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Analysis of the function of the N1 subdomain of MSCRAMMs of *Staphylococcus aureus.*

A thesis submitted for the degree of Doctor in Philosophy

by

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Declaration

I hereby declare that this thesis has not previously been submitted for a degree at this or any other university and that it is my own work except where it is duly acknowledged in the text.

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N. McCormack

Niamh McCormack

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Summary

The Microbial Surface Component Recognising Adhesive Matrix Molecules (MSCRAMMs) family of cell wall-associated proteins have recently been reclassified based on structure-function analysis. Under this new regime the MSCRAMM family is defined by the presence of at least two tandemly-linked IgG-like folded domains. Clumping factor A (ClfA) is the archetypal fibrinogen-binding MSCRAMM of *Staphylococcus aureus* and an important virulence factor. An N-terminal signal sequence directs export by the Sec pathway and the C-terminal cell wall-anchoring domain allows covalent attachment of ClfA to peptidoglycan by sortase. Region A of ClfA comprises three independently folded subdomains N1, N2 and N3. Subdomains N2N3 comprise IgG-like folds and promote fibrinogen binding. However the function of subdomain N1 has, until now, remained elusive.

The *clfA* gene was cloned into pRMC2 under the control of a tightly regulated tetracycline–inducible promoter. The plasmid was transformed into a derivative of strain Newman lacking all fibrinogen binding surface proteins. Expression of ClfA on the cell surface was dependent on the concentration of anhydrotetracycline. A mutant of pRMC2 *clfA* lacking the entire N1 subdomain was constructed but could not be expressed on the surface of *S. aureus*. However the presence of residues 211-228 of N1 was sufficient for expression of ClfA on the bacterial surface.

Loss of the N1 subdomain of ClfA did not affect fibrinogen binding. This was demonstrated by the recombinant ClfA A region protein lacking the N1 domain, surface shaving of the N1 subdomain of ClfA from *S. aureus* strain Newman by a metalloprotease and by surface expression of ClfA $\Delta N1_{40\text{-}210}$. Furthermore, the N1 subdomain is not required for the second major function of ClfA; to inhibit killing of *S. aureus* in human blood.

Attempted expression of a ClfA variant lacking the entire N1 subdomain resulted in impaired growth of *S. aureus* and accumulation of ClfA protein in the cytoplasm and cytoplasmic membrane. The presence of residues 211-228 of N1 was required to allow expression of ClfA on the bacterial surface. The importance of this region was confirmed when ClfA variants lacking residues 211-220 or 211-228 were also mislocalised to the cytoplasm and cytoplasmic membrane. However, these residues were not required for export of ClfA lacking the Ser-Asp repeats that links region A to

the wall-anchoring domain. Similarly, subdomain N1 of fibronectin binding protein B was required for export and surface display of the full length protein but not a derivative lacking fibronectin binding repeats. Surface expression of a FnBPB variant lacking the N1 subdomain was not achieved although 28 residues at the C-terminus of the N1 subdomain were restored. This suggests that a distinct region within subdomain N1 of FnBPB is necessary to mediate correct surface localisation. These results demonstrate that the N1 subdomain is dispensable on the surface of *S. aureus* but is required for export and cell wall localization of *S. aureus* surface proteins containing tandemly-arrayed IgG-like fold domains linked by unfolded repeat regions to the cell wall.

The A region of ClfA was shown previously to bind to and activate complement factor I. DNA encoding ClfA $N123_{40\text{-}559}$ and ClfA $N23_{221\text{-}559}$ and each of the individual subdomains of the A region were cloned into pGEX-4T-2 and expressed as GST-fusion proteins. Recombinant GST-tagged proteins were examined for their ability to bind factor I in serum. Loss of the N1 subdomain of ClfA did not affect factor I binding. Although the intact ClfA $N123_{40\text{-}559}$ and ClfA $N23_{221\text{-}559}$ regions can bind factor I, none of the individual subdomains were capable of promoting factor I binding. This demonstrates that the $N23_{221\text{-}559}$ region of ClfA must be intact for factor I binding.

GST-tagged recombinant ClfA variants containing amino acid substitutions that reduced affinity for fibrinogen were constructed and examined for factor I binding. No difference in affinity was observed demonstrating that fibrinogen binding is not necessary for factor I binding. Investigation of the nature of the ClfA-factor I interaction revealed that ClfA can only bind factor I in serum not purified factor I or factor I which was pulled from serum using monoclonal anti-factor I IgG. These results strongly indicate that the interaction between ClfA and factor I requires one or more co-factors to occur.

Furthermore, this study demonstrated that multiple staphylococcal surface proteins are capable of binding factor I and members of the regulators of complement activation family, factor H and C4-binding protein. These results indicate that staphylococcal surface proteins may play an important roles in survival of bacteria in human blood by modulating complement activation.

Publications

McCormack, N., Foster, T.J., Geoghegan, J.A. A short sequence within subdomain N1 of region A of the *Staphylococcus aureus* MSCRAMM clumping factor A is required for export and surface display. Microbiology. 2014 Jan 24. doi: 10.1099/mic.0.074724-0. [Epub ahead of print]

McCormack, N., Foster, T.J., Geoghegan, J.A. The N1 subdomain of ClfA is dispensable for the classic biological functions of ClfA on the surface of *Staphylococcus aureus* and its loss does not affect antigen recognition. (Manuscript in preparation)

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Key to abbreviations

Single letter amino acid code

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

Nucleotides

A	Adenine
T	Thymine
C	Cytosine
G	Guanine

Key to abbreviations	
Amp	Ampicillin
ATc	Anhydrotetracycline
aa	amino acid
BHI	brain-heart infusion medium
bp	base pair(s)
BSA	bovine serum albumin
Cm	Chloramphenicol
C4BP	C4 binding protein
DNA	deoxyribonucleic acid
dNTP	deoxy nucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
Em	Erythromycin
Fg	fibrinogen
FH	factor H
FI	factor I
Fn	fibronectin
FnBP	Fibronectin binding protein
GST	glutathione S-transferase
h	hour(s)
Ig	immunoglobulin
V_H	variable immunoglobulin heavy chain
Ck10	cytokeratin 10
Ka	Kanamycin
kb	kilobase pair
kDa	kilodalton
min	minute(s)
MSCRAMM	Microbial Surface Component Recognising Adhesive Matrix Molecule
nt	nucleotides
OD	optical density

PBS	phosphate buffered saline
Phyre	Protein Homology/anologY Recognition Engine
PCR	polymerase chain reaction
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
s	second
Tris	trishydroxymethylaminomethane
TSA	trypticase soy agar
TSB	trypticase soy broth
v/v	volume per volume
w/v	weight per volume
WT	wild-type

Chapter 1
Introduction

1.1 Biology of *Staphylococci*

1.1.1 Classification and identification

Bacteria of the genus *Staphylococcus* are Gram-positive organisms which characteristically divide in three planes to form grape-like clusters (Tzagoloff and Novick, 1977). Taxonomic studies place *Staphylococcus* in a new family, the *Staphylococcaceae*, order *Bacillales*, class *Bacilli (Becker, 2011)*. *Staphylococci* are most closely related to *Enterococcus*, *Bacillus* and *Listeria* and their genomes contain DNA of a low G+C content (30-39%) (Ludwig *et al.*, 1985, Stackebrandt and Teuber, 1988). The staphylococci are resistant to desiccation and are extremely halotolerant growing at up to 3.5 M NaCl.

