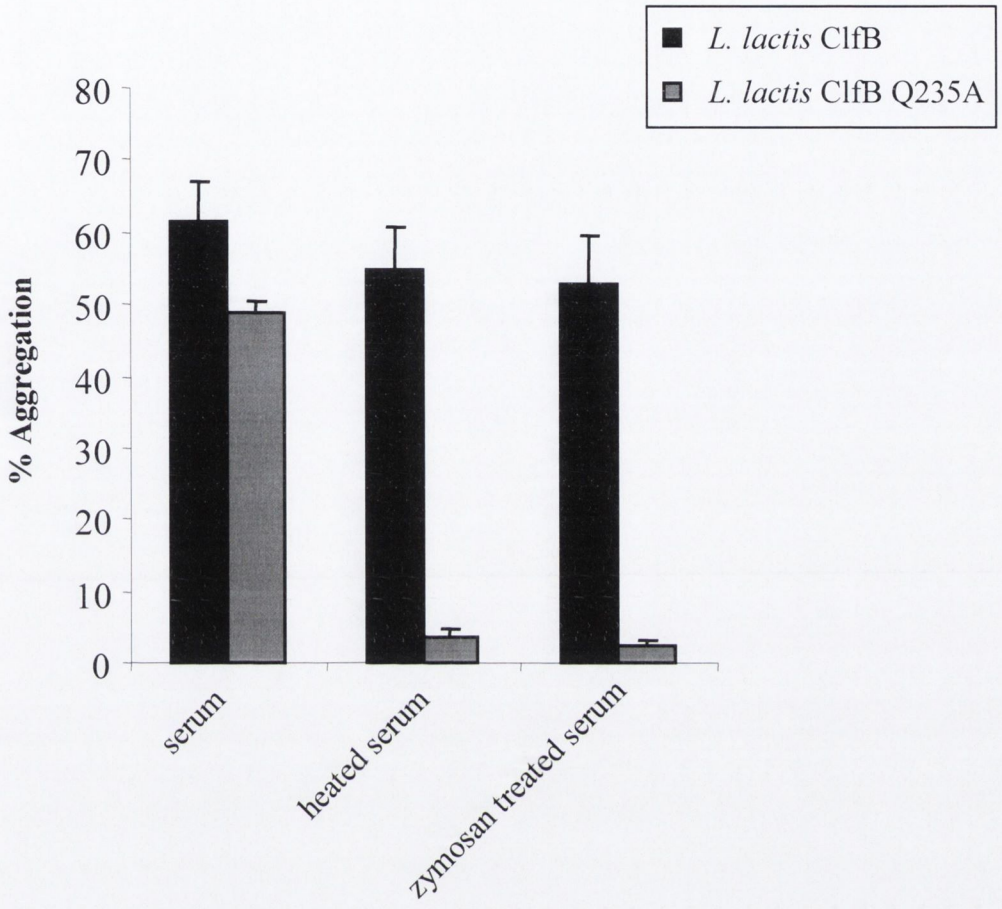


Figure 4.11. Role of plasma proteins in ClfB-promoted platelet activation

L.lactis ClfB and *L .lactis ClfB* Q235A were induced with 3.2 ng/ml of nisin and adjusted to an OD600 of 1.6. Bacterial cells were added to GFP supplemented with purified fibrinogen (Fg; 1 mg/ml) and human serum samples (10 % serum). The experiment was carried out 3 times using 3 different blood donors. Results represent the mean ± SD of light transmission (percentage aggregation) observed after 25 min.

was not affected (Figure 4.11). This shows that the non-fibrinogen binding mutant of ClfB could only promote platelet activation by a complement-dependent mechanism.

4.2.9 Specific anti-ClfB antibody is required for platelet aggregation mediated by ClfB Q235A

In order to determine if antibodies present in human serum were required for ClfB Q235A-mediated aggregation, serum was depleted of IgG by absorption with protein A- sepharose as described in section 2.18.1. Depletion of IgG from human serum was confirmed by separating serum and purified serum samples by SDS-PAGE. Bands corresponding to the heavy and light chains of IgG were absent from IgG-depleted serum samples (Figure 4.12). *L. lactis* ClfB Q235A incubated with GFP, fibrinogen and IgG-depleted serum did not support platelet activation ($p = 0.0001$). Addition of pooled human IgG restored the ability of ClfB Q235A to cause activation (62 % Figure 4.13).

Specific anti-ClfB antibodies were required for platelet activation mediated by wild type ClfB. To test if specific anti- ClfB antibodies were also required in complement-mediated activation by ClfB Q235A, absorbed human IgG samples were added to GFP and IgG-depleted serum. ELISA assays showed that IgG absorbed against *L. lactis* (pNZ8037) contained anti-ClfB antibodies (Figure 4.6). The addition of pooled human IgG absorbed with *L. lactis* (pNZ8037) to IgG-depleted serum supported platelet aggregation by ClfB Q235A (Figure 4.13). When pooled human IgG absorbed with *L. lactis* (pNZ8037*clfB*) was added to IgG-depleted serum no platelet activation occurred. The percentage aggregation was reduced to 19% ($p = 0.0080$). Thus specific anti- ClfB antibodies are required as well as complement fixation for *L. lactis* ClfB Q235A-promoted activation of platelets.

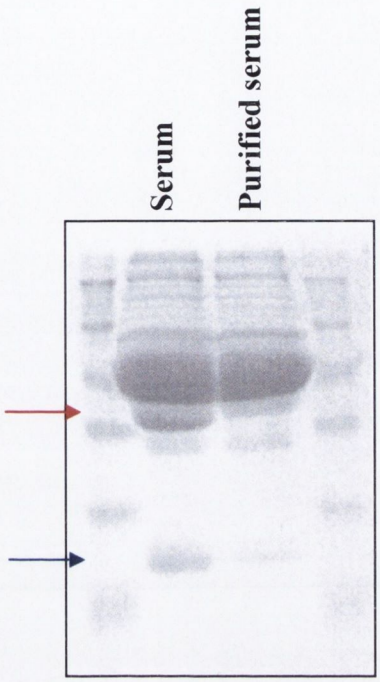


Figure 4.12. Preparation of serum purified of IgG for platelet aggregation assays

Human serum was depleted of IgG by passage over a protein A-sepharose column. Samples of serum and IgG-depleted serum were separated on 7.5 % SDS-PAGE gels and stained with Coomassie brilliant blue. Arrows highlight the bands corresponding to the IgG heavy chain (red) and light chain (blue) that were depleted by this process.

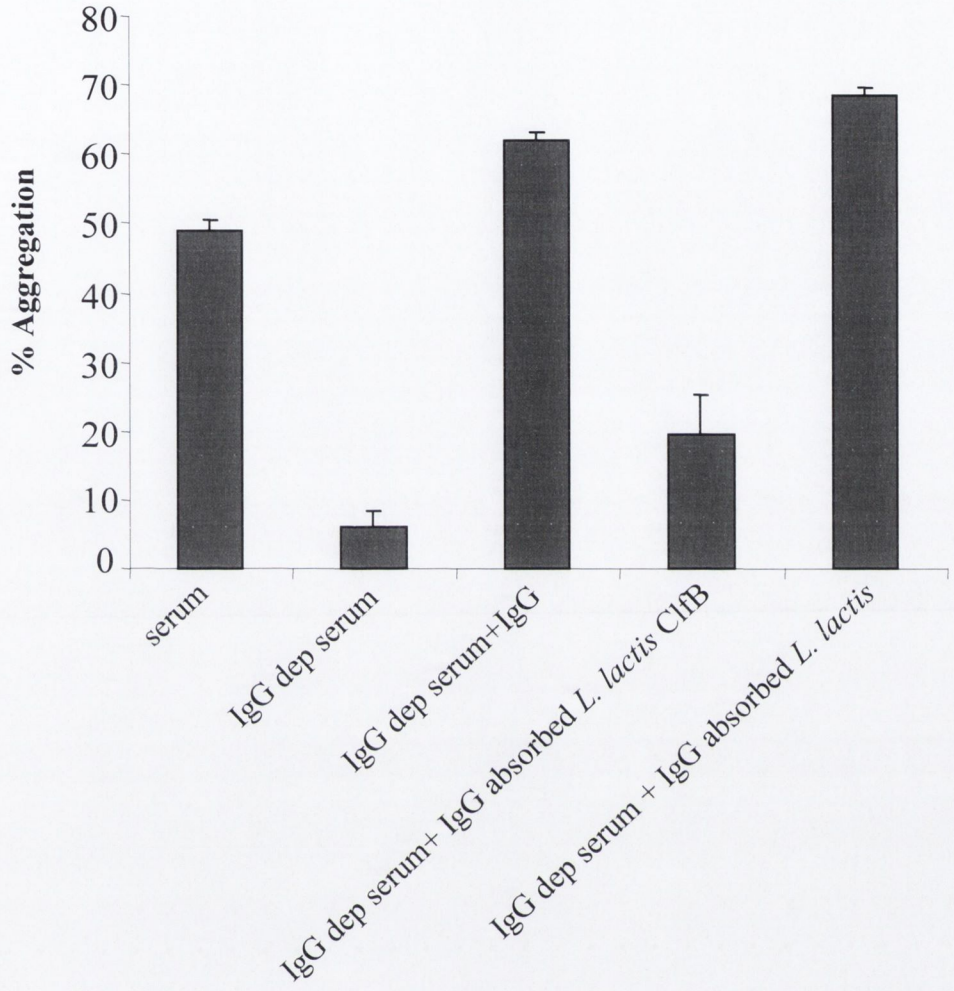


Figure 4.13. Role of ClfB-specific IgG in complement dependent platelet activation

L. lactis ClfB Q235A was induced with 3.2 ng/ml of nisin and adjusted to an OD_{600} of 1.6. Bacterial cells were added to GFP supplemented with serum and serum depleted of IgG. Pooled IgG samples and IgG sampled absorbed against *L. lactis* and *L. lactis* ClfB were added where appropriate. The experiment was carried out 3 times using 3 different blood donors. Results represent the mean ± SD of light transmission (percentage aggregation) observed after 25 min.

4.3 Discussion

Several surface proteins of *S. aureus* have been shown to be involved in the process of platelet activation and aggregation. A common mechanism for activation by ClfA and FnBPA involves an initial interaction between the bacterium and the platelet receptor GPIIb/IIIa. Bound fibrinogen and fibronectin coat the bacterial surface and crosslink the bacterium to GPIIb/IIIa on the platelet surface. The ability of ClfA to bind avidly to fibrinogen and FnBPA to bind both fibrinogen and fibronectin allows for short lag times to aggregation (Fitzgerald *et al.*, 2006b; Loughman *et al.*, 2005). When specific binding proteins are absent extended lag times for aggregation are seen such as was described for the non-fibrinogen binding mutant of ClfA and for platelet aggregation mediated by *Step. sanguis* NCTC 7863 (Ford *et al.*, 1996; Loughman *et al.*, 2005). These longer lag times are reflective of the time taken for complement assembly to occur on the bacterial surface. ClfB binds to the α-chain of fibrinogen. It was therefore surprising that longer lag times to aggregation (6 min) were caused by *L. lactis* expressing ClfB. The mean lag time was marginally shorter than that of the non-fibrinogen binding mutant of ClfA (8-10 min) (Loughman *et al.*, 2005).

The results of this study show that ClfB expressed from *L. lactis* can cause adhesion to and activation of platelets in a fibrinogen-dependent manner although binding via fibrinogen alone is not sufficient to stimulate activation. Adhesion of bacteria expressing ClfB to washed platelets was dependent on the presence of fibrinogen and could be inhibited using anti-GPIIb/IIIa antibody (Figures 4.5 and 4.7B). A non-fibrinogen binding mutant ClfB Q235A was unable to adhere to platelets in the presence of fibrinogen. The anti-GPIIb/IIIa antibody also inhibited aggregation of platelets demonstrating the importance of this receptor in initial adhesion of ClfB expressing bacteria to platelets and subsequent cross-linking of platelets following activation (Figure 4.7A). When GFP were supplemented with fibrinogen and pooled human IgG, *L. lactis* ClfB was able to stimulate platelet aggregation with a lag time of approximately 6min. Further experiments showed that specific anti-ClfB antibodies present in pooled human IgG were necessary for activation to occur (Figure 4.8). Anti-FcγRIIa (IV3) antibodies prevented aggregation of platelets mediated by *L. lactis* ClfB but not adhesion (Figure 4.7B). This indicates that this receptor is crucial for activation of platelets following adhesion. The FcγRIIa receptor is not required for subsequent aggregation which is mediated by binding of

GPIIb/IIIa on activated platelets to soluble fibrinogen in plasma. It seems that ClfB is able to cause platelet aggregation in the same manner as ClfA and FnBPA. Bound fibrinogen acts as a bridge between the platelet receptor GPIIb/IIIa and bacteria expressing ClfB facilitating initial adhesion. Specific anti-ClfB antibody binds the Fc receptor on platelets to cause activation through receptor clustering and intracellular signalling.

Since ClfB can cause aggregation in a fibrinogen-dependent manner it seems strange that the lag time to aggregation is longer than that seen for ClfA and other fibrinogen binding molecules. There are several possibilities to explain this difference. Fibrinogen is a symmetrical molecule consisting of two γ-chains, two α-chains, and two β-chains. Unlike ClfA and FnBPA and FnBPB, ClfB binds the α-chain of fibrinogen in a flexible region located between residues 221 and 391. In the case of ClfA and FnBPs the interaction with platelets occurs via a fibrinogen molecule bound at the γ-chain protruding from the end of domain D by GPIIb/IIIa and ClfA or FnBPA. This may orient fibrinogen in a manner that is more suitable for binding GPIIb/IIIa than when it is bound at the α-chain (Figure 4.14).

The affinity of ClfB and ClfA for fibrinogen has not been compared directly and it is possible that ClfB binds with a lower affinity. The αC-connectors of fibrinogen which contain the ClfB binding site are highly susceptible to proteolysis and can be cleaved into smaller fragments by plasmin and other proteases (Collet *et al.*, 2005; Weisel & Medved, 2001). A lower affinity for fibrinogen or the cleavage of the ClfB binding region in a proportion of fibrinogen molecules would explain the difference seen between *L. lactis* ClfB and *L. lactis* ClfA in fibrinogen binding assays. Across the range of inducer concentrations tested, cells expressing ClfB bound at consistently lower levels than cells expressing ClfA (Figure 4.3). Any of these factors could contribute to the longer lag time to aggregation caused by ClfB.

Platelet activation and aggregation mediated by ClfB requires a minimum level of ClfB protein to be expressed on the surface of cells. The threshold for platelet activation stimulated by *L. lactis* ClfB occurred at 0.2 ng/ml of nisin (Figure 4.4A). Below this level no activation was be seen. Increasing the number of ClfB molecules on the surface of the cell decreases lag time to aggregation. The initial adhesion of bacteria to platelets via fibrinogen seems to be important in determining lag time. Similar results have been seen with bacteria expressing ClfA and FnBPA (Fitzgerald *et al.*, 2006b; Loughman *et al.*, 2005). This study also found that a threshold number

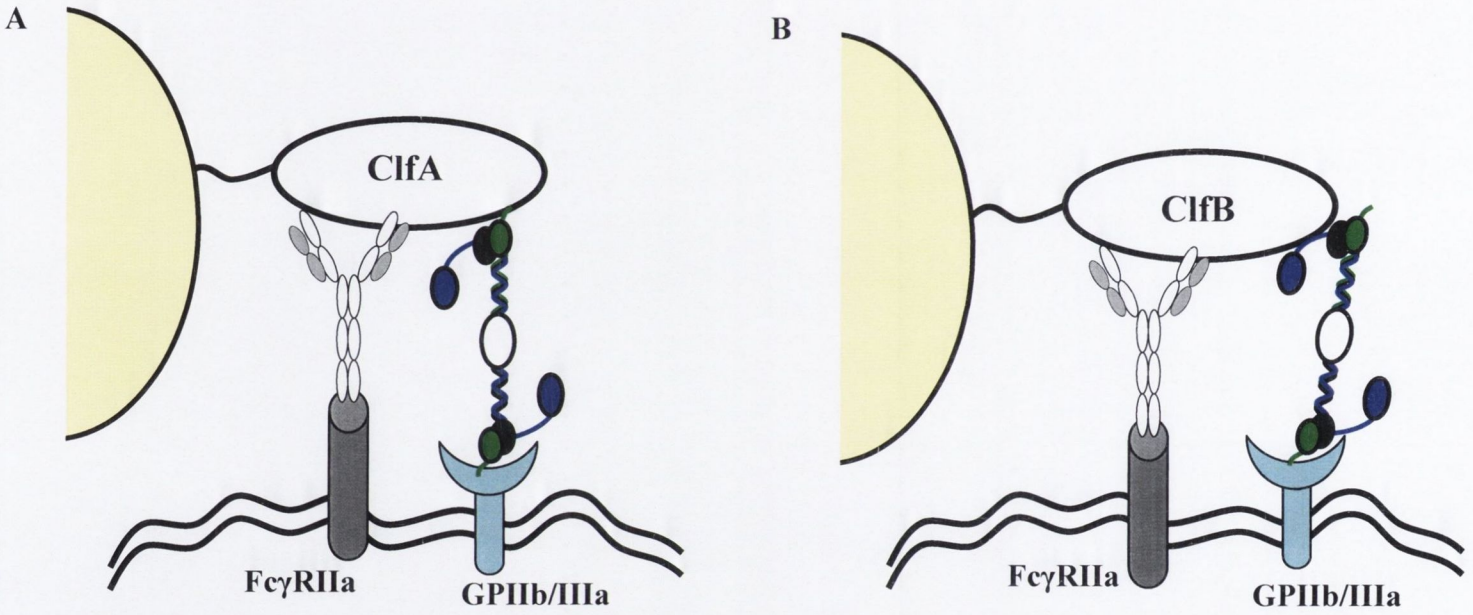


Figure 4.14. Fibrinogen-dependent platelet activation mediated by ClfA and ClfB-expressing bacteria

(A) ClfA-expressing bacteria bind the extreme C-terminus γ-chain of fibrinogen (green), facilitating adhesion to GPIIb/IIIa which also binds the γ-chain on the opposite end of the divalent fibrinogen molecule. Antibodies bound to ClfA are required for interaction with FcγRIIa. (B) ClfB-expressing bacteria bind the C-terminus of the Aα-chain of fibrinogen (blue), facilitating adhesion to platelet receptor GPIIb/IIIa. Specific-ClfB antibodies are required to interact with FcγRIIa. Platelet activation is stimulated by receptor clustering and GPIIb/IIIa activation.

of bacterial cells are needed to cause platelet activation (Figure 4.4B). At lower concentrations of bacteria the lag time to activation increased. Decreasing the level of ClfB on the surface of *L. lactis* seemed to decrease percentage aggregation, although the difference was not statistically significant. In aggregation experiments carried out with Newman strains increased lag times did not correlate with a decrease in percentage aggregation (Figure 4.2). It is possible that other surface proteins of *S. aureus* such as SdrE contribute to aggregation in this situation. Incubating platelets with *L. lactis* expressing low levels of ClfB for longer than 25 min may increase percentage aggregation.

Bacteria expressing the non-fibrinogen binding mutant ClfB Q235A were still able to cause aggregation in platelet rich plasma. The lag time to aggregation was not significantly different from that caused by the wild type protein. However experiments carried out with washed platelets and gel filtered platelets demonstrated that ClfB Q235A did not adhere to or cause aggregation of platelets in a fibrinogen-dependent manner. Instead aggregation was dependent on the presence of complement. When serum was heated to inactivate complement and added to GFP only cells expressing the wild type ClfB protein could cause aggregation. The same result was seen with zymosan-treated serum (Figure 4.11). Platelet activation mediated by ClfB Q235A also required IgG. Serum depleted of IgG did not support platelet activation by *L. lactis* ClfB Q235A. Aggregation could be restored by adding pooled IgG or IgG absorbed against *L. lactis* (pNZ8037). Adding IgG absorbed against *L. lactis* (pNZ8037*clfB*) did not restore activation (Figure 4.13). We can therefore conclude that specific anti-ClfB antibodies are required for *L. lactis* ClfB Q235A-mediated platelet activation and aggregation. These results suggest that ClfB Q235A causes aggregation in the same way as the non-fibrinogen binding mutant of ClfA (ClfA PY). Complement proteins bound to the bacterium can presumably interact with a complement receptor on the surface of the platelets. Bound anti-ClfB antibodies form another link to the platelet via the FcγRIIa receptor (Figure 4.15). It has been postulated that complement-dependent platelet activation may be common amongst IE-causing pathogens since any bacterium could cause aggregation by expressing a surface protein to which antibodies are present in the host (Fitzgerald *et al.*, 2006a). The fact that both the fibrinogen and complement dependent mechanisms of platelet aggregation identified in this study have similar lag times suggests that both mechanisms can contribute to overall activation and aggregation caused by ClfB.

This study also investigated the role ClfB plays in platelet aggregation mediated by *S. aureus* strain Newman. This strain produces a high level of coagulase which caused fibrin clot formation when cells were incubated with PRP for long periods of time. For this reason a coagulase-defective mutant of strain Newman was used. Strain Newman *coa* had the same lag time as Newman wild type (approximately 3 min). Eliminating ClfA increased lag time from 3 min to 4.67 min. Normally in the exponential phase of growth bacterial cells express high levels of FnBPA and FnBPB which cause rapid aggregation. However Newman is defective in FnBP expression (Grundmeier *et al.*, 2004). ClfA is expressed predominantly in the stationary phase of growth. Previous studies using stationary phase cells have shown that expression of ClfA caused rapid activation with a lag time of 1 min (Loughman *et al.*, 2005). ClfA is not expressed at high levels on exponential phase cells and therefore an extended lag time was seen in this study.

ClfB contributes to platelet activation and aggregation by exponentially growing cells. Lag time to aggregation was measured for strain Newman *coa clfA clfB.* In two of the donors tested Newman *coa clfA clfB* activated platelets with an extended lag time of 6.5 min compared to 4.67 min caused by Newman *coa clfA.* In the third donor Newman *coa clfA clfB* failed to activate platelets. This suggests that variable host factors such as antibody levels and abundance of platelet receptors contribute to the ability of bacteria expressing ClfB to cause activation. Overall the presence of ClfB on *S. aureus* did shorten the lag time to aggregation. It is proposed that ClfB can contribute to and amplify the platelet aggregation caused by FnBPA and FnBPB in the exponential phase of growth.

In conclusion, this study found that ClfB expressed on the surface of bacteria can adhere to and cause aggregation of platelets in a fibrinogen-dependent manner. Bacteria expressing a non-fibrinogen binding mutant ClfB Q235A were still able to cause aggregation by a complement-dependent mechanism. Both mechanisms of platelet aggregation required specific anti-ClfB antibodies to crosslink to the platelet FcγRIIa receptor and trigger activation. The two mechanisms have similar lag times and it is feasible that both contribute to the overall activation and aggregation caused by ClfB.