Staphylococcus aureus (*S. aureus*) is a non-spore-forming and non-motile spherical organism. It forms smooth, raised colonies which have a characteristic golden pigment and are haemolytic on blood agar (Becker, 2011). In addition to its distinct colony morphology *S. aureus* is distinguished from other staphylococci by the ability to ferment mannitol, expression of thermostable DNase and the elaboration of clumping factor (the cell wall-associated fibrinogen binding protein ClfA). In addition, *S. aureus* secretes the zymogen coagulase which can bind to and activate prothrombin. Prothrombin converts fibrinogen to fibrin promoting clot formation. The expression and production of extracellular coagulase differentiates *S. aureus* from coagulase-negative staphylococci (CoNS). *S. aureus* is considered more virulent than CoNS (Marston and Fahlberg, 1960, Fahlberg and Marston, 1960). However, CoNS species such as *S. epidermidis*, *S. lugdunensis* and *S. haemolyticus* can cause serious and life threatening human infections.

1.1.2 Colonisation and disease

S. aureus is a common commensal of humans. Its primary habitat in humans is the moist squamous epithelium of the anterior nares, although it may also be found at secondary sites throughout the body such as the skin, pharynx, axillae and perineum. The nares of approximately 20% of the population are persistently colonised with *S. aureus* while the remainder are intermittent low level carriers (van Belkum *et al.*, 2009). To colonise the anterior nares *S. aureus* must overcome both innate and adaptive immune responses controlled by nasal-associated lymphoid tissue (NALT). Host factors associated with immune responses are believed to play a role in determining carriage

status. However, these factors are poorly understood. Studies have reported that single nucleotide polymorphisms in the genes encoding the glucocorticoid receptor, interleukin-4, C-reactive proteins and complement cascade inhibitor proteins are associated with persistent *S. aureus* nasal carriage (van den Akker *et al.*, 2006, Emonts *et al.*, 2008, Ruimy *et al.*, 2010). In addition, the presence of haemoglobin in nasal secretions may contribute to nasal colonisation by promoting surface colonisation and preventing expression of the accessory gene regulator (Agr) quorum sensing system (Pynnonen *et al.*, 2011).

The epithelium of the anterior nares is stratified and is composed of layers of squamous cells which mature as they progress from basal layers to the exposed surface. Maturation of desquamated epithelial cells or squames involves changes in cell shape, loss of nuclei and an increase in keratinization. *S. aureus* adheres most strongly to mature desquamated epithelial cells or squames found in the superficial epithelial layer. These cells have a flattened, scale-like shape (Peacock *et al.*, 2001). Colonisation of the nares most likely depends on the ability of *S. aureus* to adhere to desquamated epithelial cells and to avoid the host immune response.

Numerous surface-associated proteins of *S. aureus* promote adhesion to desquamated nasal epithelial (squamous) cells *in vitro*. Clumping factor B (ClfB) mediates adhesion to squamous cells by binding two major protein components of the cornified envelope, cytokeratin 10 and loricrin (O'Brien *et al.*, 2002, Mulcahy *et al.*, 2012, Wertheim *et al.*, 2008). Iron-regulated surface determinant protein A (IsdA) is an important component of the haem-acquisition system which also promotes adhesion to squames (Clarke and Foster, 2008, Clarke *et al.*, 2006, Corrigan *et al.*, 2009). This maybe due to the ability of IsdA to bind to the important components of the cornified envelope loricrin, K10 and involucrin (Clarke *et al.*, 2009).ClfB and IsdA support colonisation of the nares of rodents and in the case of ClfB, humans.

The *S. aureus* surface protein G (SasG), the serine-asperate repeat proteins, SdrC and SdrD and SasX (which is expressed only by ST239) also promote adhesion of bacteria to squamous cells although their ligands have not been identified *(Corrigan et al.*, 2007, Corrigan *et al.*, 2009, Li *et al.*, 2012) and the ability of the proteins to promote nasal colinization in rodents has not been tested.

Wall teichoic acid (WTA) is an important component of the *S. aureus* cell wall and has been implicated in nasal colonisation. A mutant of *S. aureus* defective in WTA had decreased adherence to nasal cells and was unable to colonise the nares of cotton rats (Weidenmaier *et al.*, 2004). Furthermore, the WTA biosynthesis genes *tarO* and *tarK* are up-regulated during early colonisation of the nares of cotton rats indicating that WTA may play a vital role during the initiation of nasal colonisation (Burian *et al.*, 2010).

Colonisation is a known risk factor for development of a *S. aureus* infection and patients are usually infected by the isolate that they carry (Wertheim *et al.*, 2005, von Eiff C, 2001, Munoz *et al.*, 2008). However, *S. aureus* bacteraemia-related death is significantly higher in non-carriers compared to carriers. This suggests that carriers may be immunologically adapted to their own strain (Wertheim *et al.*, 2004).

S. aureus is an oportunistic pathogen responsible for a wide range of diseases and a major cause of device-related nosocomial infections, second only to the CoNS species *S. epidermidis*. Superficial skin lesions such as boils, abcesses and impetigo are the most common form of infection caused by *S. aureus,* However, if *S. aureus* enters the bloodstream bacteraemia can occur. Bacteraemia can result in dissemination to and infection of internal tissues such as bone (osteomyelitis), joints (septic arthritis), lungs (pneumonia), and heart valves (endocarditis) (Lowy, 1998).

1.2 Virulence factors

S. aureus can cause a diverse array of infections due to its ability to express a plethora of virulence factors which can be subdivided into three main categories, adhesins, evasins and toxins. Although each category performs distinct roles, it appears that most *S. aureus* infections are facilitated by coordinated expression of multiple virulence factors. Furthermore, *S. aureus* expresses virulence factors which are multifunctional, fulfilling roles in adhesin and invasion or as toxins and evasins.

Adhesion is considered to be critical for the initiation of infection. *S. aureus* expresses numerous cell wall-associated factors which promote adhesion to components of the blood and extracellular matrix. These aid *S. aureus* in adherence to implanted medical devices, damaged tissue and host cells. A wide range of both secreted and surface factors play a role in *S. aureus* evasion of both the innate and adaptive immune response.

The symptoms associated with several *S. aureus* infections are caused by toxins. Toxin-mediated diseases include Toxic Shock Syndrome (resulting from production of Toxic Shock Syndrome Toxin-1 or enterotoxins, primarily of type SeaA or SeaC) (Fraser and Proft, 2008), food poisoning (an intoxication due to ingestion of food contaminated with enterotoxins), and scalded skin syndrome in neonates and young children (caused by elaboration of epidermolytic toxin A or B) (Ladhani *et al.*, 1999).

1.2.1 Cell wall components

Gram-positive bacteria like *S. aureus* are encased within a rigid cell wall. This is composed predominantly of peptidoglycan (~60%) with the remainder being made up of wall teichoic acid (WTA), lipoteichoic acid (LTA) and cell wall-associated proteins. The main function of the cell wall is as a physical barrier which protects against environmental stresses, toxic substances and prevents lysis due to osmotic changes. The cell wall also functions as a scaffold for anchoring of surface proteins which aid bacteria in diverse biological functions (Sjoquist *et al.*, 1972, Schneewind and Missiakas, 2012).

Peptidoglycan consists of a glycan backbone made up of repeating disaccharide units composed of N-acetylglucosamine and N-acetylmuramic acid (GlcNAc-(β1→4)-MurNAc) (Ghuysen and Strominger, 1963). The glycan chains are cross linked by tetrapeptides (L-Ala-D-Glu-L-Lys-D-Ala) to the MurNAc moieties (Ghuysen *et al.*, 1965). This generates the three-dimensional molecular network which maintains the integrity of the bacterium (Ghuysen *et al.*, 1965, Tipper and Strominger, 1965). In *S. aureus* peptidoglycan interpeptide bridges link the tetrapeptide units on neighboring glycan chains. This is polymerized by translocases and transpeptidases (penicillin-binding proteins) to generate peptidoglycan strands that are crosslinked with other strands. This makes *S. aureus* peptides susceptible to cleavage by the glycyl-glycyl endopeptidase lysostaphin (Schleifer and Kandler, 1972). However, *S. aureus* peptidoglycan is resistant to cleavage by lysozyme due to acetylation of MurNAc (Bera *et al.*, 2005).

Wall teichoic acid (WTA) is covalently linked to peptidoglycan and is made up of ribitol-phosphate polymers substituted with N-acetylglucosamine and D-alanine residues (Collins *et al.*, 2002, Ward, 1981, Endl *et al.*, 1983, Brown *et al.*, 2008, Yokoyama *et al.*, 1989). Lipoteichoic acid (LTA) polymers of *S. aureus* consist of

glycerol phosphate units substituted with D-alanine. LTA is attached to the cytoplasmic membrane via a glycolipid anchor and protrudes into the cell wall (Xia *et al.*, 2010). Teichoic acids are important factors in bacterial resistance to environmental stress. WTA confers protection against fatty acids found on human skin (Kohler *et al.*, 2009). Both WTA and LTA enhance *S. aureus* resistance to heat stress and low osmolarity (Oku *et al.*, 2009, Vergara-Irigaray *et al.*, 2008). They also modulate enzyme activity and cation concentrations in the cell envelope. The net negative charge of the bacterial surface is neutralised by D-alanine substitution of WTA and L-lysine modifications of phosphatidylglycerol which promotes resistance to defensins, kinocidins and cationic antibiotics like vancomycin (Collins *et al.*, 2002, Peschel *et al.*, 2001, Peschel *et al.*, 1999, Weidenmaier *et al.*, 2005).

1.2.2 Cell wall-associated surface proteins

The cell wall of Gram-positive bacteria is the first point of contact with the surrounding environment. It acts as a scaffold for proteins that interact with the host during colonisation and infection. Proteins which are covalently anchored to the cell wall promote several major functions including adhesion, invasion, nutrient acquisition and evasion of immune responses (Foster, 2005, Foster, 2013). *S. aureus* can express up to 24 different cell wall anchored proteins which can be differentially expressed under distinct growth conditions. These proteins contain several characteristic features which identify them as cell wall-associated proteins (Fig 1.1). An N-terminal signal sequence (SS) of approximately 40 residues in length targets pre-proteins for secretion across the membrane via the general secretory pathway. The SS contains the "AXA" motif which is recoginsed and cleaved by the membrane-anchored signal peptidases (SpsA and SpsB) during or shortly after translocation (Cregg *et al.*, 1996). A sorting signal is located at the C-terminus of the protein. It comprises a sorting motif, a hydrophobic membrane-spanning domain and at the extreme C-terminus, a stretch of positively charged residues. The last two elements delay secretion across the membrane and facilitate recognition and cleavage of the sorting sequence by sortase A, a lipoprotein located on the outer face of the cytoplasmic membrane (See Section 1.2.2.2) (Schneewind *et al.*, 1993). This facilitates covalent attachment of the protein to the cell wall peptidoglycan (Mazmanian *et al.*, 2001).

1.2.2.1 Export of cell wall-associated proteins

Pre-proteins which are destined to be cell wall-associated possess an N-terminal signal sequence which targets them for export from the cytoplasm. This signal sequence contains the classic tripartite structure which identifies pre-proteins to be translocated across the cytoplasmic membrane by the general secretory (Sec) pathway *(Sibbald et al.*, 2006, DeDent *et al.*, 2008). The classical Sec signal sequence consists of an N-terminal region which is rich in positively charged amino acids, a central hydrophobic region and a C-terminal hydrophilic region which contains the Ala-X-Ala motif. This motif is recognised and cleaved by the membrane-anchored signal peptidases (SpsA and SpsB) during or shortly after translocation (Cregg *et al.*, 1996).

The Sec translocon is conserved across all three domains of life and is responsible for translocating the majority of pre-proteins from the cytoplasm. Unfolded proteins are translocated through a heterotrimeric protein channel which spans the cytoplasmic membrane. The Sec translocon comprises SecY, SecE and SecG. SecY forms the channel through which unfolded proteins pass. SecE and SecG facilitate translocation through the channel. SecE is thought to stabilize SecY in its open conformation which is necessary for translocation, while SecG increases export efficiency. Although not essential, a SecD,F,YajC complex or YidC may become transiently associated with the Sec translocon aiding translocation and (in the case of YidC) membrane insertion of proteins. The SecYEG channel facilitates both post-translational and co-translational export. In Gram-negative organisms this is facilitated by the general secretory chaperone (SecB) or the signal recognition peptide (SRP). These chaperones guide the pre-protein in an export competent state to the Sec translocon. The Sec translocon is powered by the ATPase SecA, which is thought to insert into the base of the translocase and force forward motion of the pre-protein through repeated rounds of ATP hydrolysis (Pugsley, 1993).

Although the Sec system has been well documented in Gram-negative organisms less is known about nuances of secretion in Gram-positive organisms (Schneewind and Missiakas, 2012). The Sec system in *S. aureus* consists of homologues of the canonical SecYEG translocation channel and the SecA ATPase. However no homologue of the general secretory chaperone (SecB) has ever been identified in Gram-positive organisms and there is a dearth of knowledge in the area of protein specific

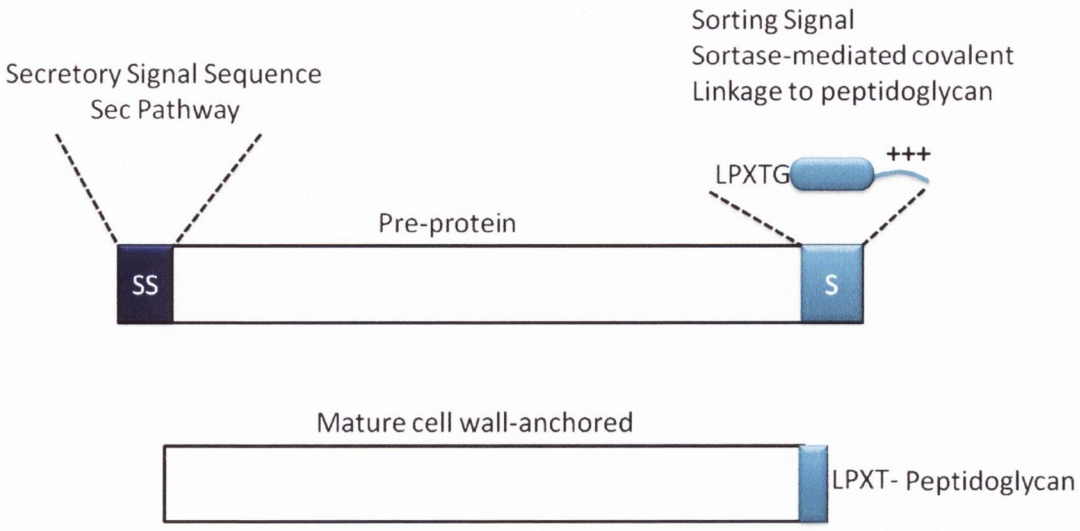


Figure 1.1 Schematic of a generic cell wall-associated protein in its precursor and mature forms.

An N-terminal signal sequence (SS) directs the pre-protein for secretion via the general secretory pathway. Upon translocation the signal sequence is removed by a signal peptidase. At the C-terminus the sorting motif (S) is located. Sortase A cleaves between the threonine and glycine residues of the LPXTG motif covalently anchoring it to the cell wall peptidoglycan. The mature cell wall anchored protein which has been secreted and sorted lacks the N-terminal signal sequence and the C-terminal portion of the sorting sequence containing the hydrophobic region and positively charged tail.

chaperones. Fig 1.2A illustrates the current model for translocation of pre-proteins by the Sec system in Gram-positive bacteria.

Some Gram-positive bacteria express an accessory Sec system composed of homologues to SecA and SecY, designated SecA2 and SecY2. The accessory secretion genes encoding these proteins, *secA2* or *secY2* are encoded on a locus along with genes encoding several accessory secretion proteins (Asps) and glycosyltransferases responsible for glycosylation of the SecA2Y2 substrates (*gtfA, gtfB*). This system is believed to be necessary to facilitate export of a subset of proteins which cannot be translocated via the canonical Sec system. SecA2 and SecY2 can form a novel translocon along with several accessory secretion proteins (Asps) (Fig 1.2B) or interact with the canonical Sec system (Feltcher and Braunstein, 2012).