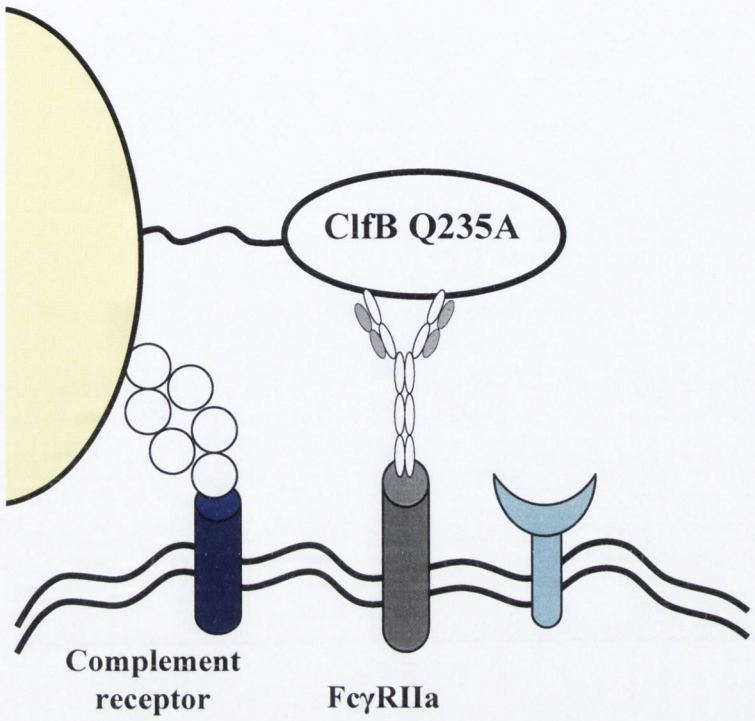


Figure 4.15. Complement-dependent platelet activation mediated by ClfB Q235A

Bactria expressing ClfB Q235A cause activation in a complement dependent manner. Assembly of complement proteins on the bacterial surface facilitates interaction with a complement receptor on the platelet surface. Anti-ClfB antibodies are required to engage platelet receptor FcγRIIa. Receptor clustering and signaling events lead to activation of GPIIb/IIIa and aggregation of platelets by cross-linking.

Chapter 5

Interaction of iron regulated surface determinants of *Staphylococcus aureus* with platelets

5.1 Introduction

The capacity of *S. aureus* to adapt to its environment makes it a successful and versatile pathogen which can cause a wide range of disease. The vast array of secreted and cell-wall anchored virulence factors produced by *S. aureus* are responsible for the pathogenesis of this organism. The expression of these factors is coordinated by two component regulatory systems (TCS), transcription factors and sigma factors which sense environmental signals such as high salt, cell density and pH. A variety of environmental signals are believed to trigger expression of virulence factors *in vivo* and one of these is the lack of available iron. Microarray studies have shown that many *S. aureus* genes regulated by the ferric-uptake regulator, Fur are expressed *in vivo* and *in vitro* under iron restricted growth conditions (Allard *et al.*, 2006).

An important defence against bacterial survival and growth inside the human host is the lack of available iron. Once inside the host, *S. aureus* expresses a unique array of proteins which allow it to capture and extract iron from host proteins. Iron is essential as a cofactor for bacterial antioxidant defence enzymes such as catalase and peroxidise. The most abundant source of iron in mammals is found intracellularly in haem-containing proteins. The porphyn haem contains one iron atom at the centre of four-ring nitrogen atoms. Haem is a cofactor of many haemoproteins such as haemoglobin and myoglobin which have roles in transport and storage of oxygen. A small percentage of iron is also found in iron binding proteins such transferrin and lactoferrin. The low solubility of iron at physiological pH is also a factor in the unavailability of this molecule to invading bacteria (Skaar & Schneewind, 2004).

S. aureus has a number of mechanisms to obtain iron in the host. In the late stages of systemic infection iron is believed to be captured by high affinity iron chelators known as siderophores. *S. aureus* secretes four different siderophores which are actively transported back into the cell via specific receptors and ABC type transporters (section 1.5.10). Transferrin can also be utilised as an iron source although studies have demonstrated that *S. aureus* preferentially utilises haem-iron (Skaar *et al.*, 2004). Haemoproteins are the most abundant source of iron in the host with 70% of total iron stores found associated with haemoglobin. *S. aureus* can capture haem from these proteins using haem-iron-uptake systems. Together the iron surface determinants (Isd) of *S. aureus* comprise a system which can capture haem from hemoproteins, transport haem across the bacterial envelope and subsequently

release iron intracellularly. The Isd genes are located on five transcriptional units and are regulated by the iron-dependent transcriptional repressor Fur. IsdA, IsdB and IsdH are cell wall anchored proteins which are exposed on the cell surface and are crosslinked to cell wall peptidoglycan by Sortase A. IsdC is partially buried in the cell wall and crosslinked to an unknown component of the cell wall envelope by Sortase B. IsdD, IsdE and IsdF are membrane transport proteins while IsdG and IsdI are cytoplasmic haem-monoxygenases which degrade haem to release iron (Figure 1.9; Skaar & Schneewind, 2004).

Surface exposed Isd proteins characteristically contain varying numbers of NEAT domains which are responsible for binding to haem and haem-containing proteins. Recent studies have outlined a mechanism for haem transfer by the Isd system. Hemoglobin is released from erythrocytes following lysis by *S. aureus* toxins. Free haemoglobin is toxic and is rapidly associated with the carrier molecule haptoglobin. Hemoglobin and haptoglobin-hemoglobin complexes are bound by the Type I NEAT domains of IsdB and IsdH, respectively. Haem is extracted by these proteins by an unknown mechanism and directly passed to the NEAT domain of IsdA. Haem is transferred from IsdA to the NEAT domain of IsdC. The location of IsdC allows it to transfer haem to IsdE, a component of the ABC transporter. Once haem is transported into the cytoplasm it can be degraded by IsdG and IsdI to release free iron which can be used as a nutrient source by bacteria (see Figure 1.9; Muryoi *et al.*, 2008; Zhu *et al.*, 2008).

IsdA is a major component of the cell wall of bacteria that have been grown in iron restricted conditions. It has been shown to interact with a number of host proteins. IsdA binds to transferrin and it has been suggested that this protein is the specific cell wall transferrin receptor. Studies carried out by Clarke *et al.* demonstrated that IsdA interacted with hemin, hemoglobin and feutin. The same study found that IsdA can also bind to fibronectin and to the Bβ and γ-chians of fibrinogen. IsdA is reported to contribute to nasal colonisation and evasion of the innate defences of the human skin (see sections 1.2 and 1.5.1) (Clarke *et al.*, 2004; Clarke *et al.*, 2007). The *isdB* gene is located 203 bp downstream to *isdA* on the chromosome. Despite its close proximity *isdB* has its own promoter and Fur consensus binding sequence. IsdB is the haemoglobin receptor on the cell-wall surface (Torres *et al.*, 2006). The *isdH* gene is isolated from the other *isd* genes on a separate region of the chromosome (Figure 1.6). The type I NEAT domains of IsdH

bind to haptoglobin-hemoglobin complexes with high affinity and haptoglobin with lower affinity (Pilpa *et al.*, 2006).

Previous studies which examined platelet aggregation mediated by *S. aureus* strains have used bacterial cells grown in complex iron enriched media such as tryptone soy broth. This study investigated platelet adhesion and aggregation mediated by *S. aureus* strain Newman grown in an iron deficient medium. RPMI medium was originally designed for the cell culture of human leukocytes but has also been utilised in many studies as an iron deficient medium for the growth of bacterial cells.

We hypothesised that the *in vivo* upregulated Isd proteins IsdA, IsdH and IsdB could be involved in interaction with platelets. These proteins are projected from the cell wall surface making them available for possible interactions with platelets. Previous studies have shown that antibodies to IsdA and IsdH are present in the serum of patients recovering of *S. aureus* infections (Roche *et al.*, 2003). Evidence that IsdA can act as an adhesin and bind to fibrinogen and fibronectin was presented by Clarke *at al.* This raised the possibility that IsdA expressing cells could interact with platelets in a fibrinogen or fibronectin dependent manner similar to cells expressing ClfA, ClfB or the FnBPs.

In order to ascertain if IsdA, IsdB or IsdH could interact with platelets, strains were constructed lacking each of these proteins. Allelic replacement mutagenesis was utilised to introduce a frameshift mutation in *isdB* on the chromosome of strain Newman. The mechanism of platelet adhesion and aggregation mediated by *S. aureus* cells grown in iron deficient media was also investigated using several strategies. The involvement of individual plasma proteins was studied using gel filtered platelets and washed platelets. Inhibitors of platelet receptors were also utilised to determine their involvement in platelet adhesion and aggregation mediated by *S. aureus* cells expressing Isd proteins.

5.2 Results

5.2.1 Platelet adhesion and aggregation mediated by *S. aureus* strain Newman grown in iron limiting conditions

Strain Newman can mediate adhesion to and aggregation of platelets via surface proteins ClfA and ClfB. The FnBPA and FnBPB surface proteins also mediate interactions with platelets; however these proteins are truncated and secreted in strain

Newman due to mutations (Grundmeier *et al.*, 2004). Western immunoblots and binding assays demonstrated that ClfA and ClfB are present on the surface of strain Newman in the stationary phase when grown in RPMI (see Chapter 6). In order to investigate the potential of Isd proteins to contribute to adhesion and aggregation of platelets without interference from other pro-aggregatory proteins this study utilised strain Newman with mutations in *clfA* and *clfB*.

Washed platelets (WP) adhered directly to microtitre wells coated with RPMI-grown Newman *clfA clfB* without the addition of exogenous fibrinogen. Adherence of WP to Newman *clfA clfB* was 2-fold higher than to immobilised fibrinogen, used as a control ($p < 0.0001$, Figure 5.1). Addition of 50 μM $FeCl_3$ to the RPMI growth medium dramatically decreased adhesion of WP to Newman *clfA clfB* compared to the same strain grown without iron ($p < 0.0001$). These results indicate that direct adherence of strain Newman to platelets is dependent on proteins induced by iron starvation.

Strains Newman and Newman *clfA clfB* were grown to stationary phase in RPMI media. Washed bacterial cells with OD_{600} values of 1.6 were added to platelet rich plasma (PRP) and light transmission from an aggregometer was measured over 25 min. Loss of ClfA and ClfB from the surface of Newman decreased the overall percentage aggregation from 71 % to 60 % ($p = 0.0112$, Figure 5.2). An apparent increase in the average lag time from 4.6 to 5.6 min was also observed in the *clfA clfB* mutant compared to RPMI-grown Newman, although this difference was not statistically significant ($p = 0.43$). Growth of Newman *clfA clfB* in the presence of iron eliminated platelet activation. It seems that ClfA and ClfB do not play a dominant role in platelet aggregation of bacteria grown in iron limiting conditions. Instead, proteins induced by iron starvation seem to be responsible for adhesion to and aggregation of platelets.

5.2.2 Aggregation of platelets by Newman *clfAclfB* grown in RPMI is not dependent on complement

Lag times to aggregation for Newman *clfA clfB* grown in RPMI varied from donor to donor with an average lag time of 5.6 min. Longer lag times are often associated with complement-mediated mechanisms of platelet activation. To further investigate the mechanism of platelet activation complement was inactivated in human serum by heat treatment as described in section 2.21. Serum depleted of IgG by passage over

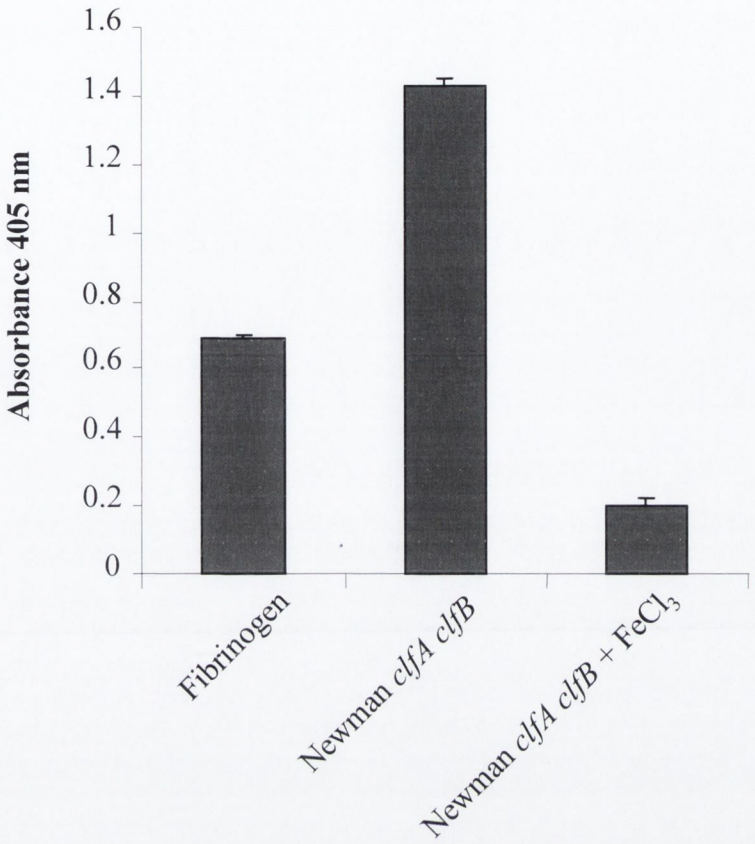


Figure 5.1. Direct adhesion to washed platelets mediated by Newman *clfA clfB* grown in RPMI

Strain Newman *clfA clfB* was grown in RPMI media with or without the addition of 50 μM $FeCl_3$. Washed bacteria (OD_{600} of 1) or 50 μg/ml fibrinogen were added to microtitre wells. Plates were incubated for 16 h at 4°C and then blocked with 1% BSA. Washed platelets were added to microtitre wells and incubated at 37°C. Adherent platelets were lysed and a substrate for the intracellular enzyme acid phosphatase was added to each microtitre well. The experiment was carried out 3 times using 3 different blood donors. Results represent the mean ± SD of adherence at 405 nm as measured by an ELISA plate reader.

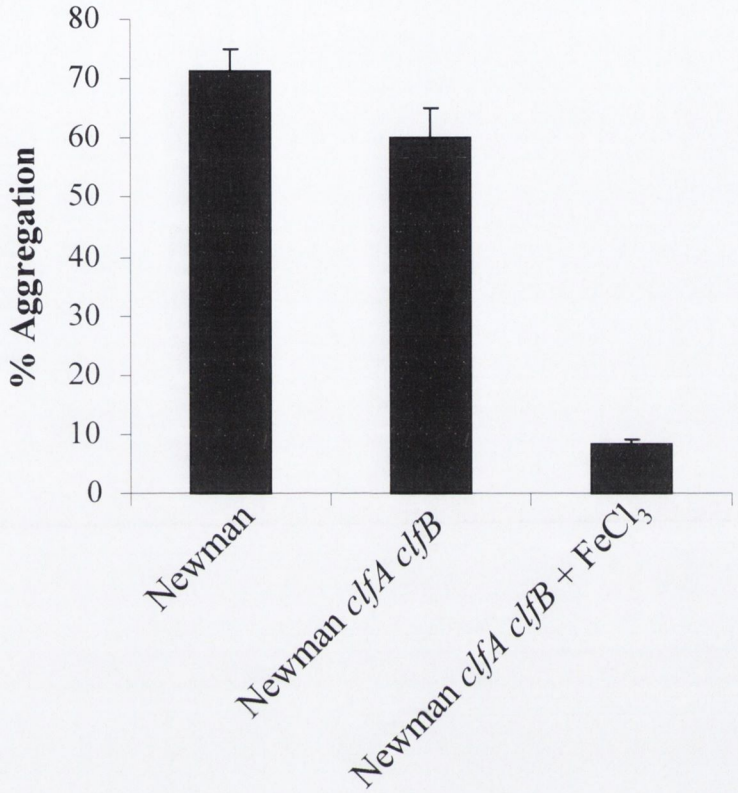


Figure 5.2. Aggregation of platelets mediated by Newman *clfA clfB* grown in RPMI

Strains Newman and Newman *clfA clfB* were grown in RPMI media with or without the addition of 50 μm $FeCl_3$. Washed bacterial cells at an OD_{600} of 1.6 were added to PRP in an aggregometer. The experiment was carried out 3 times using 3 different blood donors. Results represent the mean ± SD of light transmission (percentage aggregation) observed after 25 min.

protein A coupled to sepharose was also utilised to determine the necessity for IgG. Strain *L. lactis* expressing ClfB Q235A was used as negative control in this assay. This strain does not cause aggregation of GFP supplemented with fibrinogen and complement inactivated serum or IgG-depleted serum.

Both Newman *clfA clfB* and *L. lactis* ClfB Q235A were able to stimulate aggregation of GFP supplemented with fibrinogen and 10 % human serum (Figure 5.3). Adding heated serum or IgG-depleted serum to GFP did not support platelet activation mediated by *L. lactis* ClfB Q235A which agrees with previous results described in chapter 4. In contrast, Newman *clfA clfB* was able to cause aggregation in GFP supplemented with heated serum and IgG depleted serum. No significant difference in percentage aggregation was observed between GFP supplemented with serum, heated serum or IgG depleted serum ($p = 0.1155$ and $p = 0.2758$, respectively). Likewise, no significant difference was observed in the lag time to aggregation under these conditions. These results suggest that platelet aggregation mediated by Newman *clfA clfB* grown in iron limiting conditions is not complement-dependent and IgG does not seem to be necessary for activation.

5.2.3 Inhibition of platelet activation and adhesion mediated by RPMI grown Newman *clfA clfB* using antibodies to platelet receptors

The anti-GPIIb/IIIa monoclonal antibody abciximab inhibited aggregation mediated by RPMI grown Newman *clfA clfB*. The antibody was incubated with PRP at 37°C for 5 min prior to the addition of bacterial cells. Anti-GPIIB/IIIa reduced aggregation from 77 % to 0.67 % (Figure 5.4.B). The antibody abciximab also inhibited adherence of WP to RPMI-grown Newman *clfA clfB* (Figure 5.4.A). This indicates that adherence of bacteria grown in iron limiting conditions to platelets occurs via GPIIb/IIIa and does not involve GP1b or FcγRIIa. Aggregation of platelets also requires GPIIb/IIIa presumably for cross-linking of platelets into thrombi following adhesion and activation.

A monoclonal antibody (IV-3) that blocks the FcγRIIa platelet receptor inhibited platelet aggregation mediated by RPMI-grown Newman *clfA clfB*. Anti-FcγRIIa reduced aggregation from 77 % to 2.67 % (Figure 5.4.B). The same antibody did not inhibit adhesion of WP to microtitre wells coated with Newman *clfA clfB* (Figure 5.4.A).

A mouse monoclonal antibody that blocks the platelet receptor GP1b (AN51, Dako) did not inhibit adhesion of WP to Newman *clfA clfB* (Figure 5.4.A). However, the anti-GP1b antibody inhibited aggregation but to a lesser extent than the anti-GPIIb/IIIa and anti-FcγRIIa antibodies (Figure 5.4.B). The final aggregation values were reduced from 77 % to 20 %. The 20 % aggregation was the result of slow drift rather than the normal slope seen in aggregometer traces. The anti-GP1b antibody contains the preservative sodium azide at a concentration of 15 mM. Low concentrations of sodium azide have been reported to inhibit a thromboxane A2 pathway in human platelets. Therefore anti-GP1b antibody mediated inhibition of platelet aggregation seen in this study does not necessarily indicate a role for the GP1b receptor in activation.

5.2.4 Allelic replacement mutagenesis of *isdB*

IsdA, IsdH and IsdB are expressed on the cell surface of *S. aureus* grown in iron limiting conditions. In order to determine which of these proteins is involved in platelet adhesion and aggregation, *isd* null mutants were tested. Since *isdA* and *isdH* mutations were already available, an *isdB* null mutation was constructed by allelic replacement mutagenesis. The *isdB* mutation was constructed in a cloning vector in *E. coli* (pBlueΔ*isdB*) and subsequently introduced on a temperature sensitive shuttle plasmid (pHM1) into *S. aureus*, where allelic replacement mutagenesis was carried out (Figure 5.5).

5.2.4.1 Construction of plasmids pBlueΔ*isdB* and pHM1

Primer pairs FP*isdB* and RB*isdB* were used to amplify a 745 bp fragment of isdB coding sequence upstream of the signal sequence (Table 2.3). A 904 bp fragment of *isdB* coding sequence downstream of the signal sequence was amplified using primers FB*isdB* and RX*isdB* (Table 2.3). The upstream fragment was digested with *Pst*I and *Bam*HI while the downstream fragment was digested with *Bam*HI and *Xba*I. When the two fragments were ligated at the *Bam*HI site a + 1 frameshift was created. The ligated fragments were cloned between *Pst*I and *Xba*I sites of plasmid pBluescript II-SK and transformed into *E. coli* strain XL1-Blue. Transformants were selected on L plates containing ampicillin. DNA sequencing was used to confirm successful cloning and the resulting plasmid was named pBlueΔ*isdB* (Figure 5.5). Restriction mapping demonstrated that linearization of plasmids pBluescript II-SK and

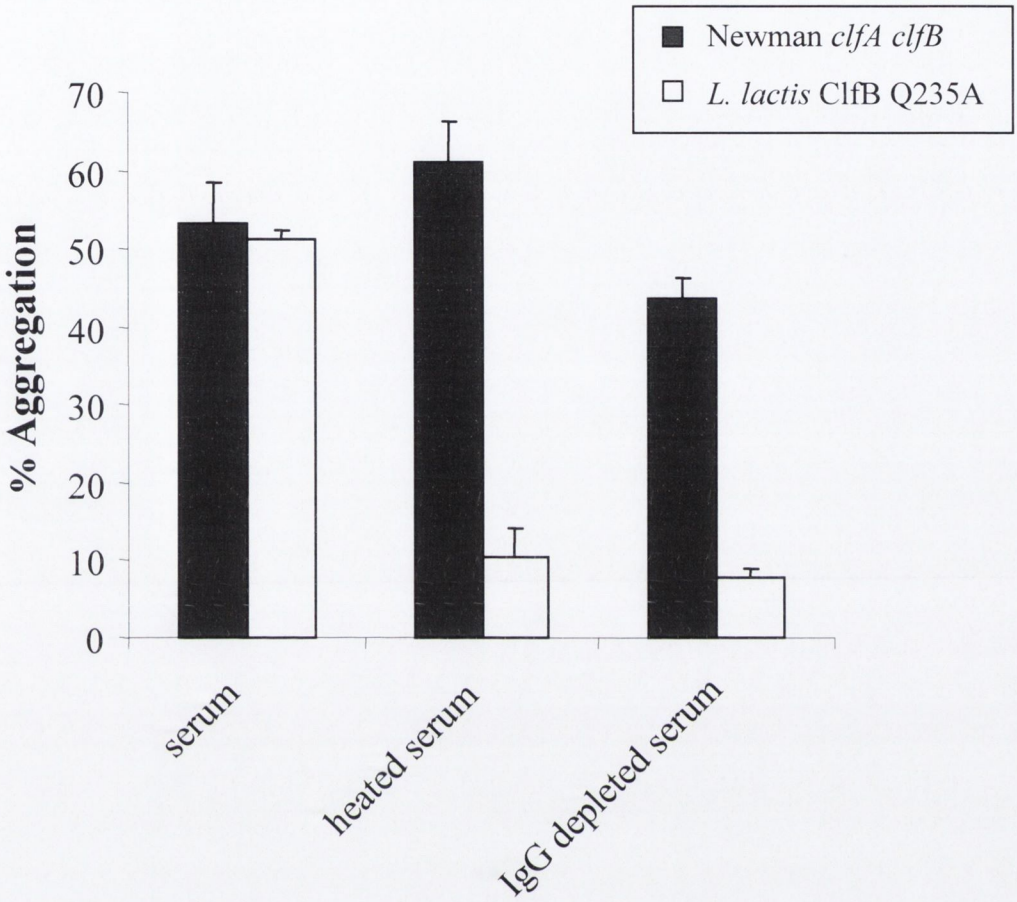


Figure 5.3. Role of complement and IgG in platelet aggregation mediated by Newman *clfA clfB* grown in RPMI

Strains *L. lactis* ClfB Q235A (grown with 3.2 ng/ml nisin) and Newman *clfA clfB* (grown in RPMI media) were washed and adjusted to an OD_{600} of 1.6. Washed bacterial cells were added to GFP supplemented with fibrinogen and 10% human serum. Serum samples were heated to 56°C for 30 min or depleted of IgG by passage over protein A coupled sepharose. The experiment was carried out 3 times using 3 different blood donors. Results represent the mean ± SD of light transmission (percentage aggregation) observed after 25 min.