In *Streptococcus gordonii*, SecA2 and SecY2 are essential for the secretion of the large glycoprotein GspB. GspB is a cell wall-associated protein which binds platelets. GspB is heavily glycosylated in the cytoplasm by the glycosyl transferases GtfA and GtfB which are encoded on the same locus as *secA2, secY2, asp1-5* and *gspB*. Due to heavy glycosylation GspB cannot be transported via the canonical Sec pathway. In addition to its 90 residue N-terminal signal sequence, the first 20 amino acids of mature GspB are required for SecA2/SecY2-mediated translocation by a process that involves also the accessory secretion proteins Asp1–5 (Bensing and Sullam, 2010, Bensing *et al.*, 2005).

S. aureus encodes the *secA2* and *secY2* genes along with genes for the accessory secretion proteins Asp1-3. SraP, a serine rich glycoprotein implicated in platelet binding is the only protein shown to be translocated by this system and is encoded within the *secA2Y2* locus (Siboo *et al.*, 2008).

Secretion of staphylococcal surface proteins is further complicated by the presence of a 'YSIRK/GS' motif present within the signal sequence. Pre-proteins which contain the 'YSIRK/GS' motif are directed for secretion to sites of peptidoglycan biosynthesis at the region of septum formation also known as the cross wall (DeDent *et al.*, 2008). Pre-proteins containing a 'YSIRK/GS' motif include the MSCRAMMs ClfA and FnBPB and the iron-regulated surface determinants IsdB and IsdH. Pre-proteins lacking this motif are targeted to the cell pole. Surface proteins targeted into the cross wall because of their 'YSIRK/GS' motif will eventually become distributed over the

entire bacterial surface. Those that do not are located only at the cell poles. Membrane proteins which contain abortive-infectivity domains, SpdABC (surface protein display), are required for the trafficking of 'YSIRK/GS' proteins into the cross wall (Frankel *et al.*, 2010). However, the mechanisms involved in trafficking pre-proteins to this cellular compartment are yet to be elucidated. Furthermore, it is unclear why proteins such as the iron-regulated surface determinants which act in concert to acquire iron for the cell are targeted to distinct locations.

During or shortly after translocation the signal sequence is cleaved by a membrane anchored signal peptidase. Once across the membrane proteins must rapidly fold to prevent degradation by cell wall-associated proteases. It is unclear how fully folded proteins can migrate through the peptidoglycan. Recently, it has been proposed that channels exist within peptidoglycan of sufficient width to allow fully folded proteins to migrate across the cell wall (Schneewind and Missiakas, 2012).

1.2.2.2 Sortase-mediated anchoring of cell wall-associated proteins

The covalent linkage of proteins to the cell wall of Gram-positive bacteria requires a specific carboxy-terminal sorting signal. In *S. aureus,* two membrane-bound lipoprotein transpeptidases known as sortase (Srt) A and B mediate sorting (Pallen *et al.*, 2001, Mazmanian *et al.*, 2001). For the majority of *S. aureus* surface proteins the C-terminal LPXTG sorting signal is recognised by SrtA. This is followed by a hydrophobic membrane-spanning domain of ~20 residues and a positively charged cytoplasmic tail. This region slows translocation of the protein across the membrane and facilitates recognition by a sortase enzyme and subsequent covalent attachment to the cell wall (Fischetti *et al.*, 1990, Mazmanian *et al.*, 2001, Navarre and Schneewind, 1999). SrtB anchors the iron-regulated surface determinant IsdC to the cell wall. The gene for SrtB (*srtB*) is encoded within the *isd* gene cluster of *S. aureus* and is only expressed under iron restricted conditions. SrtB recognizes the distinct sortase cleavage motif 'NPQTN' present in IsdC (Mazmanian *et al.*, 2002).

The active site of SrtA cleaves between the threonine and glycine residues of the 'LPXTG' motif. A thioester linked intermediate is formed by SrtA and the carbonyl group of threonine. This intermediate is resolved by covalently attaching the surface protein to the pentaglycine crossbridge of lipid II. This restores the enzyme active site. The linked surface protein is then incorporated into the cell wall envelope via the

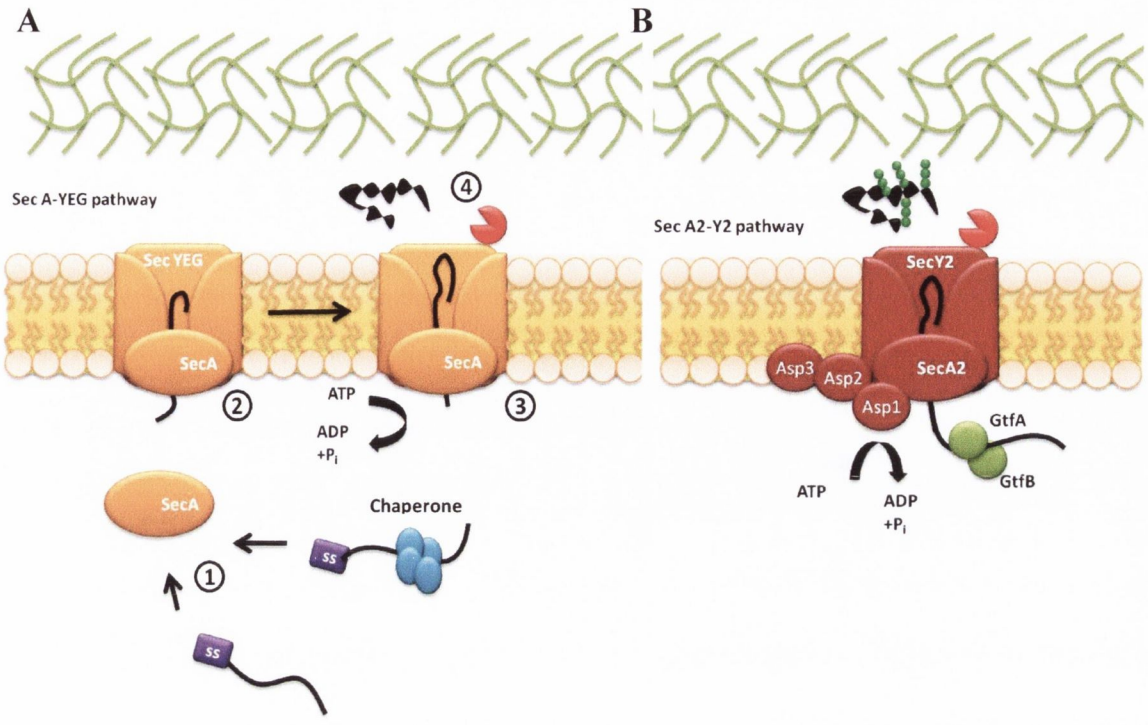


Figure 1.2 Secretion of pre-proteins by the Sec pathway

(A) Pre-proteins synthesized with N-terminal Sec signal peptides are targeted for secretion by the Sec pathway (step 1). Cytoplasmic chaperones may aid in maintaining pre-proteins in an unfolded export competent state and deliver pre-proteins to SecA. SecA delivers the preprotein to a membrane-spanning SecY, SecE and SecG complex (step 2). The signal peptide inserts into SecYEG channel. Export is powered by ATPase SecA which goes through rounds of ATP hydrolysis to promote forward movement of the unfolded preprotein through the SecY channel (step 3). During or shortly after translocation, the signal sequence is removed by the signal peptidases (SP), and the protein then adopts its mature, folded conformation (step 4). After translocation, the exported protein can be anchored to the cell wall peptidoglycan via sortase A. **(B)** The accessory Sec system proteins (Asps) promote SecA2–SecY2-mediated export by unknown mechanisms. They may serve as a scaffold for the export complex or target preproteins to the translocase. SecA2–SecY2-mediated export and glycosylation are thought to be coupled processes. Glycosyl groups (small green circles) are added to the preprotein by cytoplasmic glycosylation factors, GtfA and GtfB glycosyltransferases. SecA2 provides the energy for translocation through the channel formed by SecY2.

transglycosylation and transpeptidation reactions of cell wall biosynthesis (Ton-That *et al.*, 2000) (Fig 1.3).

Recognition of the 'LPXTG' motif by SrtA is stringent and substitutions at positions 1, 2, 4 and 5 are not tolerated (Kruger *et al.*, 2004). The N-terminal catalytic domain of SrtA in complex with an LPXTG peptide has been crystalised (Zong *et al.*, 2004, Liew *et al.*, 2004). The crystal structure revealed that proline and threonine residues of the 'LPXTG' motif form crucial hydrophobic interactions with residues near the sortase active site, holding the ligand in place. SrtA is essential in the correct anchoring of surface proteins involved in colonisation and virulence. In *S. aureus srtA* deficient mutants surface proteins containing an 'LPXTG' motif are not correctly anchored. These mutants are defective in the colonisation of the nares of mice and cotton rats (Schaffer *et al.*, 2006, Weidenmaier and Peschel, 2008) and are attenuated in animal infection models of septic arthritis and endocarditis (Jonsson *et al.*, 2002, Weiss *et al.*, 2004).

1.2.2.3 MSCRAMM family of surface proteins

Cell wall-associated proteins which adhere to components of the blood and extracellular matrix were originally defined as Microbial Surface Component Recognising Adhesive Matrix Molecules (MSCRAMMs). This definition characterized cell wall-associated proteins based on their ability to bind host proteins such as fibrinogen, fibronectin and collagen. However, in recent years it has become clear that not all surface proteins are MSCRAMMs and many MSCRAMMs promote functions other than adhesion. Recently the cell wall-associated proteins have been redefined and split into four groups based on specific motifs which were defined by structure-function analysis (Foster, 2013). Under this new regime the MSCRAMM family is now defined by the presence of at least two tandemly-linked IgG-like folded domains. The MSCRAMMs are the most abundant family of surface proteins encompassing the clumping factors A and B (ClfA, ClfB), the Sdr proteins (SdrC, D, E), the fibronectin binding proteins (FnBPA, FnBPB) and collagen binding protein (CNA). The other families of cell wall-associated proteins include the NEAT motif family, the three-helical bundle family and the G5-E domain family (Foster, 2013) (Fig 1.4). The cell wall-associated proteins for which a structure is not yet known (SasX, SraP, AdsH)

have been separated into a fifth group until both structural and functional information is available to allow correct categorization.

1.2.2.3.1 The Clf/Sdr subfamily of MSCRAMMs

The largest subfamily of MSCRAMMs are the Clf/Sdr subfamily. In addition to an N-terminal signal sequence and a C-terminal wall-anchoring domain, the Clf/Sdr family contains several structural features characteristic of the MSCRAMM family (Fig 1.4). The Clf/Sdr subfamily consist of an N-terminal ligand binding A region which is composed of three independently folded subdomains N1, N2 and N3. In all members of the Clf/Sdr family where ligands have been identified the N2 and N3 subdomains fold independently into IgG like folds and binding is mediated by the 'dock, lock, latch' mechanism. 'Dock, lock, latch' is described in detail in section 1.2.2.3.2.1. The N1 subdomain has no known function and has never been crystallized. Subdomain N1 lacks predicted secondary structure based on homology modeling and is not required for recombinant protein to bind to fibrinogen by 'dock, lock, latch'.

An early study of ClfB showed that the N1 subdomain is composed predominantly of β-sheets and could fold as a discrete unit. Furthermore, gel permeation chromatography indicated that N1 adopts a more elongated conformation than the N23 subdomains. To date this is the only structural analysis of an N1 subdomain available (Perkins *et al.*, 2001) .

The second defining feature of the Clf/Sdr subfamily is the presence of a long, flexible unstructured region linking the A region to the cell wall anchoring domain. This region (R) is composed of Ser-Asp dipeptide repeats. Due to the nature of the repeats this region is predicted to be unstructured. The R region has no known ligand and is proposed to act as a stalk projecting the ligand binding A region away from the surface of the cell (Hartford *et al.*, 1997). Recently the Ser-Asp repeat region of ClfA has been shown to be glycosylated by the glycosyltransferases SdgA and SdgB encoded on the *sdr* operon (Hazenbos *et al.*, 2013).

In the Sdr proteins, the A region and Ser-Asp repeat region border a shorter B-repeat region (110-113 residues). This region has no known function but is believed to maintain a rigid rod-like secondary structure which projects the ligand binding A region from the surface of the cell. The B repeat domains contain a Ca^{2+} binding motif and binding Ca^{2+} is important for structural integrity (Josefsson *et al.*, 1998).

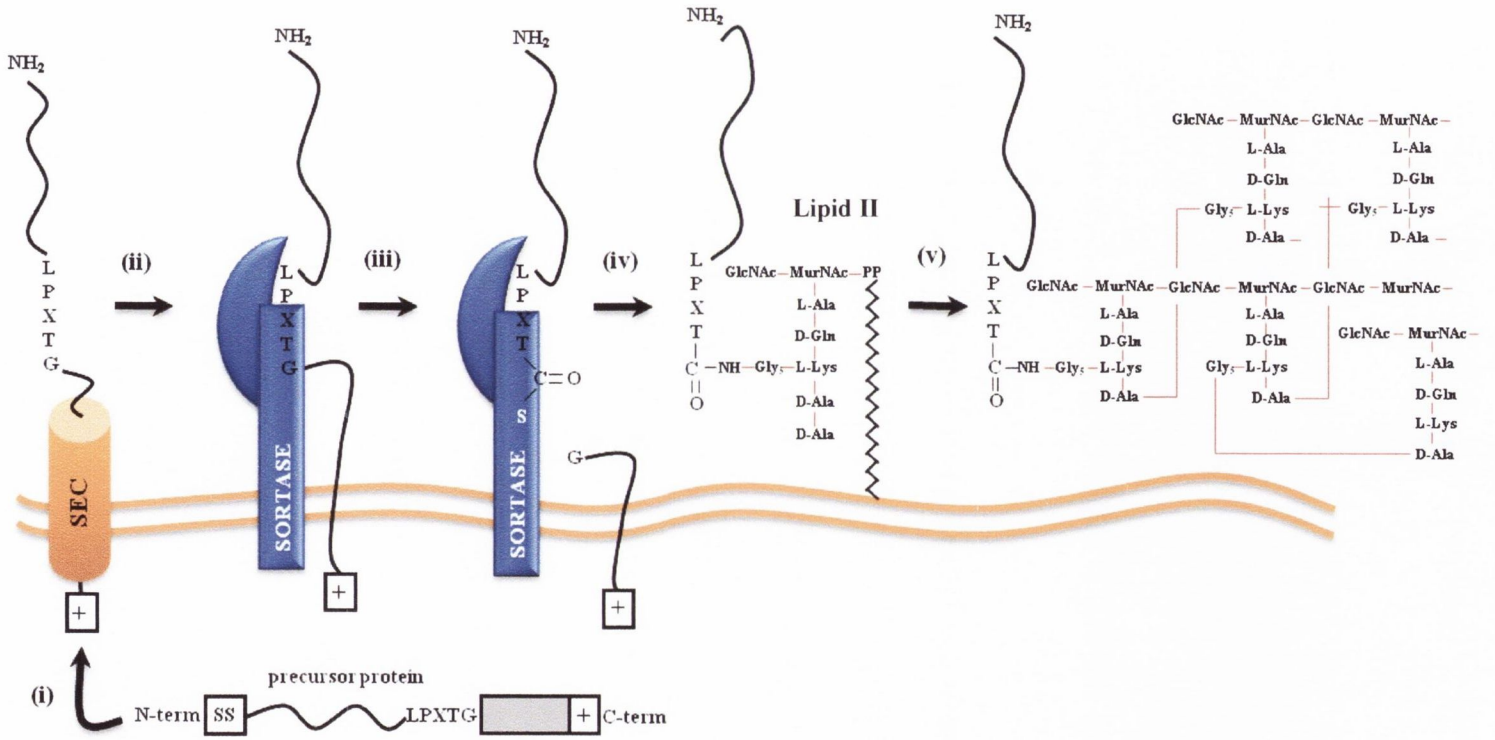


Figure 1.3. Surface protein anchoring in *Staphylococcus aureus*. (**i**) Pre-proteins are directed into the Sec pathway and the signal sequence (SS) is removed. (**ii**) The C-terminal sorting signal retains polypeptides within the secretory pathway. (**iii**) Sortase A cleaves between residues T and G of the LPXTG motif forming a thioester enzyme intermediate. (**iv**) 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 to other cell wall peptides, generating the mature murein tetrapeptide. Adapted from (Mazmanian *et al.*, 2001)