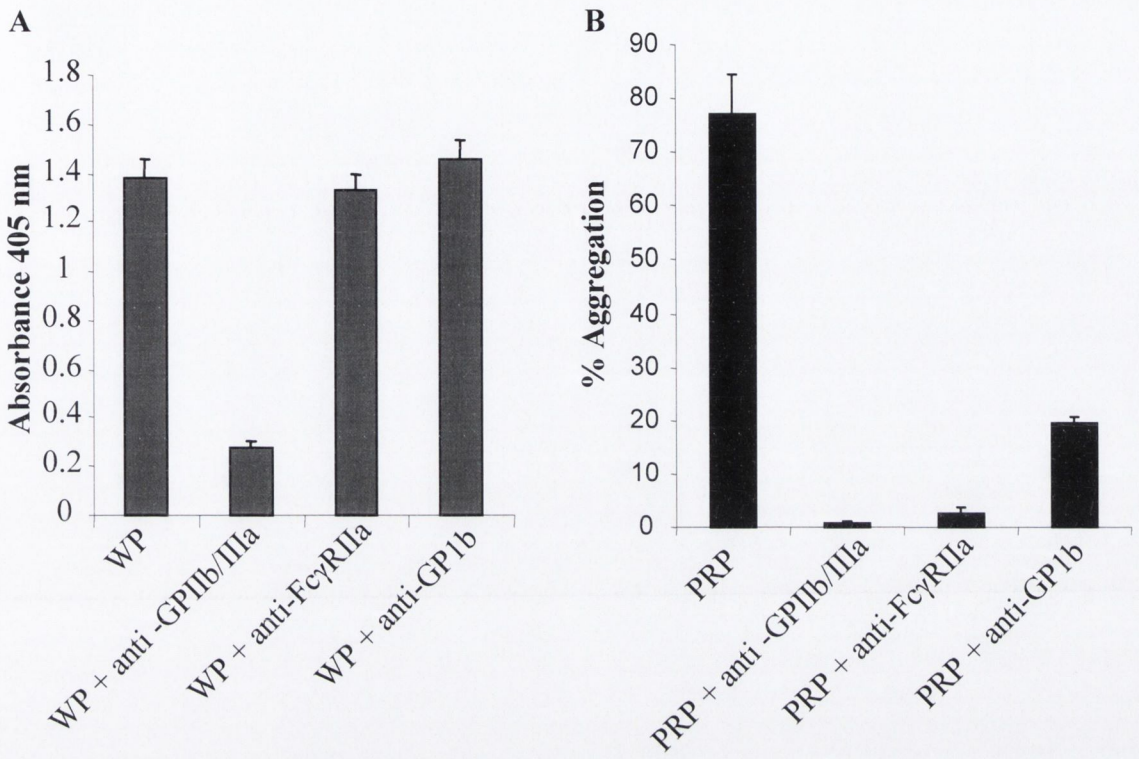


Figure 5.4. Effect of platelet receptor inhibitors on activation and adhesion mediated by RPMI-grown Newman *clfA clfB*

(A) WP were incubated for 5 min at 37°C with inhibitory antibodies to platelet receptors and added to microtitre plates coated with RPMI-grown Newman *clfA clfB.* Adherent platelets were lysed and a substrate for the intracellular enzyme acid phosphatase was added to microtitre wells. Results represent the mean ± SD of adherence at 405 nm as measured by an ELISA plate reader. (B) PRP was incubated with inhibitory antibodies as described above. Washed Newman *clfA clfB* cells grown in RPMI were adjusted to an OD_{600} 1.6 and added to PRP. Results represent the mean ± SD of light transmission (percentage aggregation) observed after 25 min. The experiments described above were carried out 3 times using 3 different blood donors.

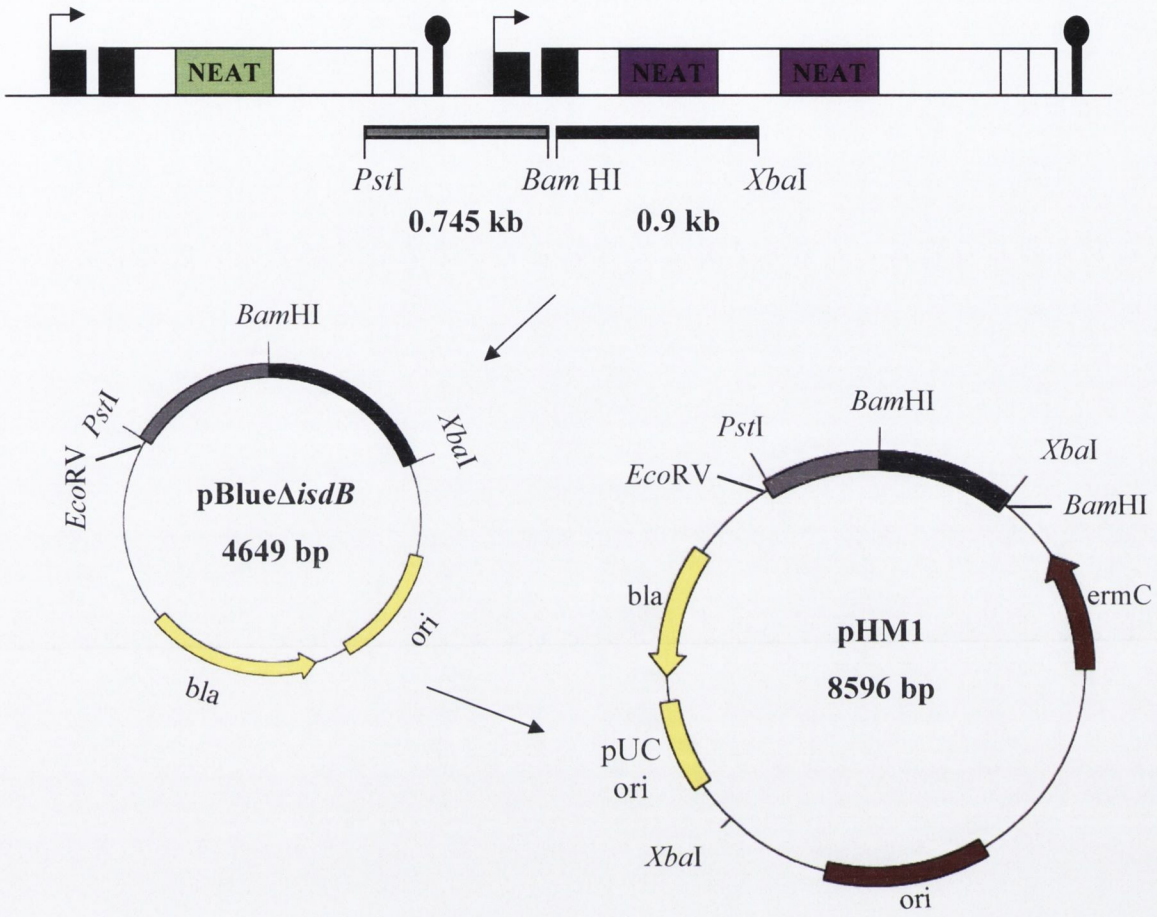


Figure 5.5. Construction of plasmids pBlueΔ*isdB* and pHM1

Fragments upstream (745 bp) and downstream (904 bp) of the *isdB* signal sequence were amplified from the genomic DNA of strain Newman. 5 bases (10-14) within the signal sequence were removed and replaced with the 6 bases of the *Bam*HI restriction site by ligation of the amplified fragments. The ligated upstream and downstream fragments were cloned in-between *Pst*I and *Xba*I sites in pBluescript II-SK to make pBlueΔ*isdB*. Plasmid pBlueΔ*isdB* was digested with *Xba*I and ligated with the *Xba*I digested temperature sensitive plasmid pTSermC. The resulting plasmid pHM1 could replicate in both *E. coli* and *S. aureus* and was used in subsequent allelic replacement mutagenesis of *isdB*.

pBlueΔ*isdB* by digestion with *Pst*I resulted in bands of 3 kb and 4.6 kb respectively. This increase in size corresponds to the combined upstream and downstream fragments. The 745 bp upstream and 904 downstream fragments could be dropped out of pBlueΔ*isdB* by double digestion with *Pst*I and *Bam*HI or *Bam*HI and *Xba*I, respectively (Figure 5.6.A).

Plasmid pBlueΔ*isdB* was digested with *Xba*I and treated with alkaline shrimp phosphatase (Roche) to minimize vector re-ligation. Plasmid pTSermC was ethanol precipitated prior to digestion with *Xba*I. The pTSermC and pBlueΔisdB plasmids were ligated and transformed into *E. coli* XL1-Blue. Transformants were selected on L agar containing ampicillin. Colonies were re-plated on L agar containing erythromycin and those that grew were screened by restriction mapping and the resulting plasmid was named pHM1 (Figure 5.5). Linearization of plasmids pBlueΔ*isdB* and pHM1 with *Eco*RV resulted in bands of 4.6 and 8.6 kb respectively. The size increase corresponds to the 3.9 kb of pTSermC. Digestion of pHM1 with *Bam*HI drops out a 904 bp fragment corresponding to the amplified downstream *isdB* fragment (Figure 5.6.B).

5.2.4.2 Allelic replacement of *isdB* in *S. aureus*

The plasmid pHM1 was electroporated into *S. aureus* RN4220 and transformants were selected for on TSA plates containing erythromycin at 28 °C. The plasmid was transduced into strains Newman and Newman *clfA* and Newman *clfA isdA* using phage 85 and grown on TSA plates containing erythromycin at 28 °C.

Temperature sensitive plasmids have replicons derived from pE194 or pT181. These can replicate in *S. aureus* at 28 °C, but not at a higher, restrictive temperature (44 °C in this study). The pHM1 plasmid was shown to be temperature sensitive by a 10^{-3}-fold lower colony count on selective agar when plates were incubated at the restrictive temperature of 44 °C. Colonies that grew at the restrictive temperature contained a copy of the plasmid integrated into the chromosomal *isdB* gene, as indicated in Figure 5.7. Plasmid excision via a second recombination event was encouraged by growth of single integrants without selection at the permissive temperature (28 °C) for replication. Rolling circle replication of chromosomally integrated plasmids is believed to stimulate plasmid excision. Loss of excised plasmids was subsequently encouraged by dilution in drug-free broth and growth at the restrictive temperature. This process was repeated twice to enrich for plasmid-free

excisants. Finally the cultures were diluted, grown on drug-free agar and screened for loss of resistance to erythromycin. The desired outcome was the retention of the mutation on the chromosome and the excision and loss of the temperature sensitive plasmid (Figure 5.7).

PCR was carried out to detect a novel *Bam*HI site which should be present in the *isdB* gene of strains containing the desired mutation. Primers FPisdB and RXisdB were used to amplify 1.6 kb of the genomic DNA of Newman, Newman *isdB*, Newman *clfA isdB* and Newman *clfA isdA isdB*. As a positive control the same primers were used to amplify 1.6 kb from the pHM1 plasmid. The PCR products were digested with *Bam*HI. A novel BamHI site was present in some of the amplimers from each strain of Newman *isdB*, Newman *clfA isdB* and Newman *clfA isdA isdB*. When digested with *Bam*HI the amplified 1.6 kb from the genomic DNA of these strains was fragmented into 750 and 904 bp bands (Figure 5.8). The 1.6 kb PCR product amplified from the negative control, Newman genomic DNA was not altered in size when digested with *Bam*HI (Figure 5.8).

5.2.5 Construction of *S. aureus* strains for platelet studies

Removal of 5 bases (10-14) of *isdB* and their replacement with 6 bases of *Bam*HI restriction site (GGATCC) caused a +1 frameshift that introduced a premature stop codon 9 amino acids into the signal sequence of isdB. This resulted in the translation of a truncated protein. Since the *isdB* null mutant was not marked with antibiotic resistance it could be combined with other mutations affecting *S. aureus* surface proteins. Strains were constructed which lacked individual Isd proteins as well as the known pro-aggregatory surface proteins ClfA and ClfB. Initially unmarked mutations were introduced into strain Newman where possible and later combined with mutations marked by antibiotic resistance insertions. It this way it would be possible to combine multiple *isd* mutations, if platelet aggregation was unaffected by single *isd* mutants.

5.2.5.1 Newman *clfA clfB isdA*

The *clfA::*Em^R mutation was introduced into strain Newman *isdA* by phage transduction with phage 85 (section 2.5). The resulting strain, Newman *clfA isdA* did not have clumping activity in the fibrinogen clumping test described in section 2.12. Phage 85 was used to introduce the *clfB::*Tc^R mutation into strain Newman *clfA isdA*.

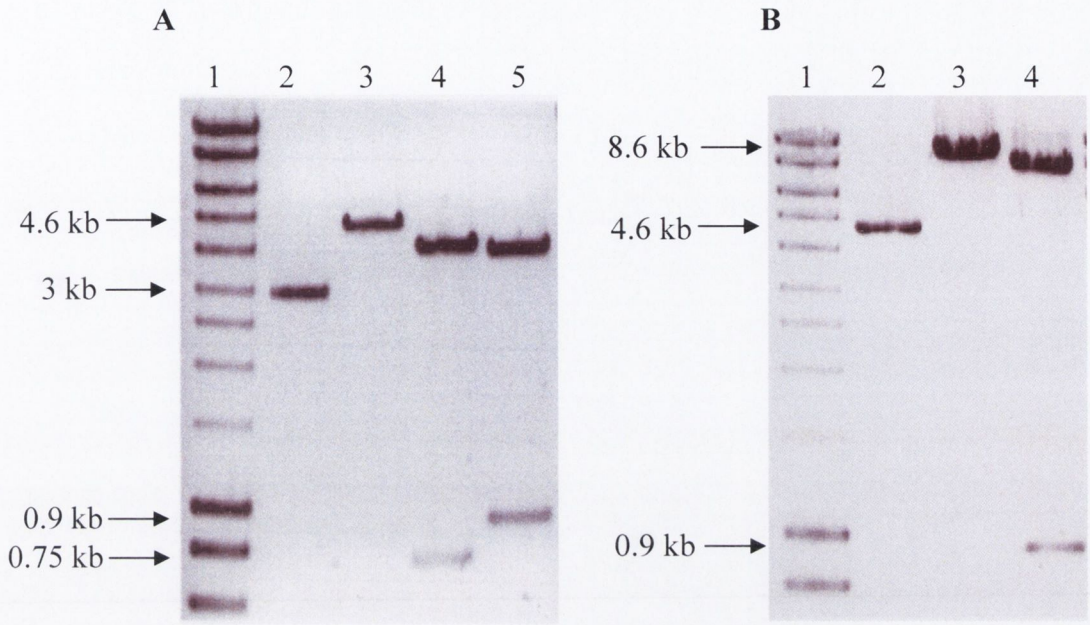


Figure 5.6. Restriction mapping of plasmids pBlueΔ*isdB* and pHM1

A. Agarose gel of restriction digests of pBlueΔ*isdB*. Lane 1, 10kb ladder Bioline; Lane 2, *Pst*I digest of pBluescript II-SK; Lane 3, *Pst*I digest of pBlueΔ*isdB*; Lane 4, *Pst*I and *Bam*HI digest of pBlueΔ*isdB*; Lane 5, *Bam*HI and *Xba*I digest of pBlueΔ*isdB*.

B. Agarose gel of restriction digests of pHM1. Lane 1, 10kb ladder Bioline; Lane 2 *Eco*RV digest of pBlueΔ*isdB*; Lane 3, *Eco*RV digest of pHM1; Lane 4, *Bam*HI digest of pHM1.

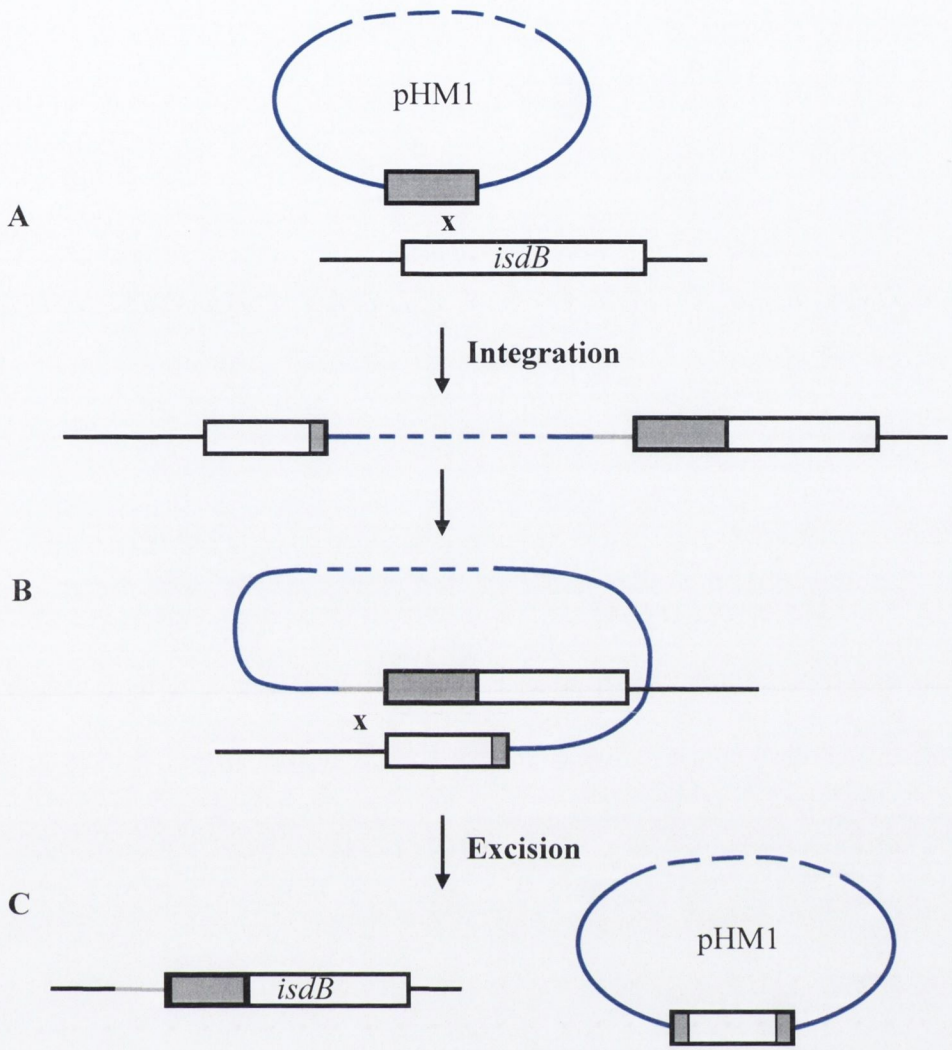


Figure 5.7. Allelic replacement mutagenesis of *isdB*

(A) The temperature-sensitive plasmid pHM1 integrated into the chromosome within the region of homology in *isdB* following growth at a restrictive temperature. (B) The plasmid was induced to excise at a temperature permissive for replication by a second cross-over event. (C). Excision resulted in the retention of the mutated allele on the chromosome. After excision the plasmid was lost by growth in non-selective media at 28°C.

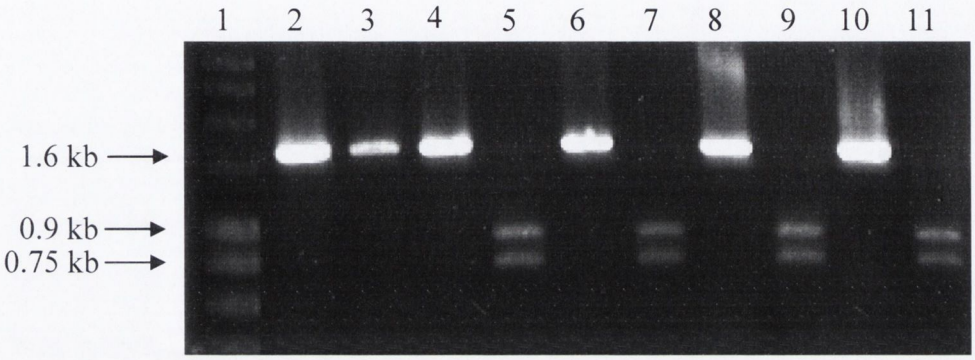


Figure 5.8. Screening for novel *Bam*HI site in the signal sequence of *isdB* mutants

Diagnostic PCR and restriction digestion of fragments amplified from Newman *isdB*. Following PCR with primers FPisdB and RXisdB products were digested with *Bam*HI. Lane 1, 10kb ladder Bioline; Lane 2, Newman PCR product; Lane 3, *Bam*HI digested Newman PCR product; Lane 4, pBlueΔ*isdB* PCR product; Lane 5, *Bam*HI digested pBlueΔ*isdB* PCR product ; Lane 6, Newman *isdB* PCR product; Lane 7, *Bam*HI digested Newman *isdB* PCR product; Lane 8, Newman *clfA isdB* PCR product; Lane 9, *Bam*HI digested Newman *clfA isdB* PCR product; Lane 10, Newman clfA isdA *isdB* PCR product; Lane 11, *Bam*HI digested Newman clfA isdA *isdB* PCR product.

Strain Newman *clfA clfB isdA* was screened by Western immunoblotting. ClfA, ClfB and IsdA were not detected in cell wall extracts of Newman *clfA clfB isdA* (Figure 5.9.A and B). IsdB was expressed from this strain although Western immunoblots consistently showed a lower level of IsdB expression from IsdA negative Newman strains (Figure 5.9.A). This difference was not apparent when strains Newman *clfA clfB* and Newman *clfA clfB isdA* were compared by whole cell immunoblotting (Figure 5.10). However, the polyclonal rabbit anti-IsdB antibody used in this study cross-reacted with several proteins larger than IsdB (approximately 150 and 200 kDa) which may mask differences in expression of IsdB when examined by whole cell immunoblotting (Figure 5.9.A). IsdH expression was detected from strains Newman *clfA clfB* and Newman *clfA clfB isdA* by western immunoblotting (Figure 5.9.A).

5.2.5.2 Newman *clfA clfB isdB*

As described in section 5.2.4.2 an *isdB* frameshift mutation was introduced into strain Newman *clfA*. Phage 85 was utilised to introduce a *clfB::*Em^R mutation into strain Newman *clfA isdB*.