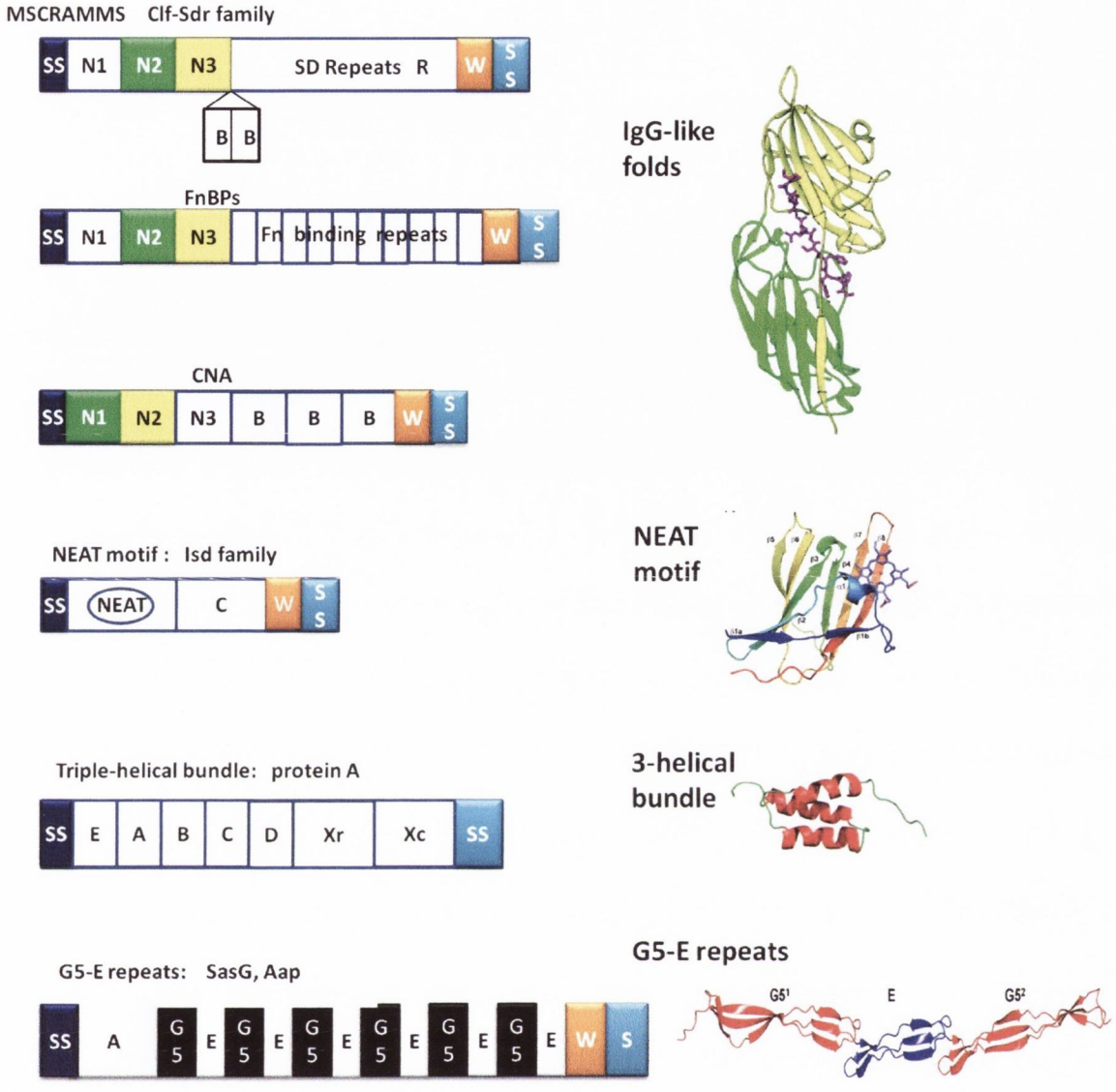


Figure 1.4 Schematic representation and ribbon diagrams of the common motif organisation of staphylococcal surface proteins.

An N-terminal signal sequence (SS) directs the pre-protein for secretion via the general secretory pathway. At the C-terminus the proline-rich wall-spanning domain W or Xc and the sorting motif (S) are located. Surface proteins are categorized based on shared structural and functional motifs. The MSCRAMMs are the largest family of surface proteins and contain an N-terminal A regions which contain two consecutive subdomains that form IgG-like folds (green and yellow boxes correspond to colour coded ribbon diagram). The NEAT motif family (Isd), triple helical bundle family (protein A) and G5-E repeat family (SasG) are also indicated. Ribbon diagrams of the known structures are also indicated to the right of each schematic.

1.2.2.3.1.1 Clumping factor A

Fibrinogen is a large glycoprotein (340 kDa) that is synthesized in the liver by hepatocytes. It is found in blood plasma and as a component of the extracellular matrix. Implanted medical devices such as catheters and prostheses can become rapidly coated by fibrinogen from plasma. This provides a surface to which *S. aureus* can adhere and colonise (Cheung and Fischetti, 1990). Fibrinogen is a hexamer composed of two identical disulfide-bonded subunits (Fig 1.5). Each subunit is formed by an Aα, Bβ and γ polypeptide chain. Fibrinogen can be subdivided into four major structural domains, 2 identical terminal D regions, a central E region, 2 αC regions and 2BβN regions (Doolittle, 1984).

ClfA is the archetypal fibrinogen binding MSCRAMM. The *clfA* gene is expressed at high levels in the stationary phase of growth from a sigma factor B-dependant promoter. Exponential phase expression of ClfA is driven by a weaker sigma 70-dependant promoter (Homerova *et al.*, 2004). Expression of ClfA promotes adherence to immobilized fibrinogen and formation of cell-aggregates in soluble fibrinogen. This is the distinguishing characteristic which confers the ability of *S. aureus* to form clumps in plasma (McDevitt *et al.*, 1994).

The boundaries of the N1, N2 and N3 subdomains of ClfA were originally defined based on similar definitions in ClfB before structures of any of the proteins were solved. The *S. aureus* zinc-metalloprotease aureolysin was found to cleave ClfB between the Ala and the Val residues at a 'SLAVA' motif during bacterial growth. The aureolysin cleavage site was designated as the boundary between N1 and N2 (McAleese *et al.*, 2001). Sequence alignment with ClfB revealed that ClfA contains a similar motif 'SLAAVA'. This predicted cleavage site was used to define the end of the N1 subdomain of ClfA as residue A_{220}.