The resulting strain Newman *clfA clfB isdB* was screened by Western immunoblotting. ClfA, ClfB and IsdB proteins could not be detected in the cell wall extracts of Newman *clfA clfB isdB* as expected (Figures 5.9A and B). The other Isd proteins of interest, IsdA and IsdH were expressed from this strain and were detected as bands of 38 kDa and 150 kDa respectively on Western immunoblots (Figure 5.9.A). Strains Newman *clfA clfB* and Newman *clfA clfB isdB* expressed similar levels of IsdA on the cell wall when compared by whole cell immunoblotting (Figure 5.11).

5.2.5.3 Newman *clfA clfB isdA isdB*

Temperature sensitive plasmid pJH1 containing a construct with a +1 frameshift in *clfA* was introduced into strain Newman *isdA*. Allelic replacement mutagenesis was carried out as described in section 5.2.4.2. Resulting colonies were screened for loss antibiotic resistance and loss of clumping activity in the fibrinogen clumping test. Temperature sensitive plasmid pHM1 containing construct with a +1 frameshift in *isdB* was introduced into the resulting strain Newman *clfA isdA* as described in section 5.2.4.2. Allelic replacement mutagenesis was carried out to produce Newman *clfA*

isdA isdB. Phage 85 was subsequently utilised to introduce the *clfB::*Em^R mutation into this strain.

The resulting strain Newman *clfA clfB isdA isdB* was screened by Western immunoblotting. ClfA, ClfB, IsdA and IsdB were not detected in the cell wall extracts of strain Newman *clfA clfB isdA isdB* (Figures 5.9.A and 5.9.B). IsdH expression was detected from this strain by western immunoblotting (Figure 5.9.A).

5.2.5.4 Newman *clfA clfB isdH*

Phage 85 was used to introduce the *isdH::*Em^R mutation into strain Newman *clfA*. The *clfB::*Tc^R mutation was introduced into the resulting strain Newman *clfA isdH*.

Strain Newman *clfA clfB isdH* was screened by Western immunobloting. ClfA, ClfB and IsdH could not be detected in the cell wall extracts of this strain (Figures 5.9.A and B). IsdA and IsdB were expressed from Newman *clfA clfB isdH*.

5.2.6 Platelet adhesion and aggregation mediated by Newman *isd* mutants

Bacterial strains were grown in RPMI medium to the stationary phase of growth, washed and normalised to an OD_{600} of 1. Washed bacterial cells were incubated in microtitre plates at 4°C for 16 h. The adherence of WP to bacterial cells coating microtitre wells was measured as described in section 2. WP adhered avidly to strain Newman *clfA clfB*. Mutations in either *isdA* or *isdH* had little or no effect on adhesion of platelets (Figure 5.12). Strain Newman *clfA clfB isdB* was unable to promote adherence of WP. Eliminating IsdA from this strain did not significantly lower adhesion (Figure 5.12). These results identify IsdB as the protein responsible for direct adhesion of bacteria to platelets under iron limiting conditions.

Newman *clfA clfB* strains lacking either IsdA or IsdH were able to promote activation and aggregation of platelets with no significant differences in percentage aggregation or lag time to activation ($p=0.46$ and $p=0.68$, respectively). Loss of IsdB resulted in strains which did not stimulate platelet activation (Figure 5.13). The Newman *clfA clfB* strain lacking both IsdB and IsdA was also unable to support platelet activation. It seems that IsdB is crucial for both adhesion to and activation of platelets by *S. aureus* grown under iron limiting conditions.

5.2.7 Plasma proteins required for platelet activation mediated by IsdB

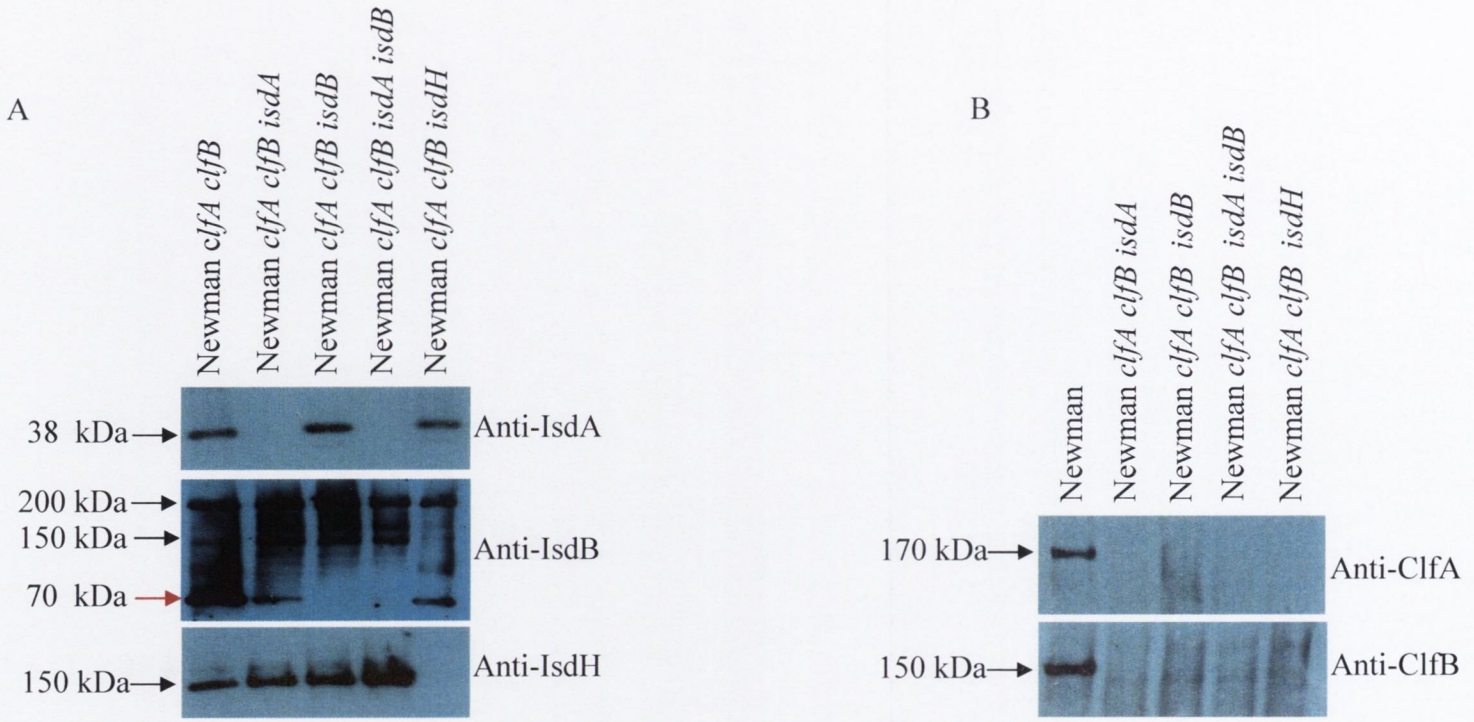


Figure 5.9. Screening of *S. aureus* Newman strains by western immonoblotting

Newman strains were grown to stationary phase in RPMI. Cell wall proteins were solubilised by lysostaphin digestion, separated on 7.5% SDS PAGE gels and electroblotted onto PVDF membranes. Membranes were probed with (A) polyclonal anti-IsdA, anti-IsdB and anti-IsdH or (B) polyclonal anti-ClfA and anti-ClfB antibody. Bound primary antibodies were detected with HRP-conjugated protein A-peroxidase. Membranes were developed by chemiluminescence and exposed to autoradiographic film. Anti-IsdB antibodies cross-react with several other surface proteins. The 70 kDa IsdB protein is indicated with a red arrow.

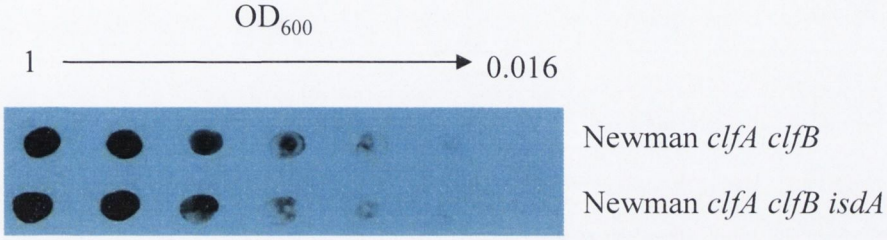


Figure 5.10. Expression of IsdB from Newman *clfA clfB isdA*

Newman strains were grown to the stationary phase in RPMI medium. Bacterial cells were washed and doubling dilutions were dotted onto nitrocellulose membrane. Membranes were probed with polyclonal anti-IsdB antibody. Bound antibody was detected with HRP-conjugated protein A-peroxidase. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

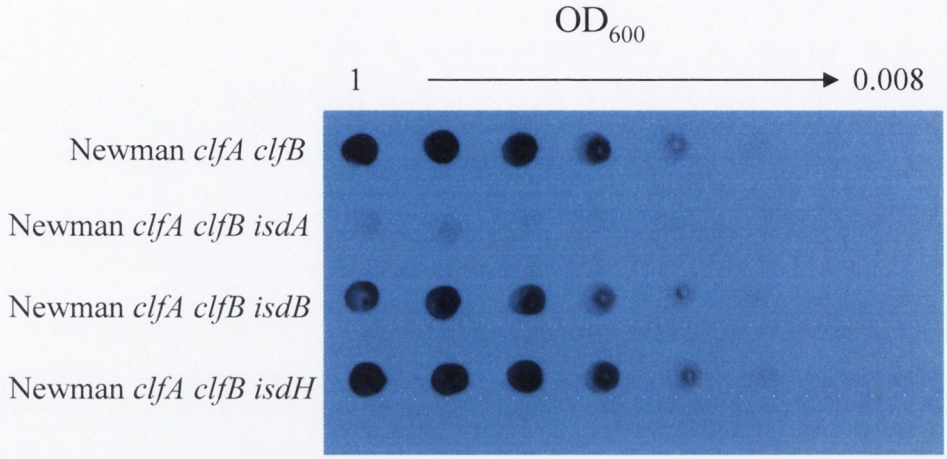


Figure 5.11. Expression of IsdA from Newman *isdB* and *isdH* mutants

Newman strains were grown to the stationary phase of growth in RPMI medium. Bacterial cells were washed and doubling dilutions were dotted onto nitrocellulose membrane. Membranes were probed with polyclonal anti-isdA and HRP-conjugated protein A-peroxidase. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

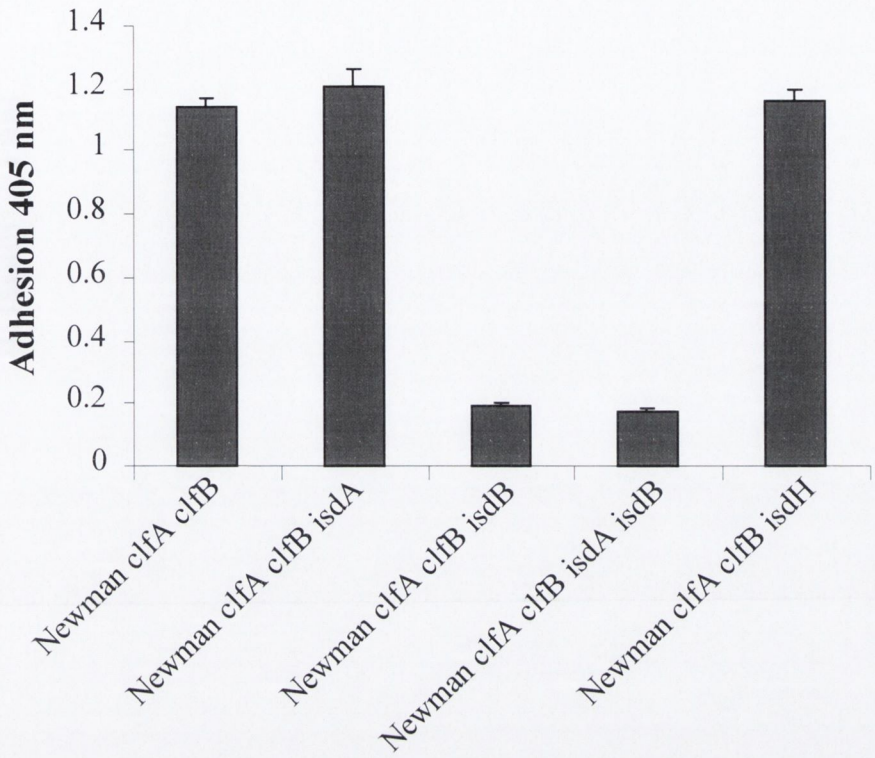


Figure 5.12. Adhesion of washed platelets to Newman strains lacking Isd proteins

Newman strains were grown in RPMI medium to the stationary growth phase. Washed bacterial cells (OD_{600} of 1) were incubated on microtitre plates at 4°C for 16 h. WP were added to the microtitre wells. Adherent platelets were lysed and a substrate for the intracellular enzyme acid phosphatase was added to each microtitre well. The experiment was carried out 3 times using 3 different blood donors. Results represent the mean ± SD of adherence at 405 nm as measured by an ELISA plate reader.

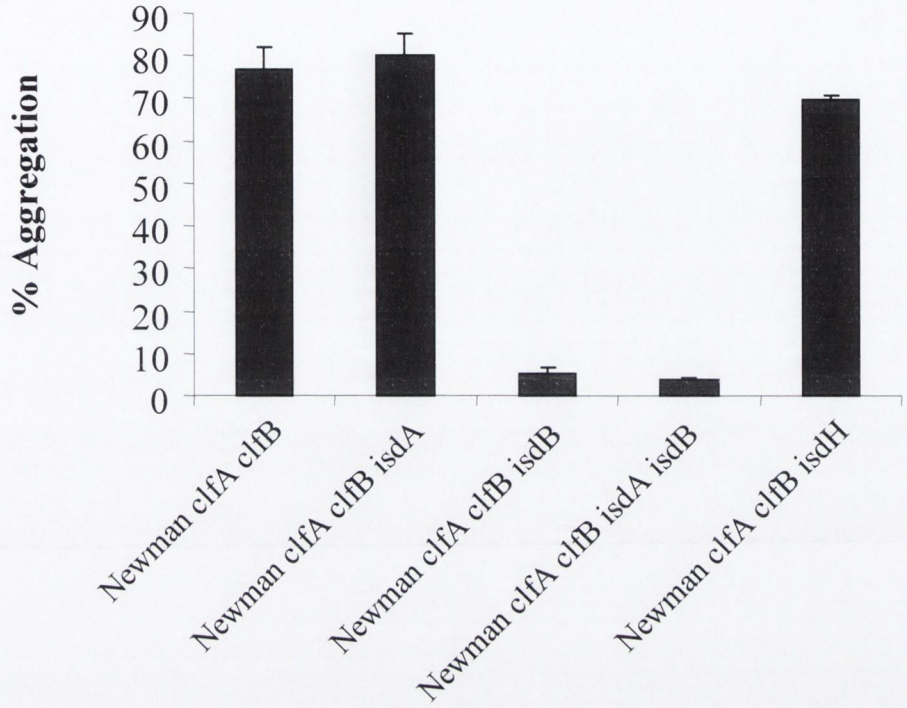


Figure 5.13. Aggregation of platelets by Newman strains lacking Isd proteins

Newman strains were grown in RPMI medium to the stationary phase of growth. Washed bacterial cells were adjusted to an OD_{600} of 1.6 and added to PRP. The experiment was carried out 3 times using 3 different blood donors. Results represent the mean ± SD of light transmission (percentage aggregation) observed after 25 min

Strains Newman *clfA clfB* and Newman *clfA clfB isdB* were grown in RPMI to the stationary phase of growth, washed and resuspended to an OD_{600} of 1.6. Bacterial cells were added to GFP supplemented with purified fibrinogen lacking contaminating IgG present in commercial supplies (see section 2.18). Strain Newman expressing IsdB was able to stimulate aggregation of GFP supplemented with fibrinogen alone (Figure 5.14). The addition of exogenous IgG seemed to decrease the mean lag time to activation from 4.33 min to 2.33 min. This difference was not found to be statistically significant ($p = 0.121$) due to considerable variations in lag time between donors. Strain Newman *clfA clfB isdB* was unable to cause activation of GFP supplemented with fibrinogen alone or fibrinogen and IgG together.

5.2.8 Construction of plasmids pCU1 *isdB* and pCU1 *isdAB*

Primers p*CisdB*F and p*CisdB*R were used to amplify the *isdB* coding sequence including the upstream promoter and Fur consensus sequence from strain Newman genomic DNA. The *isdB* coding sequence is located 203 bp downstream of the *isdA* coding sequence on the *S. aureus* chromosome. Primers p*CisdA*F and p*CisdB*R were used to amplify *isdA* and *isdB* including the upstream promoter and Fur box of both genes. The 2.3 kb *isdB* and 3.6 kb *isdAB* coding sequence was cloned between the *Pst*I and *Xba*I sites of plasmid pCU1 (Figure 5.15A). Plasmids pCU1 *isdB* and pCU1 *isdAB* were sequenced and screened by restriction mapping.

Digests of plasmids pCU1, pCU1 *isdB* and pCU1 *isdAB* with *Pst*I and *Xba*I were carried out. Digestion of pCU1 with *Pst*I and *Xba*I produced a 4.9 kb band (Figure 5.15B). Plasmids pCU1 *isdB* and pCU1 *isdAB* digested with the same enzymes produced bands of 4.9 kb and additional 2.3 kb and 3.6 kb bands respectively (Figure 5.15B).

5.2.9 Expression of IsdA and IsdB from plasmid pCU1 and interaction with platelets

Plasmids pCU1 and pCU1 *isdAB* were transduced into strain Newman *clfA clfB isdA isdB* using phage 85. Plasmid pCU1 *isdB* was introduced by phage transduction into strain Newman *clfA clfB isdB*. Western immunoblots and whole cell immunoblots were carried out to confirm expression of Isd proteins.

IsdA could be detected as a band of 38 kDa (Figure 5.16.A) in the cell wall extract of strains Newman *clfA clfB* and Newman *clfA clfB isdA isdB* (pCU1 *isdAB*). This

correlates with the reported full length form of this protein. IsdA was not detected in the cell wall extract of strain Newman *clfA clfB isdA isdB* (pCU1). Whole cell immunoblots demonstrated that IsdA is expressed from the pCU1 *isdAB* plasmid at wild-type levels (Figure 5.16.B).

IsdB could be detected as a 70 kDa band (Figure 5.16.C) in the cell wall extract of Newman *clfA clfB*, Newman *clfA clfB isdB* (pCU1 *isdB*) and Newman *clfA clfB isdA isdB* (pCU1 *isdAB*). This is the expected full length form of this protein. Whole cell immunoblots showed approximately equal IsdB expression from wild-type and pCU1 *isdB* and pCU1 *isdAB* complemented strains (Figure 5.16.D).

The defect in adhesion of WP to Newman *clfA clfB isdB* was partially restored by expression of IsdB from pCU1 *isdB* (Figure 5.17.A). A 3-fold increase in adhesion was seen when compared to strain Newman *clfA clfB isdA isdB* containing the empty pCU1 plasmid. However, expressing IsdB from pCU1 promoted 2-fold lower adhesion ($p = 0.0032$) of platelets than when IsdB was expressed from the *isdB* gene in Newman *clfA clfB*. When IsdA and IsdB were expressed together from the pCU1 plasmid adhesion of WP to strain Newman *clfA clfB isdA isdB* was fully restored to wild-type levels.

Activation of platelets could not be restored by expression of IsdA and IsdB from the pCU1 plasmid (Figure 5.17.B). The percentage aggregation was not significantly different in Newman *clfA clfB isdA isdB* containing the empty pCU1 plasmid and the strains complemented with IsdB alone or IsdA and IsdB together. The Isd proteins are part of a complex iron uptake system. The inability to complement the capacity of the strains described to fully adhere or activate platelets may be a result of disrupting the organisation of these proteins on the cell wall surface.

5.2.10 Expression of IsdB from *S. epidermidis*

The Gram positive bacterium *S. epidermidis* does not express Isd proteins. Plasmids pCU1 and pCU1 *isdB* were electroporated into *S. epidermidis* strain TU3298 as described in section 2.4.2. Western immonoblots were carried out to detect expression of IsdB from the pCU1 plasmid in *S. epidermidis*. IsdB was present as a 70 kDa band (Figure 5.18.A) in the cell wall extracts of Newman *clfA clfB* and *S. epidermidis* (pCU1 *isdB*). IsdB was not detected in cell wall extracts of strain *S. epidermidis* (pCU1) as expected. Whole cell immunoblots were carried out to

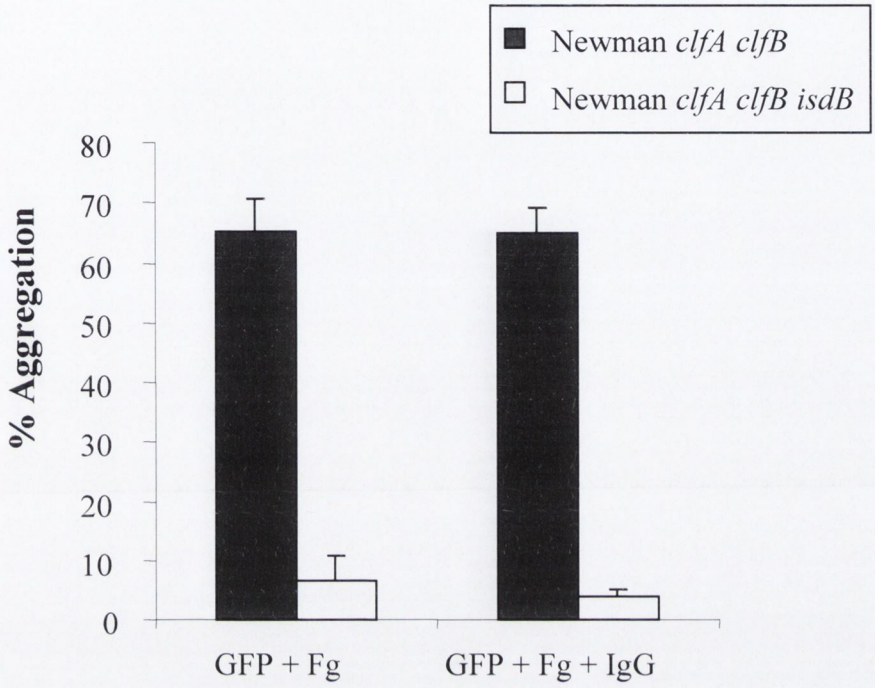


Figure 5.14. Plasma proteins required for IsdB mediated platelet aggregation
Newman strains were grown in RPMI medium to the stationary growth phase. Washed bacterial cells (OD_{600} of 1.6) were added to GFP substituted with 1 mg/ml fibrinogen. Pooled human IgG was added to GFP and fibrinogen as indicated. The experiment was carried out 3 times using 3 different blood donors. Results represent the mean ± SD of light transmission (percentage aggregation) observed after 25 min

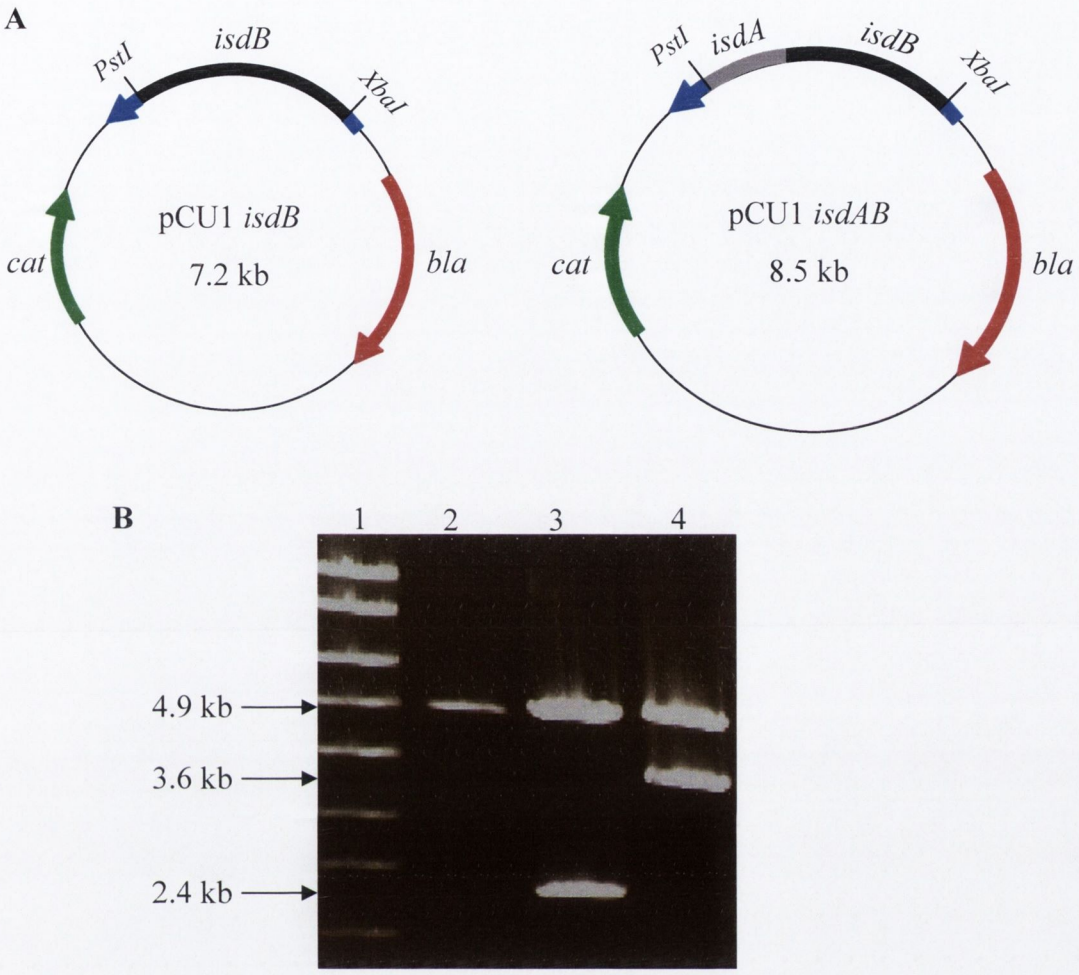


Figure 5.15. Construction of plasmids pCU1 *isdB* and pCU1 *isdAB*

(A) Plamid maps of pCU1 *isdB* and pCU1 *isdAB.* The 2.3 kb *isdB* and 3.6 kb *isdAB* coding sequence was cloned between *PstI* and *XbaI* sites within the *lacZ* gene of pCU1. The *bla* gene confers ampicillin resistance in *E. coli* while the *cat* gene confers chloramphenicol resistance in *S. aureus.* (B) Agarose gel of restriction digests of pCU1 *isdB* and pCU1 *isdAB*. Lane 1, 10kb ladder Bioline; Lane 2, *Pst*I and *Xba*I digest of pCU1; Lane 3, *Pst*I and *Xba*I digest of pCU1*isdB*; Lane 4, *Pst*I and *Xba*I digest of pCU1 *isdAB.*

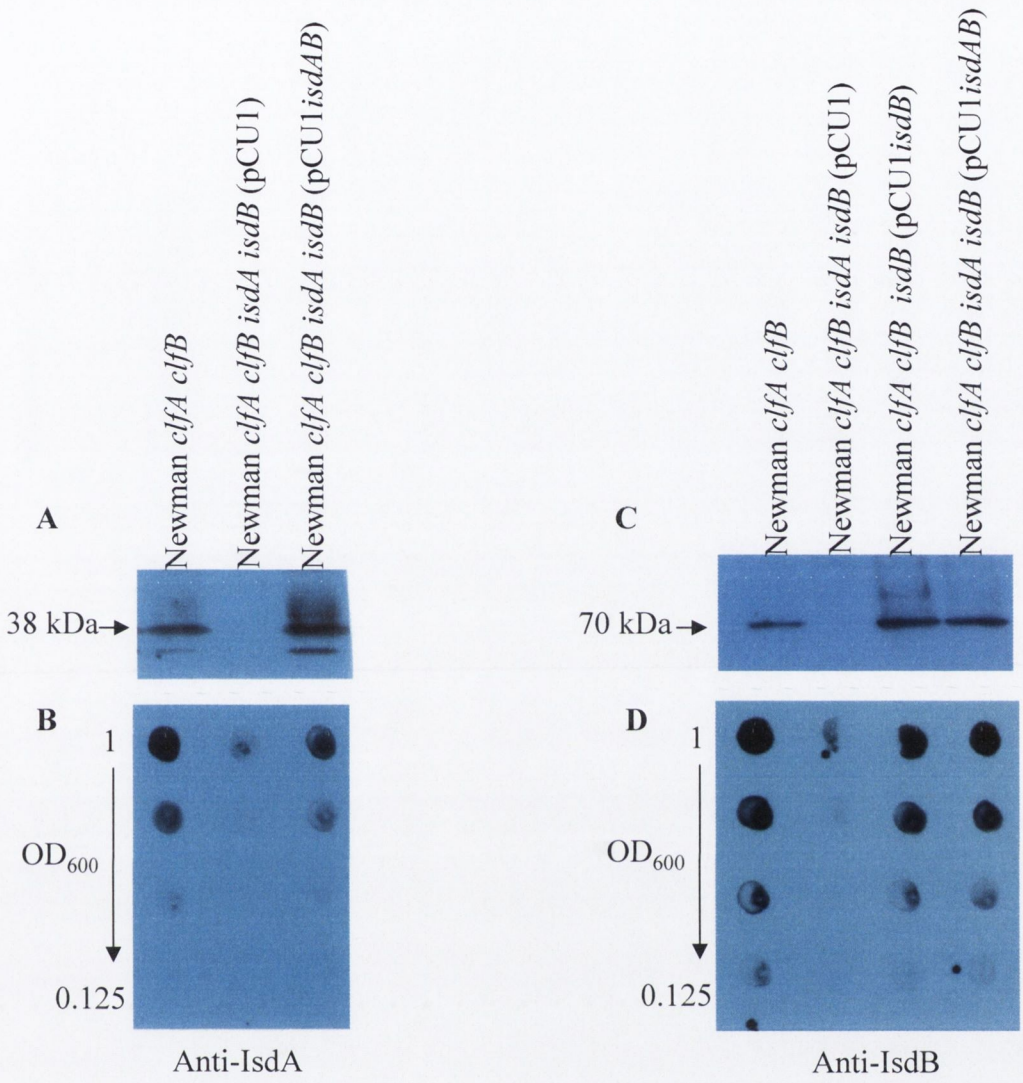


Figure 5.16. Expression of IsdA and IsdB from pCU1 in Newman strains

Newman strains were grown to the stationary phase in RPMI medium. (A and C) Cell wall proteins were solubilised by lysostaphin digestion, separated on 7.5% SDS PAGE gels and electroblotted onto PVDF membranes. (B and D) Bacterial cells were washed and doubling dilutions were dotted onto nitrocellulose membrane. Membranes were probed with (A and B) polyclonal anti-IsdA (C and D) polyclonal anti-IsdB. Bound antibodies were detected with HRP-conjugated protein A-peroxidase. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

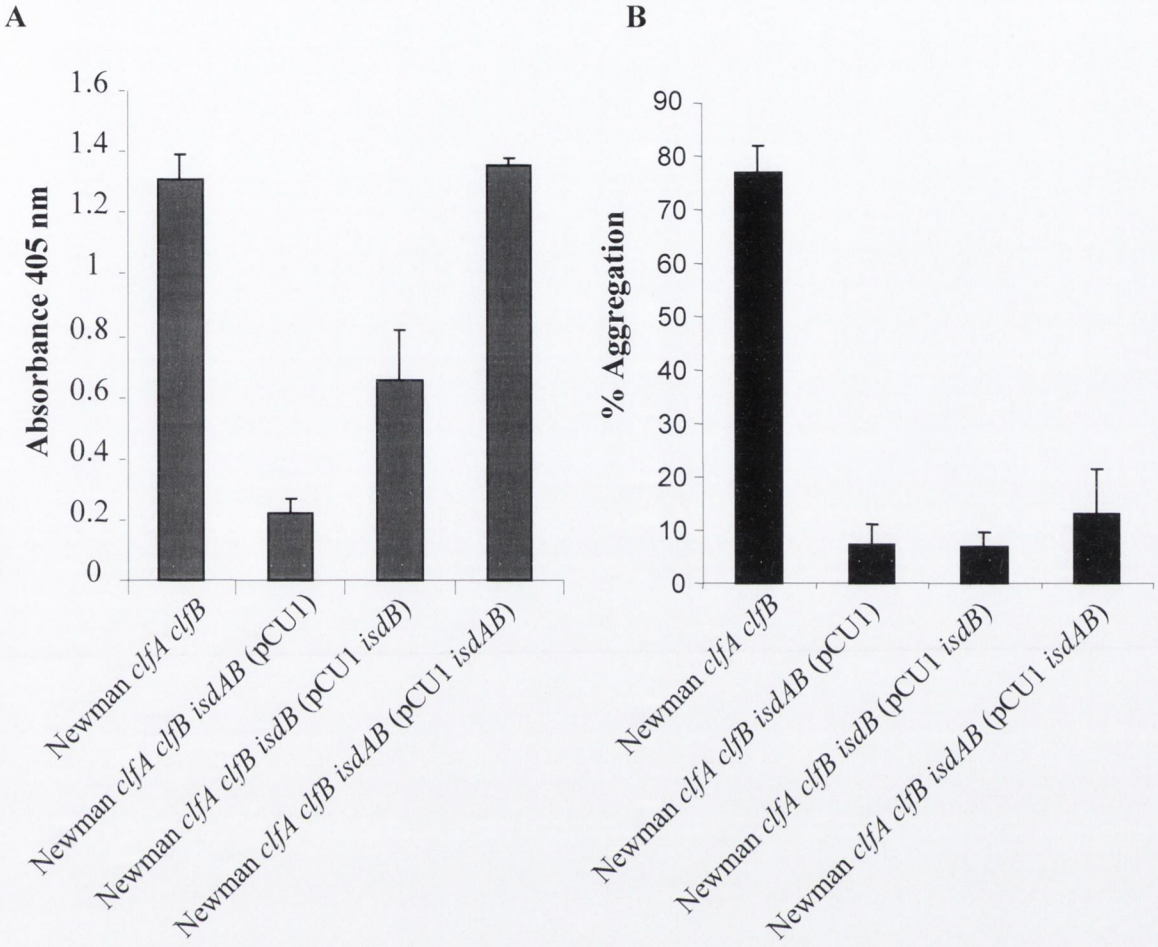


Figure 5.17. Complementation of Isd mutations in *S. aurues* Newman strains

RPMI grown Newman *clfA clfB* and pCU1 complemented *isd* mutants were (A) coated on microtitre plates and subsequently incubated with WP (B) incubated with PRP in an aggregometer for 25 min. (A) Adherent platelets were lysed and a substrate for the intracellular enzyme acid phosphatase was added. Results represent the mean ± SD of adherence at 405 nm as measured by an ELISA plate reader. (B) Results represent the mean ± SD of light transmission (percentage aggregation) observed after 25 min. The experiments described above were carried out 3 times using 3 different blood donors.

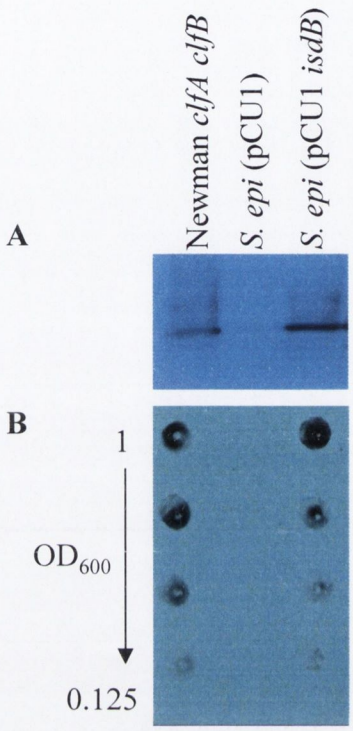


Figure 5.18. Expression of IsdB from *S. epidermidis*

Newman *clfA clfB, S. epidermidis* (pCU1) and *S. epidermidis* (pCU1 *isdB*) were grown to the stationary phase of growth in RPMI medium. (A) Cell wall proteins were solubilised by lysostaphin digestion, separated on 7.5% SDS PAGE gels and electroblotted onto PVDF membranes. (B) Bacterial cells were washed and doubling dilutions were dotted onto nitrocellulose membrane. Membranes were probed with polyclonal anti-isdB and HRP-conjugated protein A-peroxidase. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

compare expression of IsdB from Newman *clfA clfB* and *S. epidermidis* (pCU1 *isdB*). Expression of IsdB from these strains appeared similar (Figure 5.18.B).

S. epidermidis (pCU1) and *S. epidermidis* (pCU1 *isdB*) were tested for their ability to support adherence of WP. Platelet adhesion assays were carried out as described in section 2.22. Although a marginal increase ($p = 0.0032$) in adhesion of WP to *S. epidermidis* occurred when IsdB was expressed from this strain, it can not be considered to promote adhesion when compared to Newman *clfA clfB* (Figure 5.19.A). *S. epidermidis* expressing IsdB was also unable to cause platelet activation (Figure 5.19.B).

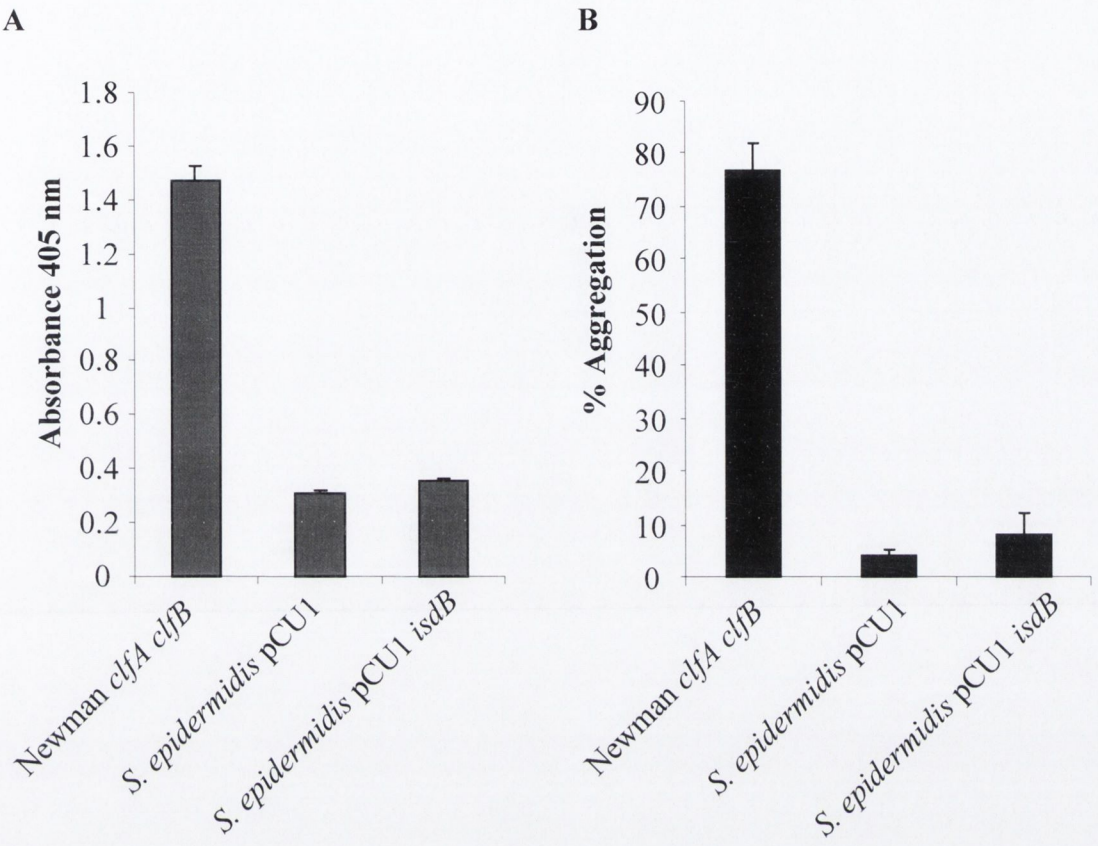


Figure 5.19. Adhesion to and aggregation of platelets by *S. epidermidis* strains

RPMI-grown Newman *clfA clfB, S. epidermidis* (pCU1) and *S. epidermidis* (pCU1 *isdB*) were incubated (A) on microtitre plates for 16 h (B) with PRP in an aggregometer for 25 min. (A) WP were added to microtitre plates coated bacterial cells. Adherent platelets were lysed and a substrate for the intracellular enzyme acid phosphatase was added to microtitre wells. Results represent the mean ± SD of adherence at 405 nm as measured by an ELISA plate reader. (B) Results represent the mean ± SD of light transmission (percentage aggregation) observed after 25 min. The experiments described above were carried out 3 times using 3 different blood donors.

5.3 Discussion

S. aureus bacteraemia can lead to the development of cardiovascular infections such as infective endocarditis (IE). Bacteria circulating in the blood stream are exposed to iron restricted conditions and express a unique array of surface proteins. *S. aureus* surface proteins ClfA, FnBPA, FnBPB and ClfB are known to trigger platelet activation. The studies that identified roles for these proteins in platelet activation were carried out with bacteria grown in complex iron enriched media. This study investigated platelet adhesion and aggregation mediated by bacteria that were grown in an iron deficient medium (RPMI) to mimic more closely the *in vivo* environment that bacteria are exposed to when circulating in the blood stream.

ClfA and ClfB were present on the cell wall of *S. aureus* cells that had been grown in RPMI (see Chapter 6). These two proteins are known to cause both adhesion to and activation of platelets when expressed from cells grown in iron rich media. Under iron restricted growth conditions strain Newman was able to cause platelet activation with a lag time of 4.6 min. Strains expressing high levels of ClfA have shorter lag times to activation (1-2 min; Loughman *et al.*, 2005). Expression studies described in Chapter 6 found a very low level of ClfA on RPMI-grown cells. It has been demonstrated that lag times to aggregation are dependent on expression levels of pro-aggregatory proteins. Therefore, ClfA expressed from RPMI-grown cells may contribute less to platelet aggregation than when expressed at high levels from cells grown in iron rich broth. ClfB is expressed maximally in the exponential growth phase in iron rich media (McAleese *et al.*, 2001). RPMI grown *S. aureus* cells expressed a constant, high level of ClfB in all growth phases tested (see Chapter 6). It therefore seems likely that ClfB makes a bigger contribution to platelet activation in iron limiting conditions. Future studies should eliminate ClfA and ClfB individually from the *S. aureus* cell surface to ascertain their contributions to platelet aggregation in iron limiting conditions. Eliminating ClfA and ClfB together from strain Newman reduced total percentage aggregation and extended lag time to activation slightly (Figure 5.2). The rest of the experiments described by this study were carried out in a ClfA and ClfB defective background so that the contribution of Isd proteins could be studied in isolation. Previous studies carried out with *S. aureus* strains grown in iron rich media have shown that FnBPA and FnBPB are potent platelet activators on cells from the exponential phase of growth (Fitzgerald *et al.*, 2006). The FnBP proteins are not expressed by the strain used in this study due to mutations that result in secretion

of truncated forms of each protein (Grundmeier *et al.*, 2004). It would be interesting to asses the contribution these two proteins make to platelet aggregation mediated by *S. aureus* grown in iron-deficient conditions.

Initial adhesion of bacteria to platelets is required to trigger subsequent platelet activation. *S. aureus* surface proteins ClfA and ClfB mediate adhesion to platelets via a fibrinogen bridge. The results of this study strongly indicate that under iron restricted growth conditions the adhesion of *S. aureus* to platelets is mediated directly by IsdB interacting with platelet receptor GPIIb/IIIa. Bacteria expressing ClfA and ClfB require the addition of fibrinogen to adhere to platelets via GPIIb/IIIa. *S. aureus* strains expressing IsdB adhered to platelets without the addition of fibrinogen (Figure 5.1). A mutant lacking IsdB on the cell wall surface did not support adhesion of platelets (Figure 5.12). Expression of Isd proteins is inhibited from wild-type strains grown in the presence of iron. Platelet adhesion to *S. aureus* cells was inhibited by growth of bacteria in the presence of iron. Therefore direct adhesion to platelets was specific to iron restricted conditions and was dependent on the expression of IsdB. IsdA and IsdH could be eliminated individually without affecting adhesion to platelets indicating that these proteins are not involved in binding (Figure 5.12). A function blocking antibody that binds to platelet receptor GPIIb/IIIa completely inhibited adhesion to platelets. In contrast antibodies to platelet receptors FcγRIIa and GP1b had no effect indicating that they do not play a role in IsdB mediated adhesion to platelets (Figure 5.4.A). Further studies could be carried out to determine if recombinant IsdB can interact with platelets and purified GPIIb/IIIa.

IsdB was also found to play a crucial role in platelet activation and aggregation mediated by RPMI-grown bacteria. The ability of *S. aureus* to activate platelets was eliminated by the addition of iron to the bacterial growth medium which prevents expression of Isd proteins (Figure 5.2). Furthermore Newman strains lacking IsdB were not able to stimulate platelet activation (Figure 5.13).

Strains lacking IsdA seemed to have lower levels of IsdB on their cell surface (Figure 5.9.A). However this had no effect on platelet adhesion or activation. Strain Newman *clfA clfB isdA* was able to cause platelet activation a with similar lag time and percentage aggregation as strain Newman *clfA clfB*. Strains lacking IsdH also activated platelets without significant effects on percentage aggregation or lag time (Figure 5.13). Therefore expression of IsdB from *S. aureus* under iron restrictive conditions appears to be crucial for both adhesion to and activation of platelets.

Platelet studies were also carried out with strains where IsdB was expressed from the multicopy plasmid pCU1. Western and whole cell immunoblots indicated that expression of IsdA and IsdB from plasmids pCU1 *isdB* and pCU1 *isdAB* was similar to wild type levels seen in *S. aureus* (Figure 5.16). Adhesion of platelets to Newman strains lacking IsdB was partially restored by expressing IsdB from pCU1. Platelet adhesion mediated by Newman strains lacking both IsdA and IsdB could be restored fully by expression of IsdA and IsdB together from pCU1 (Figure 5.17.A). This indicates that IsdA may play an accessory role in platelet adhesion while expression of IsdB is crucial for adhesion to platelets.