The X-ray crystal structure of ClfA N23 has been solved both as an apo-protein and with the fibrinogen peptide ligand bound (Ganesh *et al.*, 2008). The fibrinogen γ-chain peptide binds in a hydrophobic trench formed between the separately folded N2 and N3 subdomains by the 'dock, lock, and latch' mechanism (Ponnuraj *et al.*, 2003). The minimum ligand binding domain necessary for fibrinogen adherence spans residues 229-545. This has resulted in two distinct definitions for the C-terminal end of subdomain N1. The first was defined by the predicted aureolysin cleavage motif spans

residues 40-220, and the second defined by the minimum ligand binding domain spans residues 40-228.

The A region of ClfA binds to an unfolded peptide sequence at the extreme C-terminal residues of the γ-chain of fibrinogen (McDevitt *et al.*, 1997). This is the same region of fibrinogen that is recognised by the human platelet integrin GpIIb/IIIa (Farrell *et al.*, 1992). ClfA can interact with and stimulate activation of human platelets by two distinct mechanisms. (1) ClfA forms a fibrinogen bridge between the bacterium and the platelet receptor GpIIb/IIIa. ClfA-specific immunoglobulin interacts with the platelet immunoglobulin Fc receptor (FcγRIIa). Both components are necessary for platelet activation to occur (Fig 1.6A). (2) The second mechanism occurs independently of fibrinogen, instead requiring complement and specific anti-ClfA immunoglobulin (Fig 1.6B). Platelet activation by *S. aureus* is considered to be an important contributing factor in the development of infective endocarditis (Loughman *et al.*, 2005). The biological functions of ClfA which have been validated in animal models for infection are summarised in Table 1.1.

1.2.2.3.2 Fibrinogen binding by MSCRAMMs

1.2.2.3.2.1 The 'dock, lock and latch' mechanism of fibrinogen binding

The 'dock, lock, latch' mechanism was originally proposed for SdrG of *S. epidermidis*. The crystal structure for the N23 region of SdrG has been solved both as an apo-protein and also in complex with a synthetic fibrinogen 15-mer β-chain peptide (Ponnuraj *et al.*, 2003). The apo-protein folds as an open structure containing a ligand-binding trench formed between the IgG-like folds of the N2 and N3 subdomains in the A region. The peptide binds in the hydrophobic trench formed between the N2 and N3 subdomains and adopts an extended conformation interacting with residues in both the N2 and N3 subdomains. Substitution of key residues in SdrG identified S338 and D339 as crucial for interaction with residues F11 and S12 of the fibrinogen β-chain peptide, respectively (Ponnuraj *et al.*, 2003).

Upon ligand binding, the trench is no longer exposed and the protein-peptide complex structure adopts a closed conformation. Comparison of the apo-protein and the bound-ligand form led to the proposal of the 'dock, lock, latch' mechanism (Figure 1.7). The docked ligand is stabilised by residues within the trench with the ligand. This

Table 1.1 Role of cell wall-associated proteins in infection

Role in infection	Cell wall-associated protein	Mechanism	Reference
Endocarditis	ClfA	Adhesion to thrombus	(Moreillon *et al.*, 1995)
	FnBPA	Adhesion to thrombus. Invasion of adjacent endothelium	(Que *et al.*, 2005)
	ClfB	Adhesion to thrombus	(Entenza *et al.*, 2000)
	SraP	Adhesion to platelets. Colonization of thrombus	(Siboo *et al.*, 2005)
Mastitis	FnBP	Invasion of mammary gland epithelial cells	(Brouillette *et al.*, 2003)
Pneumonia	Protein A	Increased inflammation of lung epithelium	(Gomez *et al.*, 2004)
Foreign body infection	FnBP	MRSA biofilm promoted adhesion to intra-aortic patch	(Vergara-Irigaray *et al.*, 2009, Arrecubieta *et al.*, 2006)
Ocular keratitis	CNA	Enhanced colonization and infection	(Rhem *et al.*, 2000)
Septic death Survival in blood	ClfA, Protein A, IsdH, AdsA SasX	Reduced opsonophagocytosis	(Cheng *et al.*, 2009)
Kidney abscess	AdsA, ClfA, Protein A, IsdH	Increased survival in blood stream prior to kidney infection	(Cheng *et al.*, 2009)
	IsdA, IsdB, SdrD	Initiation of abscess. Iron acquisition Initiation of abscess, unknown function	
	ClfB, SrdE, SasG	Development of abscess	
Septic arthritis	ClfA, Protein A	Improved survival in blood prior to invasion of joint	(Josefsson *et al.*, 2001) (Palmqvist *et al.*, 2002)
	CNA	Enhanced survival in blood & adhesion to joint cartilage	(Patti *et al.*, 1994)

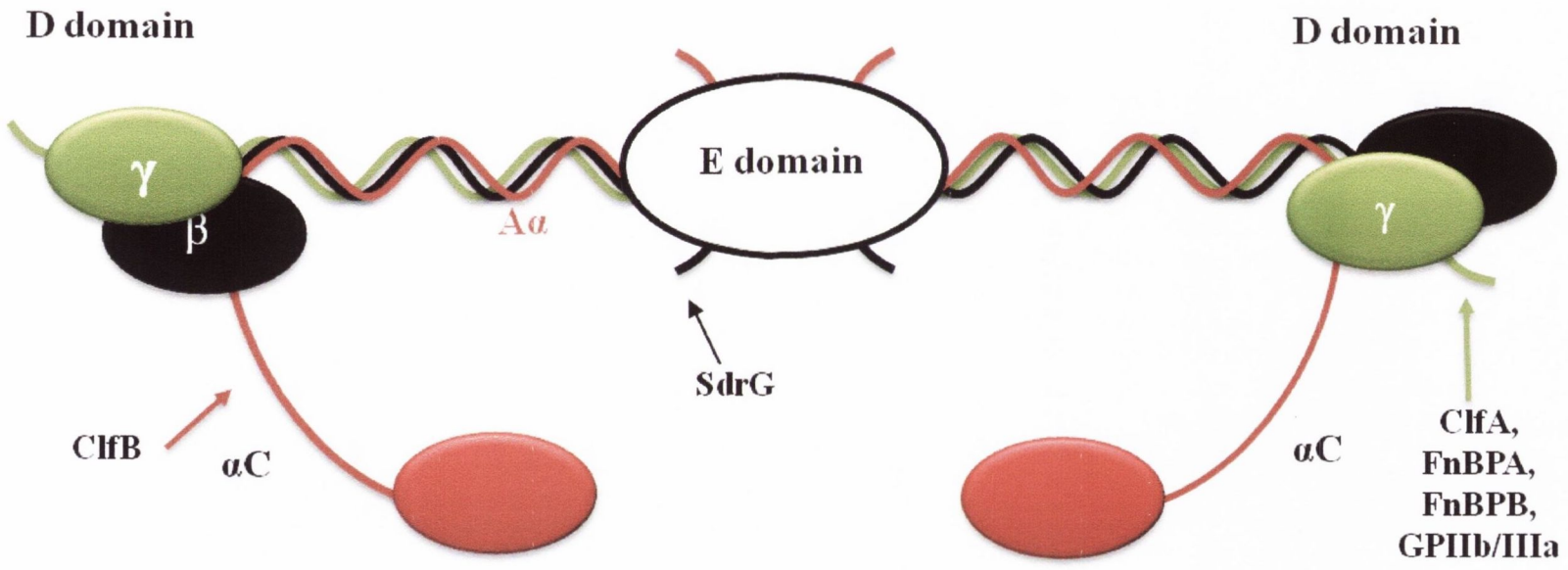


Figure 1.5. Structure of human fibrinogen.