In contrast activation of platelets could not be restored in strains lacking IsdB by expression of this protein from pCU1. Expression of both IsdA and IsdB from pCU1 also had no effect on activation by Newman strains lacking IsdA and IsdB (Figure 5.17.B). Introducing pCU1 *isdB* into *S. epidermidis* marginally increased adhesion. However activation of platelets was not supported by this strain (Figure 5.19). The level of IsdB expression from *S. epidermidis* was similar to that seen in *S. aureus* when compared by Western and whole cell immunoblotting (Figure 5.18). Previous studies carried out in this laboratory have found that complementing the phenotypes of the iron-regulated IsdH protein was also problematic (Visai *et al.*, 2008). Together the Isd proteins of *S. aureus* are involved in haem acquisition and transport of iron through the cell wall. Studies have shown that IsdB binds to haemoglobin, extracts haem and transfers it directly to IsdA. Haem is believed to be passed from the surface exposed Isd proteins to IsdC in a sequential manner through protein-protein interactions. Efficient transfer of haem would require the Isd proteins to be located close to each other so that the functional NEAT domains can interact (Muryoi *et al.*, 2008; Zhu *et al.*, 2008). Perhaps the failure to complement platelet activation by expression from pCU1 is due to expression of IsdB out of its usual context and without the other Isd proteins. Further studies are necessary to investigate whether the Isd proteins are co-located on the cell wall surface of *S. aureus*. Site-directed mutagenesis could also be utilised to introduce point mutations into *isdB* which affect function but not expression of the IsdB protein, leaving the Isd system intact on the cell surface. It is not clear why adhesion to platelets could be partially complemented by expression of IsdB from pCU1 while activation could not. Coexpression of IsdA and IsdB from pCU1 increased adhesion to platelets. Activation may require expression of IsdB with additional proteins to securely cross-link bacteria to platelets.

Such "accessory factors" would not be directly involved in platelet interactions and could not stimulate activation when expressed in the absence of IsdB.

Strain Newman *clfA clfB* caused platelet aggregation with an average lag time of 5.6 min. Longer lag times to activation are generally associated with complement-dependent platelet activation mechanisms and reflect of the time taken for complement fixation to occur on the bacterial cell surface. Despite the longer lag times, aggregation was not complement dependent. Newman strains expressing IsdB could cause activation in heat treated serum in which complement proteins were inactivated (Figure 5.3). To further investigate the mechanism of platelet activation experiments were carried out with GFP. Bacteria expressing either ClfA or ClfB require fibrinogen and specific IgG to cause platelet aggregation. The only plasma factor necessary for platelet activation mediated by strain Newman *clfA clfB* expressing IsdB was fibrinogen (Figure 5.14). Since initial adhesion to platelets is direct and does not require fibrinogen the necessity for fibrinogen in subsequent events must be for cross-linking of platelets into aggregates. IgG did not seem to be directly required for activation since the addition of fibrinogen alone to GFP was sufficient to allow activation. However, there was a trend towards a faster lag time in the presence of IgG. This needs to be investigated further with more donors. Newman strains expressing IsdB could cause activation of GFP supplemented with IgG-depleted serum without affecting lag time or percentage aggregation (Figure 5.3).

A function-blocking anti-GPIIb/IIIa antibody completely inhibited activation of platelets by RPMI-grown Newman *clfA clfB* strains. The same antibody also fully inhibited adhesion to platelets (Figures 5.4.A and B). This strongly indicates a crucial role of platelet receptor GPIIb/IIIa in platelet adhesion and activation mediated by *S. aureus* in iron-deficient conditions.

Although antibodies to platelet receptors FcγRIIa and GP1b did not inhibit adhesion of Isd expressing bacteria to platelets, both inhibited activation of platelets (Figure 5.4.B). IgG does not seem to have direct role in either platelet adhesion or activation mediated by RPMI-grown Newman strains. Adhesion to platelets was direct and not inhibited by anti-FcγRIIa. The only plasma factor required for activation was fibrinogen, most likely for the aggregation phase. The inhibitory effect of anti-FcγRIIa antibodies on platelet activation therefore seems contradictory. Previous studies have reported similar findings for platelet aggregation mediated by SrpA of *S. sanguis*. SrpA mediated direct adhesion of *S. sanguis* to platelets through

binding GP1b and triggered activation without the requirement for IgG. However, aggregation was inhibited by anti-FcγRIIa. Inhibition was postulated to be a result of co-localisation of GP1b and FcγRIIa on platelets resulting in steric hindrance of Gp1b by anti-FcγRIIa antibodies (Kerrigan *et al.*, 2002; Sullam *et al.*, 1998). A similar situation may lead to the inhibition of aggregation by anti-FcγRIIa antibodies seen in this study. Activation of platelets is accompanied by a complex chain of signalling events, conformational changes in receptors and clustering of receptors. Complexes of GP1b, GPIIb/IIIa and CD9 have been identified on the platelet surface and clustering of the GP1b complex has been shown to activate GPIIb/IIIa (Kasirer-Friede *et al.*, 2002; Longhurst *et al.*, 1999). Due to the complex interplay that exists between these receptors upon activation the use of platelet inhibitors to determine the involvement of individual receptors can be problematic. The anti-GP1b antibody (AN51, Dako) used in this study contained the preservative sodium azide, a known inhibitor of the thromboxane A2 pathway in human platelets. Thromboxane A2 is synthesised by platelets in response to activation and released to stimulate activation of nearby resting platelets (Jackson *et al.*, 2003). Therefore the inhibitory effects of the anti-GP1b antibody could be due to inhibition of thromboxane A2 pathway. Future experiments with this antibody should utilise equal concentrations of sodium azide in negative controls to determine if this receptor is actually involved.

The results of this study point to a novel mechanism of platelet activation for *S. aureus* expressing Isd proteins where IsdB possibly supported by IsdA binds directly to the resting form of platelet integrin GPIIb/IIIa. Microarray studies have demonstrated that expression of Isd proteins including IsdB is up-regulated *in vivo* and *S. aureus* circulating in the blood may initiate interactions with platelets via IsdB.

Chapter 6

Expression of *Staphylococcus aureus* clumping factors A and B under iron and nutrient limiting conditions

6.1 Introduction

Expression of cell surface associated virulence factors by *S. aureus* is generally accepted to take place during the exponential phase of growth while secreted proteins and toxins are expressed when the cells approach stationary phase. This temporal switch in protein expression is triggered by an increase in cell density following the exponential growth phase and the resulting activation of the accessory gene regulator, *agr* (Bronner *et al.*, 2004). Sequential expression of virulence factors from *S. aureus* in this manner has been established *in vitro* with bacteria grown in complex media.

The *agr* system is a cell density sensing mechanism. In the exponential growth phase transcription from the P2 promoter of the *agr* locus results in the RNAII transcript. This transcript encodes a two component regulatory system composed of the sensor, AgrC and response regulator, AgrA (Lina *et al.*, 1998). This system is activated by the extra-cellular accumulation of the secreted auto-inducing peptide (AIP) in the mid- to post-exponential growth phase (Ji *et al.*, 1995). Activated AgrA increases transcription from the P2 and P3 promoters. The transcript of P3 is RNAIII, a global regulator which represses expression of cell associated proteins and activates expression of toxins and enzymes (Figure 1.10) (Novick, 2003).

Studies carried out by McAleese *et al.* determined that transcription of *clfB* ceases in the late exponential growth phase. This correlates with the observation that the 150 kDa ClfB protein is maximally expressed in the exponential growth phase. As bacteria enter the stationary phase of growth, ClfB is degraded by the metalloprotease aureolysin into a non-functional truncate of 120 kDa. Cleavage occurs at a SLAVA motif located at the end of the N1 domain of ClfB. Loss of ClfB in the post-exponential growth phase is also mediated by the dilution of existing protein as the cells grow and divide while transcription of *clfB* has ceased. Shedding of ClfB into the growth medium by autolysis also contributes to the loss of ClfB from post-exponentially growing cells (McAleese *et al.*, 2001). Transcription of *clfB* is not directly affected by Agr or the staphylococcal accessory regulator, SarA. However, SarA represses expression of several proteases including aureolysin. Increased expression of aureolysin in *sarA* mutant strains results in the presence of the truncated, non-functional form of ClfB on the cell surface earlier in the exponential growth phase (McAleese *et al.*, 2001; McAleese & Foster, 2003).

The repressor of toxins, Rot, belongs to the SarA family of proteins. These proteins have a highly conserved motif (KYRXXXDER) located at the C-terminus.

Rot was identified by McNamara *et al.* as a repressor of alpha toxin in the exponential growth phase. Later studies found that Rot is a global regulator which repressed expression of secreted toxins and enzymes while also activating certain cell associated proteins expressed in the exponential growth phase, including ClfB. Rot therefore seems to be an antagonist of *agr* regulation. By promoting expression of adhesins, Rot is believed to contribute to the early stages of infection and colonisation. Transcription of Rot occurs throughout all growth phases. However, its expression is believed to be inhibited by RNAIII post-transcriptionally. Studies have reported that the translation of *rot* is inhibited by the base pairing of RNAIII with the ribosome binding region of *rot* mRNA. RNase III may subsequently cleave *rot* mRNA (Boisset *et al.*, 2007; Geisinger *et al.*, 2006). Other studies have suggested that RNAIII acts to neutralise Rot protein rather than inhibiting translation of *rot* mRNA (McNamara *et al.*, 2000). In the post-exponential growth phase when the *agr* system is active, Rot is inactivated partly by RNAIII. This leads to increased exotoxin production and repression of cell-associated factors such as ClfB. Aureolysin, the metalloprotease that cleaves ClfB into a non-functioning truncate is repressed in the early exponential growth phase by high levels of SarA and Rot. In the post-exponential growth phase inhibition of Rot by RNAIII occurs and decreased expression of SarA results in expression of aureolysin. Therefore ClfB is expressed at high levels in its intact form in the exponential growth phase (Figure 6.1).

In contrast to ClfB, ClfA is expressed predominantly in the stationary phase of growth by *S. aureus* cells in complex broth media. Although ClfA is subject to proteolytic cleavage at the N1 domain in a similar manner to ClfB, loss of the N1 domain does not affect fibrinogen-binding activity (McDevitt *et al.*, 1997). Stationary phase *S. aureus* cells expressing ClfA can promote clumping in soluble fibrinogen and adherence to immobilised fibrinogen. *S. aureus* strains with mutations affecting the alternative sigma factor, SigB were found to express low levels of ClfA (Kullik *et al.*, 1998). Sigma factors are involved in the initiation of transcription in response to various stimuli. SigB is one of three sigma factors of *S. aureus* (section 1.4.3). SigB is involved in the stress response and studies have shown that it is activated when cells are exposed to heat shock, alkaline shock or when grown in the presence of $NaCl_2$ or $MnCl_2$ (Pane-Farre *et al.*, 2006). The *sigB* locus encodes a number of post-translational regulators of SigB. The genes located directly upstream of the *sigB* gene in *S. aureus* encode for an anti-sigma factor, RsbW, a phosphatase RsbU and the

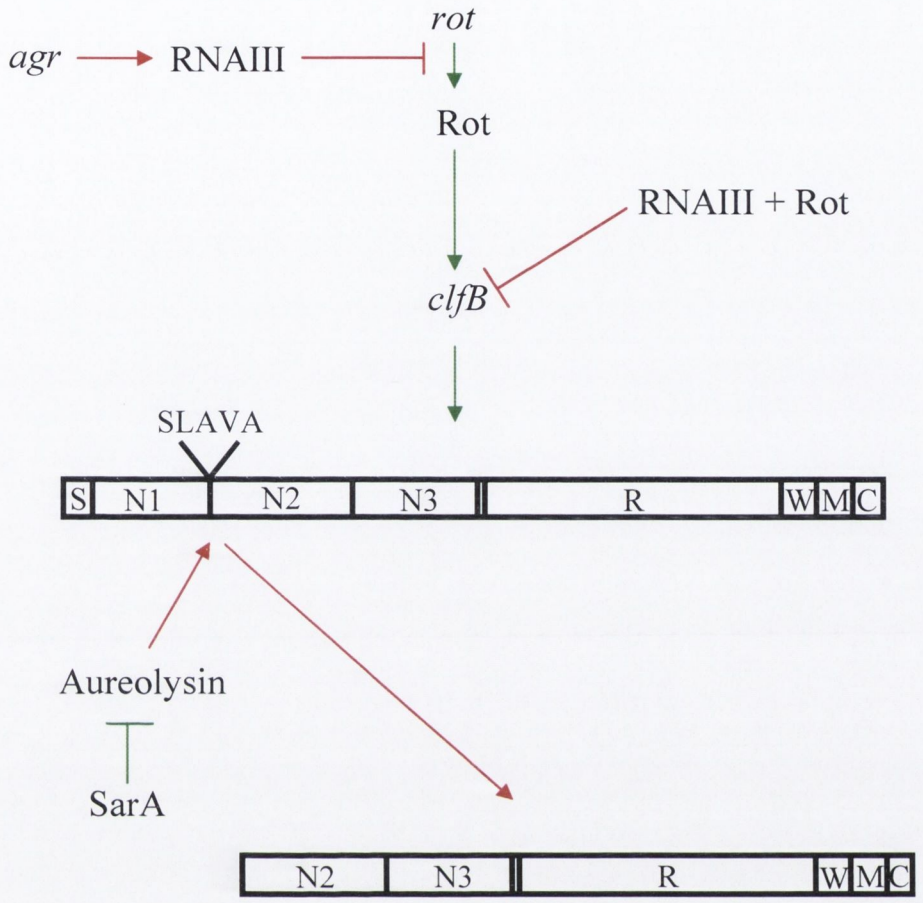


Figure 6.1. Expression of ClfB in exponential and post-exponential growth phases

Transcription of *clfB* is positively activated by Rot in the early exponential growth phase while SarA represses the expression of aureolysin. In the post-exponential phase of growth RNAIII inhibits translation of *clfB* mRNA or RNAIII acts to neutralise Rot protein. Decreased levels of SarA are present in the post-exponential growth phase which relieves repression of *aur*. Aureolysin degrades ClfB at the SLAVA motif to produce a non-functioning truncate of ClfB. (Green = exponential, red = post-exponential)

substrate for RsbU, RsbV. In its dephosphorylated RsbV can bind competitively to RsbW to release SigB (Miyazaki *et al.*, 1999; Palma & Cheung, 2001). Differential transcription from the *rsbUVWsigB* operon has been proposed to regulate the level of free SigB in *S. aureus*. In the exponential phase of growth transcription of the entire operon results in basal expression of SigB from *S. aureus*. In the stationary growth phase a short transcript that encodes SigB only is induced at high levels and raises the concentration of free SigB in cells (Kullik & Giachino, 1997). Previous studies have shown that in the stationary phase of growth ClfA is expressed from a SigB dependent promoter. Weaker expression of ClfA occurs in the exponential growth phase and is dependent on transcription from a SigA-dependent promoter (Higgins, 2005).

The ClfA and ClfB proteins of *S. aureus* cause fibrinogen-dependent platelet activation. ClfB contributes to activation mediated by exponential growth phase *S. aureus* while ClfA is the dominant pro-aggregatory protein in the stationary growth phase (Loughman *et al.*, 2005). The study described in the previous chapter investigated platelet aggregation mediated by *S. aureus* cells grown in RPMI medium. This medium is deficient in iron and other nutrients which results in the slow growth of bacteria. RPMI medium was used to reflect the slow growth of bacterial cells and iron poor conditions which are present *in vivo*. The study described in this chapter was primarily undertaken to investigate expression of ClfA and ClfB under iron limited conditions and the effect this might have on platelet interactions.

6.2 Results

6.2.1 Growth of *S. aureus* in iron and nutrient limiting conditions

A 16 h culture of strain Newman grown in RPMI medium was washed and diluted into fresh RPMI to an OD_{600} of 0.05. The cell density of the cultures was monitored by recording OD_{600} values of samples taken at various time points over 32 h. The doubling time in RPMI medium for strain Newman was 82 min. The final yield of bacterial cells after growth for 31 h was an $OD_{600\ nm}$ of 1.25 (Figure 6.2).

Bacteria grown in RPMI are starved of nutrients as well as iron since this is a nutrient poor medium. Chelex 100 resin (sodium form, Sigma) contains paired iminodiacetate ions which act as chelating groups by binding polyvalent metal ions. This resin was used to remove Fe^{2+} and Fe^{3+} ions from TSB as described in previous studies and section 2.1.2 Successful depletion of Fe^{2+} and Fe^{3+} was confirmed by detection of the iron regulated surface determinant, IsdH from cells cultured in

Chelex-treated TSB. Cell wall extracts were prepared from stationary phase Newman cells grown in RPMI, TSB and Chelex-treated TSB by lysostaphin digestion. Solubilised cell wall proteins were separated by SDS-PAGE and electroblotted onto PVDF membranes. Membranes were probed with polyclonal anti-isdH antibody. IsdH was detected as a band of 150 kDa in the cell wall extracts of strain Newman grown in RPMI medium. No IsdH could be detected from cells grown in TSB and very little by cells grown in Chelex-treated TSB (Figure 6.3). This indicated that TSB treated with Chelex still contained enough iron to suppress expression of IsdH. To further deplete iron from Chelex-treated TSB, 2,2'-dipyridyl, a high-affinity chelator of Fe^{2+} which also binds with lower affinity to Fe^{3+}, was added at concentrations of 10, 50, 200 and 400 μM. Previous studies have used 2,2'-dipyridyl to create iron poor conditions in complex media. High levels of IsdH could be detected on the cell wall surface of cells grown in response to increasing concentrations of 2,2'-dipyridyl. Since IsdH is only expressed under iron deficient conditions this indicated that TSB had been depleted of iron. However, at the 400 μM of 2,2'-dipyridyl expression of IsdH was still lower than that seen in RPMI medium (Figure 6.3).

The treatment of TSB with Chelex resin and the addition of 2,2'-dipyridyl resulted in a increase doubling times and a decrease in the final yield of cells following growth for 32 h (Figure 6.2). Strain Newman had a doubling time of 42 min when grown in TSB and the optical density at 600 nm reached 8.2 after 31 h of growth. The same strain grown in Chelex-treated TSB had a doubling time of 45 min and the final yield of cells was decreased 1.7 fold compared to Newman grown in TSB. The addition of 50 μM 2,2'-dipyridyl did not affect the doubling time or growth yield. The addition of 200 and 400 μM 2,2'-dipyridyl increased the doubling time to 58 and 70 min and decreased the final yield of cells 3-fold and 4.6-fold respectively compared to cells grown in TSB. The decrease in growth rates of cells grown in Chelex-treated TSB reflects the loss of iron from the growth medium which is essential for growth of bacteria.

6.2.2 Expression of ClfA from RPMI-grown *S. aureus* strain Newman

Strain Newman was grown in TSB and RPMI medium supplemented with $FeCl_3$ at final concentrations of 20, 50 and 100 μM and western immunblots were carried out to detect expression of ClfA. Cell wall extracts of stationary growth phase Newman cells (approximate OD_{600} of 8 and 1.2 in TSB and RPMI respectively) were

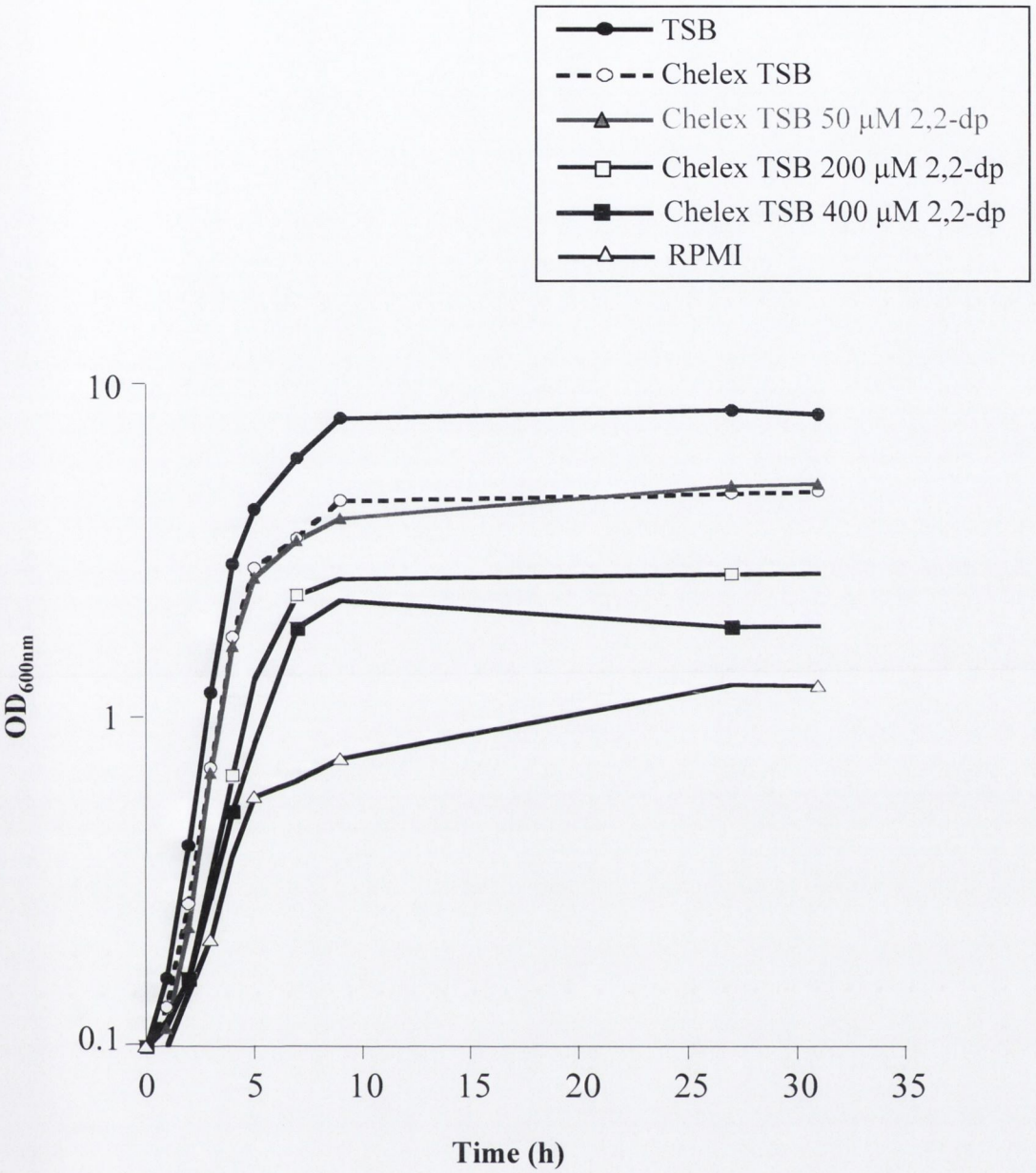


Figure 6.2. Growth of *S. aureus* strain Newman in iron limiting conditions

A 16 h cultures of strain Newman grown in TSB, RPMI, Chelex treated TSB and Chelex treated TSB supplemented with 2,2 dipyridyl at the indicated concentrations were diluted in fresh medium and grown at 37°C with shaking (200rpm), over 31 h. Growth was monitored by measuring cell density of samples taken at indicated time points.

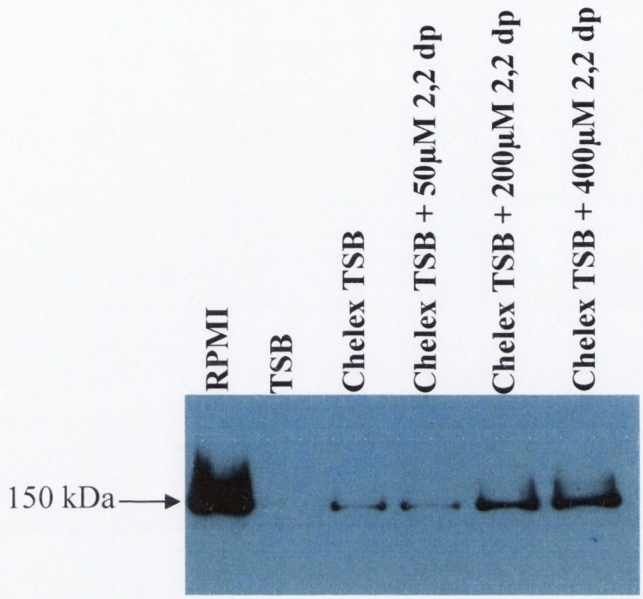


Figure 6.3. IsdH expression from strain Newman grown in iron limiting conditions

S. aureus strain Newman was grown to the stationary phase in RPMI, TSB, and TSB treated with Chelex resin supplemented with 2,2 dipyridyl as indicated. Cell wall proteins were solubilised by lysostaphin digestion, separated on 7.5% SDS PAGE gels and electroblotted onto PVDF membranes. Membranes were probed with polyclonal rabbit anti-IsdH antibody and HRP-conjugated protein A. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

prepared by lysostaphin digestion. Cell wall extracts were separated by SDS-PAGE and electroblotted onto PVDF membranes which were probed with polyclonal anti-ClfA antibody.

Expression of ClfA was greatly reduced in bacterial cells grown in RPMI medium compared to cells grown in TSB. The addition of iron to the RPMI growth medium in the form of $FeCl_3$ increased ClfA expression. A progressive increase in ClfA expression could be seen with the addition of 20 and 50 μM $FeCl_3$. The addition of 100 μM Fe Cl_3 did not further increase ClfA expression (Figure 6.4). Even with the addition of iron to the RPMI growth medium, expression of ClfA was dramatically reduced compared to that seen in TSB.

Western immunoblots were also carried out to detect ClfA expression in TSB depeleted of iron. Growth of Newman cells in Chelex-treated TSB did not have a dramatic impact on ClfA expression. The addition of 2,2 dipyridyl to Chelex-treated TSB reduced ClfA expression (Figure 6.5A). Whole cell immunoblots indicated that strain Newman grown in the presence of 50 and 200 μM 2,2 dipyridyl had 2-fold and 32-fold decreased ClfA expression, respectively (Figure 6.5B). At 400μM ClfA could still be detected by Western immunoblotting. However, whole cell immunoblotts showed a minimal amount of ClfA on the bacterial cell surface. These results indicated that a high level of ClfA expression is dependent on the availability of iron to bacterial cells.

6.2.3 Adherence to fibrinogen by strain Newman grown in RPMI

Adherence of RPMI-grown Newman strains to immobilized fibrinogen was assessed and compared to that of Newman strains grown in TSB. ClfB is expressed by strain Newman and can bind to fibrinogen, therefore a mutant defective in ClfB (Newman *clfB*) was utilised to assess the contribution of ClfA alone to fibrinogen binding. Newman is defective in the FnBPA and FnBPB proteins which also mediate binding to fibrinogen (Grundmeier *et al.*, 2004). Washed bacterial cells were resuspended to an OD_{600} of 1 and added to microtitre plate wells coated with fibrinogen. Adherence was measured by crystal violet staining as described in section 2.15.

Newman *clfB* grown in RPMI showed reduced adherence to fibrinogen in comparison to the same strain grown in TSB (Figure 6.6). This correlates with the decreased ClfA expression seen with RPMI-grown cells. RPMI grown Newman *clfA*

clfB adhered weakly to fibrinogen indicating that the adhesion of RPMI grown Newman *clfB* to fibrinogen was mediated by ClfA. The level of adhesion of RPMI-grown Newman *clfB* to fibrinogen was increased to that of TSB-grown cells by the addition of 50 μM $FeCl_3$ to the growth medium (Figure 6.6). This correlates with the increased expression of ClfA from RPMI-grown cells upon the addition of $FeCl_3$.

6.2.4 Expression of ClfA from RPMI-grown Newman cells is dependent on SigB

Previous studies have shown that high levels of expression of ClfA in rich, complex media such as TSB is dependent on SigB. To assess the role of SigB in expression of ClfA in iron limiting conditions strain Newman *sigB* was tested. Cell wall extracts of Newman and Newman *sigB* cells grown to stationary phase in RPMI were prepared by lysostaphin digestion, separated by SDS-PAGE and electroblotted onto PVDF membranes. Membranes were probed with polyclonal anti-ClfA antibody.

As described previously ClfA was expressed weakly by RPMI-grown Newman cell and expression could be increased by the addition of $FeCl_3$. Expression of ClfA could not be detected from RPMI grown Newman *sigB* even in the presence of 50 μM $FeCl_3$ (Figure 6.7). This result indicates that expression of ClfA in iron-limiting conditions is controlled by the transcription factor SigB.

6.2.5 Expression of ClfB in iron limiting media

Strain Newman was grown in TSB and RPMI to early exponential, early stationary and stationary growth phases. Western immunblots were carried out to detect expression of ClfB. ClfB was detected in the cell wall extracts of strain Newman grown to the exponential growth phase in TSB. In the early stationary phase ClfB was detected weakly while in the stationary phase no ClfB was present in cell wall extracts (Figure 6.8A). This corresponds to the results of previous studies which showed that ClfB is expressed maximally in the early exponential growth phase after which it is gradually lost from the cell wall following cessation of transcription in the post-exponential phase. ClfB is also cleaved by aureolysin resulting in a 120 kDa non-functional protein. The full length ClfB protein of 150 kDa was detected in cell wall extracts of RPMI-grown cells in the exponential, early stationary and stationary growth phases (Figure 6.8A). Strains 8325-4 and SH1000 grown in RPMI also had ClfB present in cell wall extracts taken from the growth phases outlined. Strain 8325-

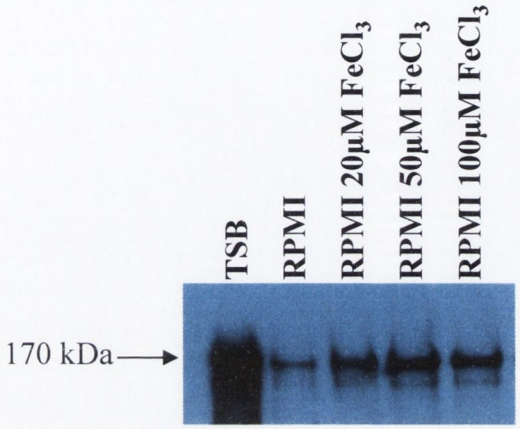


Figure 6.4. ClfA expression from strain Newman grown in iron limiting conditions

S. aureus strain Newman was grown to the stationary growth phase in TSB, RPMI and RPMI supplemented with $FeCl_3$ as indicated. Cell wall proteins were solubilised by lysostaphin digestion, separated on 7.5% SDS PAGE gels and electroblotted onto PVDF membranes. Membranes were probed with polyclonal rabbit anti-ClfA antibody and HRP-conjugated protein A. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

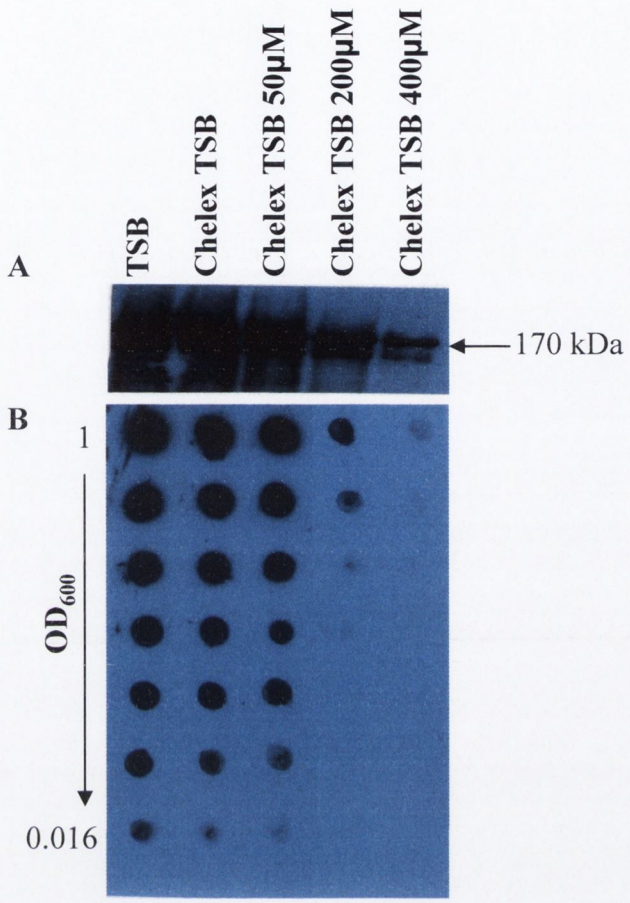


Figure 6.5. ClfA expression from strain Newman grown in TSB depleted of iron

S. aureus strain Newman was grown to the stationary phase in TSB and Chelex-treated TSB. The iron chelator 2,2 dipyridyl was added to Chelex-treated TSB as indicated. (A) Cell wall proteins were solubilised by lysostaphin digestion, separated on 7.5% SDS PAGE gels and electroblotted onto PVDF membranes. (B) Doubling dilutions of bacterial cells were dotted onto nitrocellulose membrane. Membranes were probed with polyclonal rabbit anti-ClfA antibody and HRP-conjugated protein A. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

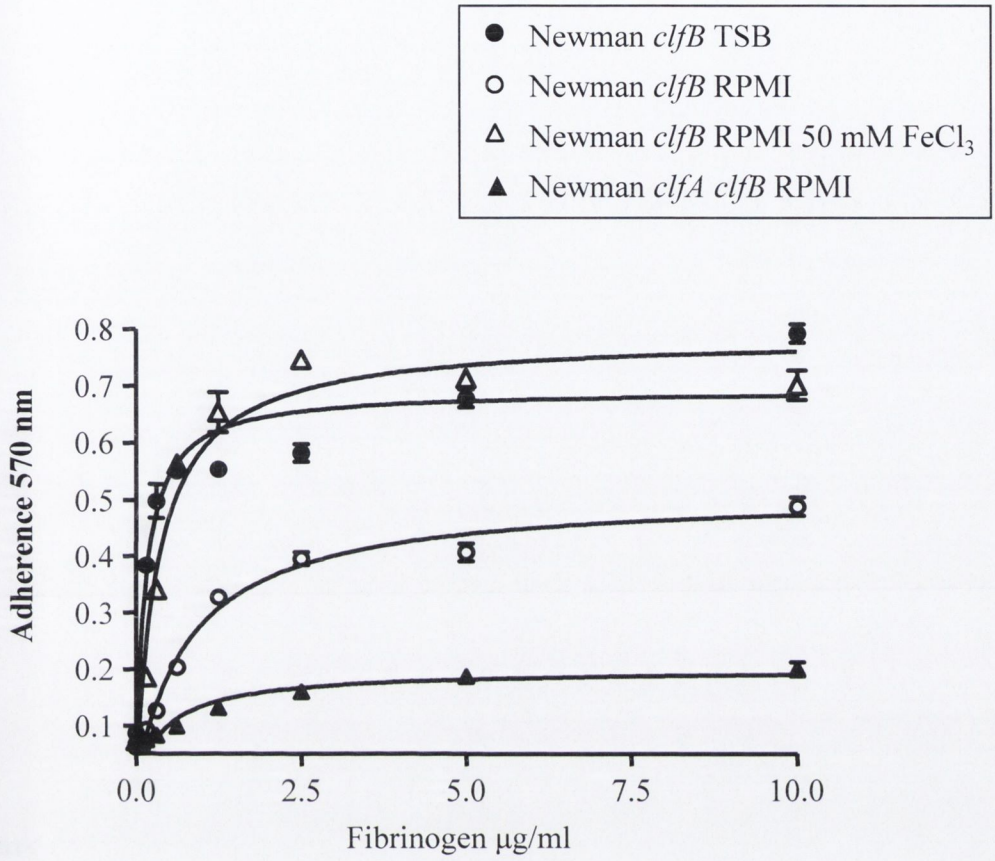


Figure 6.6. Adherence of Newman *clfB* grown in iron limiting conditions to fibrinogen

S. aureus strains Newman *clfB* and Newman *clfA clfB* were grown to stationary phase in TSB, RPMI and RPMI supplemented with $FeCl_3$ as indicated. Washed cells were adjusted to $OD_{600\ nm}$ of 1.0. Adherence of washed cells to ELISA plates coated with fibrinogen was assessed by crystal violet staining. Absorbance at 570 nm was determined. Values represent the means ± standard deviation of triplicate wells. The experiment was performed three times with similar results.

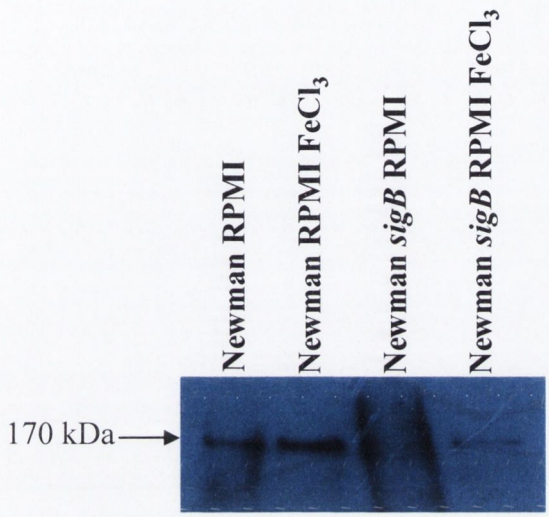


Figure 6.7. ClfA expression from strains Newman and Newman *sigB* grown in iron limiting conditions

S. aureus strains Newman and Newman *sigB* were grown to the stationary phase in RPMI and RPMI supplemented with $FeCl_3$ as indicated. Cell wall proteins were solubilised by lysostaphin digestion, separated on 7.5% SDS PAGE gels and electroblotted onto PVDF membranes. Membranes were probed with polyclonal rabbit anti-ClfA antibody and HRP-conjugated protein A. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

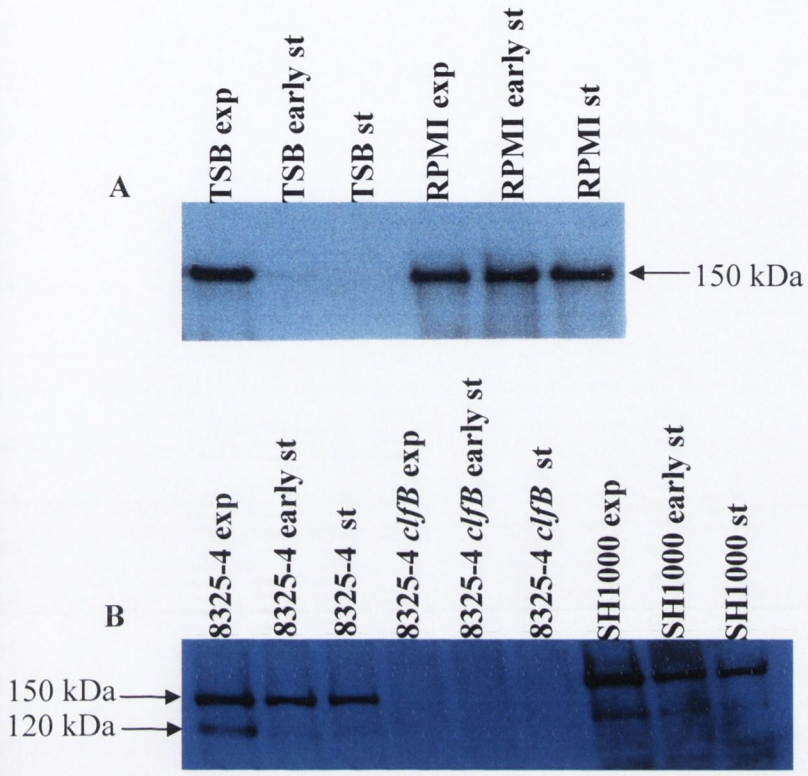


Figure 6.8. ClfB expression from strain Newman grown in iron limiting conditions

(A) *S. aureus* strain Newman was grown to exponential (exp), early stationary and stationary (st) growth phases in TSB and RPMI as indicated. (B) 8325-4, 8325-4 *clfB* and SH1000 were grown in RPMI medium to exponential (exp), early stationary and stationary (st) growth phases as indicated. Cell wall proteins were solubilised by lysostaphin digestion, separated on 7.5% SDS PAGE gels and electroblotted onto PVDF membranes. Membranes were probed with polyclonal rabbit anti-ClfB antibody and HRP-conjugated protein A. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

4 has an 11-base-pair deletion in *rsbU*. This affects SigB expression and the response to cellular stress. The absence of RsbU in 8325-4 had no discernible effect on expression of ClfB. Strain 8325-4 *clfB* lacked ClfB protein as expected (Figure 6.8B).

ClfB was not detected in cell wall extracts prepared from stationary phase Newman cells grown in Chelex-treated TSB with increasing concentrations of 2,2 dipyridyl (Figure 6.9A). The addition of $FeCl_3$ to RPMI-grown Newman cells did not increase expression of ClfB (Figure 6.9B). This indicated that presence of ClfB in the stationary phase in RPMI is not due to iron deprivation.

6.2.6 Adhesion of *S. aureus* Newman cells grown to exponential and stationary phase to cytokeratin 10

Adherence of RPMI-grown Newman *clfA* cells to immobilized cytokeratin 10 was assessed and compared to that of Newman *clfA* grown in TSB. Cells expressing ClfA are known to adhere weakly to cytokeratin 10 (E. Walsh, personal communication). Strain Newman *clfA* was utilised to assess adherence to cytokeratin10 mediated by ClfB only. Washed bacterial cells at an OD_{600} of 1 were added to microtitre plate wells coated with murine cytokeratin 10. Adherence was measured by crystal violet staining as described in section 2.15.

Newman *clfA* cells from the stationary phase of growth in RPMI adhered to cytokeratin 10 in a dose-dependent and saturable manner (Figure 6.10). RPMI-grown Newman *clfA clfB* did not adhere to cytokeratin 10 indicating that the adhesion of Newman *clfA* to cytokeratin 10 is mediated by expression of ClfB. Newman *clfA* grown to stationary phase in TSB bound poorly to cytokeratin 10 compared to the same strain grown in RPMI.

In the exponential phase strain Newman *clfA* grown in RPMI and TSB adhered to cytokeratin 10 in a dose-dependent and saturable manner at similar levels (Figure 6.11). RPMI-grown Newman *clfA clfB* did not adhere detectably to cytokeratin 10. These results imply that ClfB expressed from RPMI grown cells in exponential and stationary phase is functional.

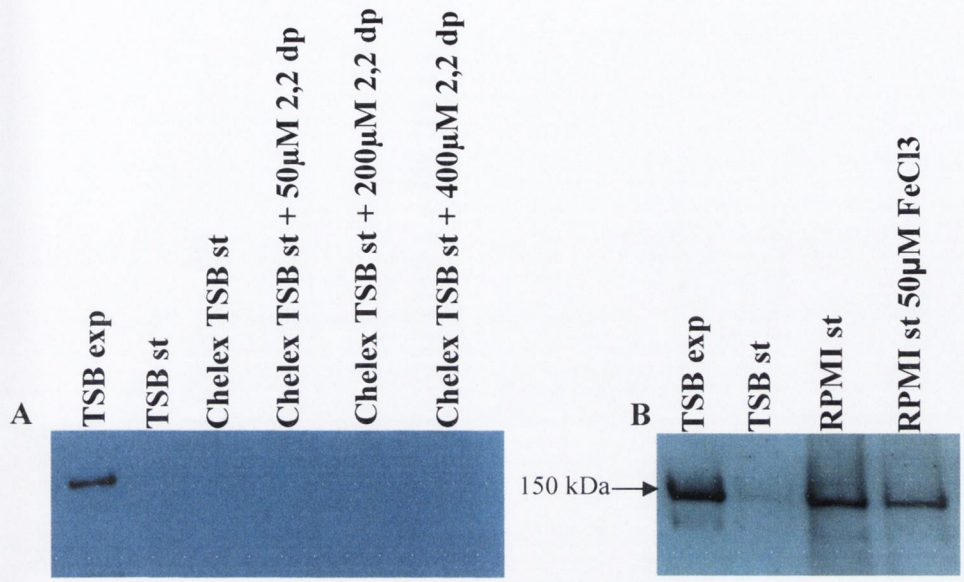


Figure 6.9. ClfB expression from strain Newman grown in TSB depleted of iron

(A) *S. aureus* strain Newman was grown to the exponential phase (exp) in TSB and to the stationary (st) phase in TSB and Chelex-treated TSB medium. The iron chelator 2,2 dipyridyl was added to Chelex-treated TSB as indicated. (B) *S. aureus* strain Newman was grown to the exponential and stationary phase in TSB and to the stationary phase in RPMI with or witout the addition of $FeCl_3$. Cell wall proteins were solubilised by lysostaphin digestion, separated on 7.5% SDS PAGE gels and electroblotted onto PVDF membranes. Membranes were probed with polyclonal rabbit anti-ClfB antibody and HRP conjugated protein A peroxidase. Membranes were developed by chemiluminescence and exposed to autoradiographic film.

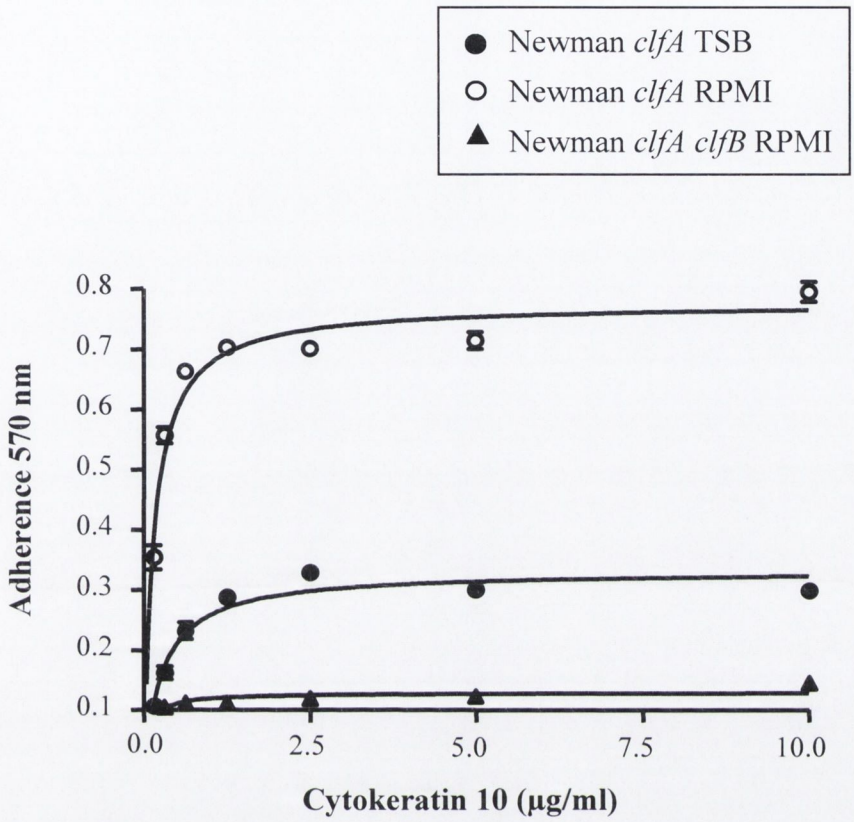


Figure 6.10. Adherence of stationary phase RPMI-grown Newman strains to cytokeratin 10

S. aureus strains Newman *clfA* and Newman *clfA clfB* were grown to the stationary growth phase in TSB and RPMI as indicated. Washed cells were adjusted to OD_{600} of 1.0 in PBS and added to cytokeratin 10-coated microtitre plates. Cells were allowed to adhere for 2 h at 37°C. Adherent cells were detected by crystal violet staining and absorbance at 570 nm was determined using an ELISA plate reader. Values represent the means ± standard deviation of triplicate wells. The experiment was performed three times with similar results.

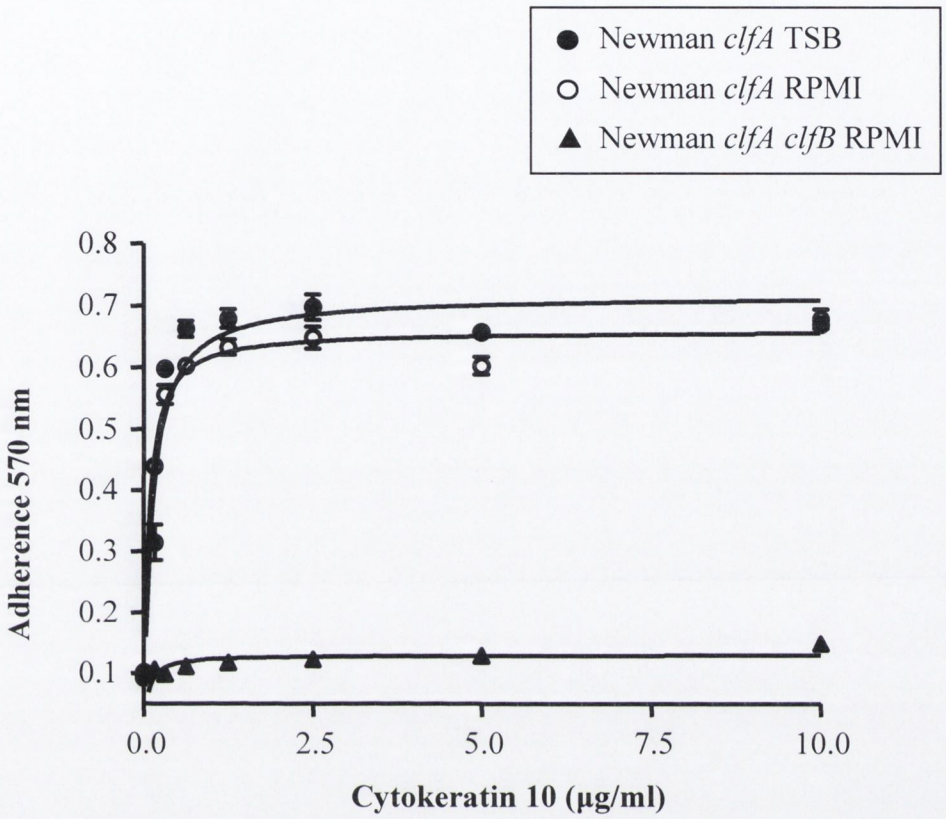


Figure 6.11. Adherence of exponential phase RPMI-grown Newman strains to cytokeratin 10

S. aureus strains Newman *clfA* and Newman *clfA clfB* were grown to the exponential phase in TSB and RPMI as indicated. Washed cells were adjusted to OD_{600} of 1.0 in PBS and added to cytokeratin 10-coated microtitre plates. Cells were allowed to adhere for 2 h at 37°C. Adherent cells were detected by crystal violet staining and absorbance at 570 nm was determined using an ELISA plate reader. Values represent the means ± standard deviation of triplicate wells. The experiment was performed three times with similar results.

6.3 Discussion

ClfA and ClfB are cell wall-associated proteins of *S. aureus* that contribute to physiologically significant processes such as platelet aggregation, evasion of phagocytosis and nasal colonisation (Higgins *et al.*, 2006; O'Brien *et al.*, 2002; Schaffer *et al.*, 2006). The expression of these proteins by *S. aureus* cells grown in complex media has been well characterised. This study investigated the expression of these two proteins by cells grown in iron and nutrient limiting conditions which more closely resemble conditions that occur *in vivo*.

Western immunoblotting consistently showed that RPMI-grown *S. aureus* cells expressed less ClfA on their cell surface than the same cells grown in a complex medium such as TSB. The level of ClfA expression could be increased by the addition of iron to RPMI. However, expression was still lower than from the same strains grown in TSB (Figure 6.4). ClfA-dependent fibrinogen binding was increased by addition of iron to RPMI-grown *S. aureus* cells which corresponded to the increase in ClfA expression (Figure 6.6). The expression of ClfA was also examined from cells grown in a rich medium from which iron was removed using high affinity iron chelators. Western and whole cell immunoblots showed a proportional decrease in ClfA expression when iron was depleted from TSB (Figure 6.5). Therefore the availability of iron seemed important for high levels of ClfA expression.

Previous studies have shown that the high level of ClfA expression in the post-exponential phase of growth is dependent on SigB (Higgins, 2005). This study also found that ClfA expression from post-exponential, RPMI-grown *S. aureus* is dependent on SigB. A *sigB* mutant did not express ClfA even when iron was added to the growth medium (Figure 6.7). A decrease in SigB and ClfA expression *in vivo* has been noted in some earlier studies (Allard *et al.*, 2006; Goerke *et al.*, 2005; Wolz *et al.*, 2002). *S. aureus* strains grown in implanted tissue cages in guinea pigs had decreased SigB activity and *clfA* expression compared to strains grown in broth cultures *in vitro*. RT-PCR determined that the expression of *clfA* mRNA increased gradually *in vivo* with bacterial samples taken at 8 days expressing 4-fold more ClfA than samples taken at day 2 (Goerke *et al.*, 2005). Allard *et al.* reported that SigB was downregulated *in vivo* but not *in vitro* from *S. aureus* cells grown in iron deficient medium. That study concluded that factors other than iron starvation were responsible for downregulation of SigB *in vivo* (Allard *et al.*, 2006). The analysis of growth of *S. aureus* in RPMI medium carried out by this study demonstrated that cell

density was low compared to that seen in TSB (Figure 6.2). Agr is a cell density sensing mechanism and bacteria growing slowly in iron and nutrient limiting conditions may not reach the cell density which would usually accompany the transition from Agr regulation to stationary phase SigB regulation. Iron is essential for the growth and survival of *S. aureus*. The addition of iron to the RPMI growth medium may simply increase growth and cell density sufficient to allow an increase in SigB expression.

Similarly the slow growth and the low cell density may explain the expression of ClfB in stationary phase RPMI-grown cells. This study found ClfB to be expressed at the same level by cells grown in RPMI in exponential and stationary phase (Figure 6.8). ClfB-dependent adherence of RPMI-grown *S. aureus* to cytokeratin 10 was indistinguishable in exponential and stationary growth phases indicating that the protein was intact and had not been degraded by aureolysin (Figures 6.10 and 6.11). This was seen in Western immunoblots of Newman cell wall extracts in which ClfB was visible as a band of 150 kDa with very little of the 120 kDa truncated protein (Figure 6.8A). This differs from the pattern of expression of this protein which was established for cells growing in complex media. In these conditions ClfB is expressed maximally in the exponential phase after which transcription ceases (McAleese *et al.*, 2001). The altered expression of ClfB seen in RPMI does not appear to be a result of iron starvation. No increase in ClfB expression was seen when iron was added to *S. aureus* cells grown in RPMI and ClfB was not detected on cells grown in TSB from which iron was depleted (Figure 6.9). A faster rate of growth and a higher cell density were observed in cells grown in this medium which may be sufficient to trigger regulation of *clfB* transcription and induction of aureolysin. The interaction of RNAIII with Rot and increased expression of aureolysin would contribute to the loss of functional ClfB on the cell surface of growing cells under these conditions. Increased *clfB* transcription *in vivo* has been observed in studies using mouse tissue cage models. This phenomenon was only seen in the *in vivo* conditions and did not occur in iron depleted medium *in vitro* (Allard *et al.*, 2006).

Since ClfA and ClfB were able to bind to their ligands when expressed from RPMI-grown cells their capacity to trigger activation of platelets was also investigated. As discussed in the previous chapter eliminating ClfA and ClfB from strain Newman grown under iron restricted conditions reduced the percentage aggregation and extended the lag time to activation. The lag time of 4.6 min caused

by RPMI-grown Newman indicates that ClfA must be weakly expressed as strains expressing high levels of ClfA have a very short lag time to activation (1-2 min). Since a threshold level of protein expression is required to trigger platelet activation, the low level of ClfA expressed from RPMI-grown cells may not be enough to induce platelet activation. The longer lag times to activation are similar to those reported for ClfB expressing *S. aureus* cells. ClfB and IsdB seemed to play greater role in platelet activation in RPMI-grown bacteria than in TSB grown bacteria.

Bacteria grown in complex media undergo a change in protein expression in the mid- to post-exponential phase of growth as a result of accumulation of extracellular AIP that at a specific concentration triggers the *agr* response. This is responsible for the early exponential phase expression of cell surface proteins and the late and post-exponential growth phase expression of secreted toxins and enzymes. *In vivo* the slow growth of bacteria due to lack of iron and other nutrients may result in the small number of bacteria present in the blood stream with Agr in the off state, resulting in prolonged expression of cell surface proteins. Bacteremic conditions are probably mirrored in planktonic *S. aureus* cells grown in a nutrient and iron poor medium such as RPMI. An adhesive phenotype would facilitate initial colonisation and binding to platelets during early stages of an infection mediated by proteins such as ClfB and FnBPA and B. The accumulation of high numbers of bacteria in confined sites such as in endocardial vegetations or in abscesses may result activation of *agr* and SigB (Arvidson & Tegmark, 2001). Bacteria growing on glass beads *in vivo* are thought to mimic sessile bacteria such as those found in thrombi. *S. aureus* grown in these conditions had increased expression of ClfA (Wolz *et al.*, 2002).

Experimental endocarditis models have shown that expression of ClfB and ClfA by bacteria contributes to pathogenesis *in vivo* (Entenza *et al.*, 2000; Moreillon *et al.*, 1995). Function blocking antibodies to ClfA administered in combination with vancomycin prevented colonisation of heart valve vegetations in an experimental endocarditis model. Inhibition of heart valve colonisation was postulated to occur due to increased clearance of bacteria from the blood stream. The inhibition of fibrinogen binding mediated by the function blocking antibodies was also thought to reduce bacterial interactions with platelets (Vernachio *et al.*, 2003). High antibody titres for ClfA, ClfB and FnBPA have also been detected in the serum of patients recovering from infective endocarditis (Rindi *et al.*, 2006). Although this observation does not confirm that these proteins are involved in platelet interactions taken with other data it

supports their involvement in the development of IE. Therefore there seems to be data to support an *in vivo* role for ClfA and ClfB in platelet interactions. Studies have reported that SigB has a limited effect in pathogenesis in a rat endocarditis model although it seems necessary for *S. aureus*-induced arthritis and sepsis in mice (Entenza *et al.*, 2005; Josefsson *et al.*, 2001). The model for septic arthritis is more complex than experimental endocarditis with much longer periods of bacterial growth *in vivo*. Since SigB and ClfA are reported be expressed late in infection, studies which allow longer times for bacterial growth would more accurately assess the roles these two factors play *in vivo*. Future studies of platelet and bacterial interactions should take into account *in vivo* conditions which result in different expression of surface proteins from *S. aureus*.

Chapter 7
Discussion

7.1 Discussion

S. aureus is an opportunistic pathogen that colonises the desquamated epithelium of the anterior nares. Carriage of *S. aureus* is associated with an increased risk of blood stream infections and it has been reported that *S. aureus*-bacteraemia can predominantly be attributed to an endogenous source (von Eiff *et al.*, 2001; Wertheim et al., 2005). Bacteraemia develops when *S. aureus* gains access to the blood stream at sites of infection such as wounds, ulcers or abscesses. Increased use of intravascular devices and invasive procedures in hospitals has lead to an increase in bacteraemic infections in recent years (Moreillon & Que, 2004).

S. aureus is frequently associated with wound infections. It can inhibit platelet activation and delay wound healing through secretion of a fibrinogen binding protein, Efb. This protein binds directly to platelets and inhibits their activation (Shannon & Flock, 2004). Once *S. aureus* gains access to the blood stream Efb does not seem play a role in interaction with platelets. Studies have shave shown that Efb does not contribute to virulence in a rat endocarditis model (Palma *et al.*, 1996). During bacteraemia pro-aggregatory virulence factors promote bacterial adhesion to and activation of platelets (Fitzgerald *et al.*, 2006).

Several endovascular infections result from the interaction of bacteria with platelets. Different outcomes occur depending on the ability of bacteria to either adhere to or activate platelets. Localised platelet adhesion and activation lead to thrombus formation. If thrombi are deposited on heart valves or if the thrombus itself develops on damaged heart valve tissue, infective endocarditis can develop. *S. aureus* is now acknowledged to be the leading cause of IE (Moreillon & Que, 2004). Bacteria that activate platelets without adhering may cause more diffuse activation of platelets leading to development of thrombocytopenia and disseminated intravascular coagulation. Bacteraemic infection has also been postulated to contribute to atherosclerosis, myocardial infarction and stroke (Fitzgerald *et al.*, 2006).

A common mechanism for platelet activation has been identified for bacterial proteins capable of binding to the blood glycoprotein fibrinogen. ClfB of *S. aureus* is a multi-functional surface protein that can bind to fibrinogen and cytokeratin 10 (Ni Eidhin *et al.*, 1998; O'Brien *et al.*, 2002b). ClfB is known to bind to the α-chain of fibrinogen. This study identified the binding site within the α-chain as tandem repeat 5 of the αC-connector. Tandem repeat 5 is likely to form an Ω loop similar to those found within the ClfB binding site in cytokeratin 10. Previous studies reported that

ClfB interacts with lorocrin, a protein associated with the outer envelope of cornified epithelial cells (E. Walsh, personal communication). Lorocrin also has Ω loops within its structure suggesting that ClfB binds to this common structural motif found in several host proteins. Other ligands containing Ω-loops may also promote ClfB binding. How a loop structure rather than a linear peptide interacts with ClfB is unknown. Walsh *et al.*, (2004) noted a similarity between the C terminal sequence of the latching peptide of ClfB (YGGGSADGDSA) and the Ω loops of cytokeratin 10. It was postulated that Ω loops might mimic the latch peptide and bind within the latching cleft in domain N2 of ClfB. However the same study presented evidence that the binding site for fibrinogen lies between the N2 and N3 domains of ClfB which is supported by the results of this study (Walsh *et al.*, 2004).

The fibrinogen binding proteins of *S. aureus* are predicted to interact with fibrinogen by the dock, lock and latch model (Deivanayagam *et al.*, 2002). Evidence found in this study supports this mechanism for ligand binding by ClfB. Changes to ClfB that reduced binding to fibrinogen are located in the putative binding trench between domains N2 and N3. Substituting a residue (N526) predicted to be involved in the function of the latching peptide reduced binding to fibrinogen (Ponnuraj *et al.*, 2003). Since the same residues of ClfB appear to be involved in the interaction with both fibrinogen and cytokeratin 10, these two ligands are likely to occupy overlapping binding sites in ClfB. Solution of the X-Ray crystal structure of ClfB in complex with its ligands will conclusively determine the binding mechanism.

The potential to bind to fibrinogen contributes to the ability of surface proteins such as ClfA and FnBPA to interact with platelets (Fitzgerald *et al.*, 2006b; Loughman *et al.*, 2005). Previous studies have shown that ClfB causes aggregation of platelets when expressed by *L. lactis* and that ClfB contributes to the pathogenesis of experimental endocarditis in rats (Entenza *et al.*, 2000; O'Brien *et al.*, 2002a). This study established that bacteria expressing ClfB mediate adhesion to platelets via receptor GPIIb/IIIa in a fibrinogen-dependent manner. Subsequent activation requires specific anti-ClfB antibodies that bind the Fc receptor on platelets and trigger activation through receptor clustering. Typically bacteria expressing fibrinogen-binding proteins cause rapid aggregation of platelets. Longer lag times to aggregation are caused by ClfB. This may be due to the fact that potent platelet aggregators such as ClfA interact with the γ-chain and not the αC-connector of fibrinogen which is highly susceptible to proteolysis (Weisel & Medved, 2001).

Fibrinogen-dependent platelet activation appears to be common. *S. aureus* expressing ClfA or FnBPA mediate platelet activation in this manner as does FbsA of *S. agalactiae* (Fitzgerald *et al.*, 2006b; Loughman *et al.*, 2005; Pietrocola *et al.*, 2005). The M protein of *S. pyogenes* also causes fibrinogen-dependent platelet activation (Sjobring *et al.*, 2002). M protein released from the bacterial cell surface forms complexes with fibrinogen. When these complexes contain antibodies to M protein activation of platelets is triggered. Fibrinogen bound to M protein can engage the resting form of GPIIb/IIIa. It is likely that bacterial bound fibrinogen also interacts with the resting form of GPIIb/IIIa allowing platelet activation to occur in the circulation and not just at sites of tissue damage. Once microthrombi form in the circulation they can be deposited at locations distal to the original infection site. *In vivo* aggregated platelets co-localised with *S. pyogenes* and M protein. This supports the hypothesis that platelet activation by bacteria can lead to thrombus formation *in vivo* (Shannon *et al.*, 2007).

Bacteria expressing the non-fibrinogen binding mutant ClfB Q235A were able to cause aggregation in a complement dependent manner which required bound anti-ClfB antibodies to trigger platelet activation. Bacteria expressing the non-fibrinogen binding ClfA PY protein and certain strains of *S. sanguis* mediate platelet activation in the same manner (Ford *et al.*, 1996; Loughman *et al.*, 2005). It has been postulated that complement-dependent platelet activation may be a common mechanism amongst IE-causing pathogens since any bacterium could cause aggregation by expressing a surface protein to which antibodies are present in the host (Fitzgerald *et al.*, 2006a).

Previous studies have shown that stationary phase *S. aureus* cells mediate platelet aggregation via ClfA while in the exponential growth phase the FnBPs and ClfB contribute to platelet aggregation (Fitzgerald *et al.*, 2006b; Loughman *et al.*, 2005). *In vivo*, *S. aureus* expresses a unique array of proteins including the iron regulated surface determinants which capture haem-iron from host proteins. This study found that IsdB plays a crucial role in platelet activation mediated by bacteria grown in iron- and nutrient-limiting conditions which reflect the growth environment *in vivo*. The adhesion of *S. aureus* to platelets appeared to be mediated directly by IsdB interacting with platelet receptor GPIIb/IIIa. Activation did not require IgG although an anti-FcγRIIa antibody inhibited aggregation. Inhibition of platelet aggregation by anti-FcγRIIa antibodies may be due to steric hindrance or inhibition of receptor clustering events that accompany platelet activation.

Expression of IsdB from a heterologous host, *S. epidermidis* did not support platelet adhesion or aggregation indicating that additional factors may be involved. It is not clear why adhesion to platelets by *S. aureus* lacking IsdB could be partially complemented by expression of IsdB from pCU1 while activation could not. Co-expression of IsdA and IsdB from pCU1 increased adhesion to platelets indicating that IsdA may play an accessory role. Activation may require expression of IsdB with additional proteins to securely cross-link bacteria to platelets. Alternatively, correct expression of IsdB may require co-expression of the Isd system components. These proteins are believed to cluster on the bacterial cell surface to facilitate haem acquisition (Watanabe *et al.*, 2008). Expression of Isd proteins in isolation from the other Isd system components may explain the failure by this study and others to fully complement the phenotypes of mutants defective in individual Isd proteins.

The novel interaction of IsdB with platelets identified in this study provides the basis for future investigations into the exact mechanism of platelet activation involved. The potential of IsdB to bind to GPIIb/IIIa should be further investigated by determining whether purified IsdB protein can support binding to GPIIb/IIIa. The NEAT domains of IsdB are important for interaction with haemoglobin and haem. In order to determine if these domains are also involved in the interaction with platelets point mutations could be introduced into domains NEAT1 and NEAT2. The co-location of Isd proteins should also be investigated to clarify why platelet aggregation can not be complemented by expression of IsdB from plasmids.

In this study platelet activation was measured by light transmission aggregometry. Bacteria are used at high concentrations in this procedure which does not reflect the *in vivo* situation where bacterial densities would be much lower. However, *in vivo* platelet activation can be a self-perpetuating process since activated platelets secrete potent platelet activators such as thromboxane A_2, ADP and serotonin. These activators can activate resting platelets in the vicinity (Fitzgerald *et al.*, 2006). Therefore the interaction between bacterial surface proteins and platelets characterised in this study may contribute to endovascular disease *in vivo*. Following initial adhesion and activation of platelets by bacteria expressing ClfB and IsdB platelet activation could be amplified by secreted activating factors.

IsdB mediated platelet activation may be of relevance *in vivo* particularly if other pro-aggregatory molecules are expressed at lower levels than when bacteria are grown in rich broth. Although ClfB was expressed at constant high levels from *S. aureus*

grown in RPMI, expression of ClfA was dramatically reduced compared to that seen from cells grown in rich broth. Temporal expression of surface proteins by *S. aureus* has been well established *in vitro*. *In vivo* the slow growth of bacteria due to lack of iron and other nutrients results in low bacterial cell density in the blood stream. Under these conditions it is likely that Agr is inactive allowing prolonged expression of cell surface adhesins such as ClfB and the FnBPs. Adhesion to sites of vascular damage by adhesins may facilitate initial colonisation and thrombus formation during early stages of an infection.

Delayed expression of high levels of ClfA is most likely due to slow growth and low cell densities which delay expression of stationary phase proteins (Higgins, 2005; Kullik *et al.*, 1998). *In vivo* ClfA expression has been detected at late stages of infection. Accumulation of high numbers of bacteria in confined sites such as in endocardial vegetations may result in expression of ClfA facilitating thrombus growth by platelet recruitment (Arvidson & Tegmark, 2001).

Multiple surface expressed proteins of *S. aureus* can mediate activation of platelets. This study has characterised the interaction between ClfB and platelets and identified a novel, direct interaction between IsdB and platelets. Proteins such as IsdB whose expression is induced by the harsh growth environment *in vivo* may be important in mediating platelet activation and thrombus formation in the human host.

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