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

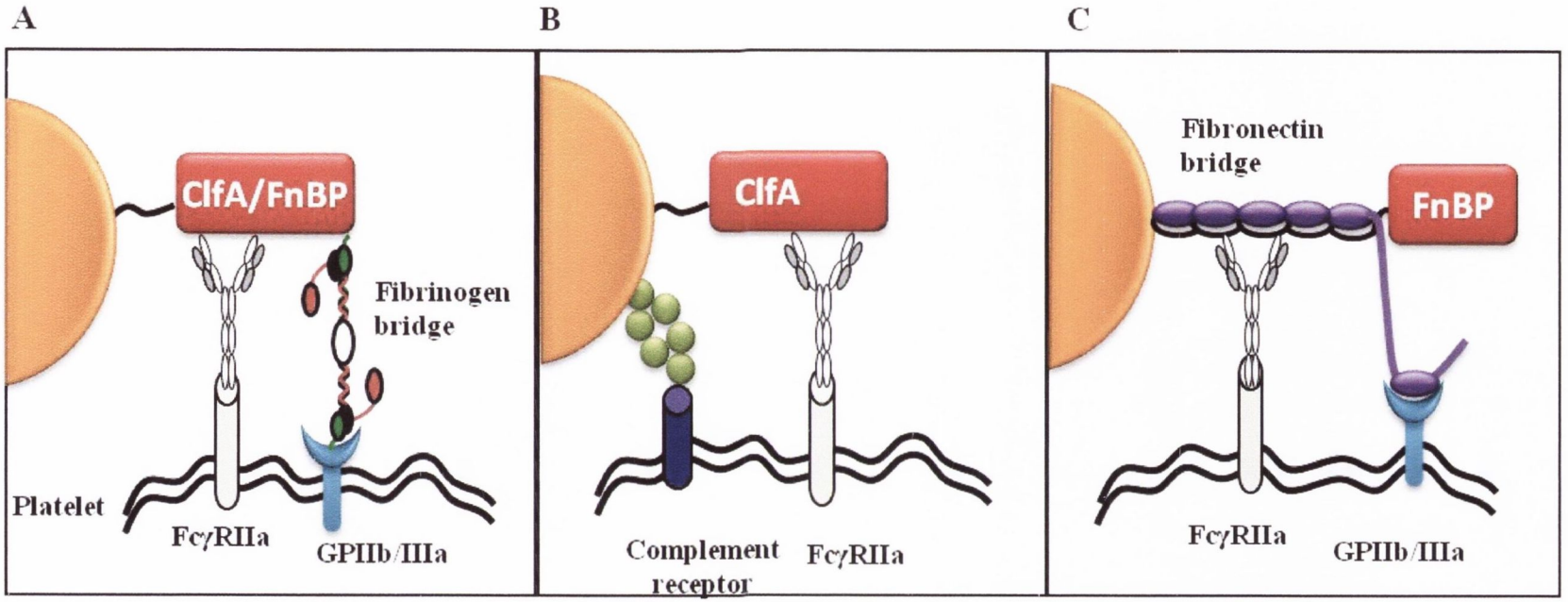


Figure 1.6 Mechanisms of MSCRAMM mediated interaction with platelets

(A) Fibrinogen mediated platelet activation. *S. aureus* expressing fibrinogen binding MSCRAMMs 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 (green circles) on the bacterial surface which cross-links bacteria to a complement receptor on platelets. Specific antibodies are required to engage platelet receptor FcγRIIa. **(C)** Fibronectin mediated platelet activation. *S. aureus* expressing FnBPs can become cross-linked to platelets via surface bound fibronectin (purple ovals). Activation also requires specific antibodies to crosslink bacteria to the platelet Fc recpetor.

interaction induces a conformational change where a C-terminal extension in the N3 subdomain is reoriented to cross over the ligand binding trench (Fig 1.7, blue β-strand). This extension covers the bound ligand, locking it in place. The final latching step is β-strand complementation. A short C-terminal latching peptide inserts between β-strands in the N2 subdomain (the latching trough) creating a new β-strand (G′′) in the N2 subdomain (Fig 1.7 red β-strand).

This mechanism and the importance of the locking and latching steps was proven experimentally for SdrG. A variant of SdrG which was constrained in a closed form by a disulfide bond formed by engineered Cys residues in the β-strands E and G′′ at the base of the latching trench was unable to bind fibrinogen or the β-peptide (Bowden *et al.*, 2008). This demonstrated that the flexibility of the C-terminal extension is essential for 'dock, lock, latch'. Furthermore a truncate of recombinant SdrG lacking the C-terminal latching extension was unable to bind (Ponnuraj *et al.*, 2003).

The crystal structure of ClfA both in complex with the synthetic fibrinogen γ-chain peptide and as an apo-protein confirmed that ClfA employs a variation of the 'dock, lock, latch' mechanism (Ganesh *et al.*, 2008). A variant of recombinant ClfA forced into a closed, "lock latch" conformation where the C-terminal extension of N3 was held at the base of the latching trough by a disulfide bond retained the ability to bind the fibrinogen γ-chain peptide. This suggests that in contrast to SdrG, an open conformation not be required by ClfA as the γ-chain peptide can still gain access to the ligand binding trench. In addition, in SdrG the bound peptide formed an anti-parallel β-sheet with the C-terminal extension of ClfA, whereas in ClfA the bound peptide formed a parallel β-sheet. Ribbon diagrams of the N23 subdomains of SdrG, ClfA and ClfB are shown with their ligands docked in the binding trench to illustrate the conserved mechanism of 'dock, lock, latch' (Fig 1.8).

1.2.2.3.2.2 A second fibrinogen binding site within ClfA

ClfA has a higher affinity for whole fibrinogen than for the γ-chain peptide which mimics the C-terminal residues that bind to the trench in "dock, lock, latch". Binding of recombinant ClfA A regions to fibrinogen could only be inhibited by 60-70% by the γ-chain peptides, whereas in contrast FnBPA was inhibited from binding to fibrinogen by 100% (Wann *et al.*, 2000). These observations suggest that the A region

of ClfA contains a second fibrinogen binding site. The second binding site was mapped to the top of the N3 subdomain and overlaps with part of the epitope recognised by the monoclonal ClfA antibody 12-9. 12-9 is a function blocking monoclonal antibody which recognizes an epitope at the top of N3 distinct from the γ-chain binding trench. This antibody inhibited recombinant ClfAN23 binding to fibrinogen, but not to a His-tagged γ-chain peptide. Single alanine substitutions in residues located in this region of the N3 subdomain reduced fibrinogen binding significantly. This led to the proposal of a revised model where ClfA initially interacts with the extreme C-terminus of the γ-chain of fibrinogen by the 'dock, lock, latch' mechanism. The second binding site then interacts with a site within the D-domain of fibrinogen (Ganesh VK, (manuscript in preparation)). A schematic of the proposed mechanism is illustrated in Fig 1.9.

1.2.2.3.3 The fibronectin binding protein subfamily of MSCRAMMs

The second subfamily of MSCRAMMs are the fibronectin binding protein family (FnBPs). FnBPs are multifunctional proteins which mediate adherence to fibrinogen, elastin and fibronectin. FnBPA and FnBPB are encoded by the closely linked and independently transcribed *fnbA* and *fnbB* genes (Jonsson *et al.*, 1991). Expression of FnBPs occurs in the exponential phase of growth in most laboratory strains. However FnBP expression has been detected in late stationary phase in BH1CC, a clinical isolate of *S. aureus* which is deficient in proteases (Geoghegan *et al.*, 2013).

In addition to an N-terminal signal sequence and a C-terminal wall anchoring and sorting domain, the FnBP family shares key structural features with the Clf/Sdr family (Fig 1.4). The A region is composed of three independently folded subdomains N1, N2 and N3 (Deivanayagam *et al.*, 2002). The N1 subdomain lacks predicted secondary structure and has no known function. The N2 and N3 subdomains adopt IgG-like folds which promote binding to both fibrinogen and elastin most likely by 'dock, lock, latch' (Keane *et al.*, 2007).

The A region of FnBPs is linked to the cell wall anchoring domain by a long, flexible unstructured repeat region. This region is composed of multiple (10/11) tandemly arrayed motifs which promote binding to the N-terminal type I repeats of fibronectin by the β-tandem zipper mechanism described in section 1.2.2.3.3.1 (Schwarz-Linek *et al.*, 2003).

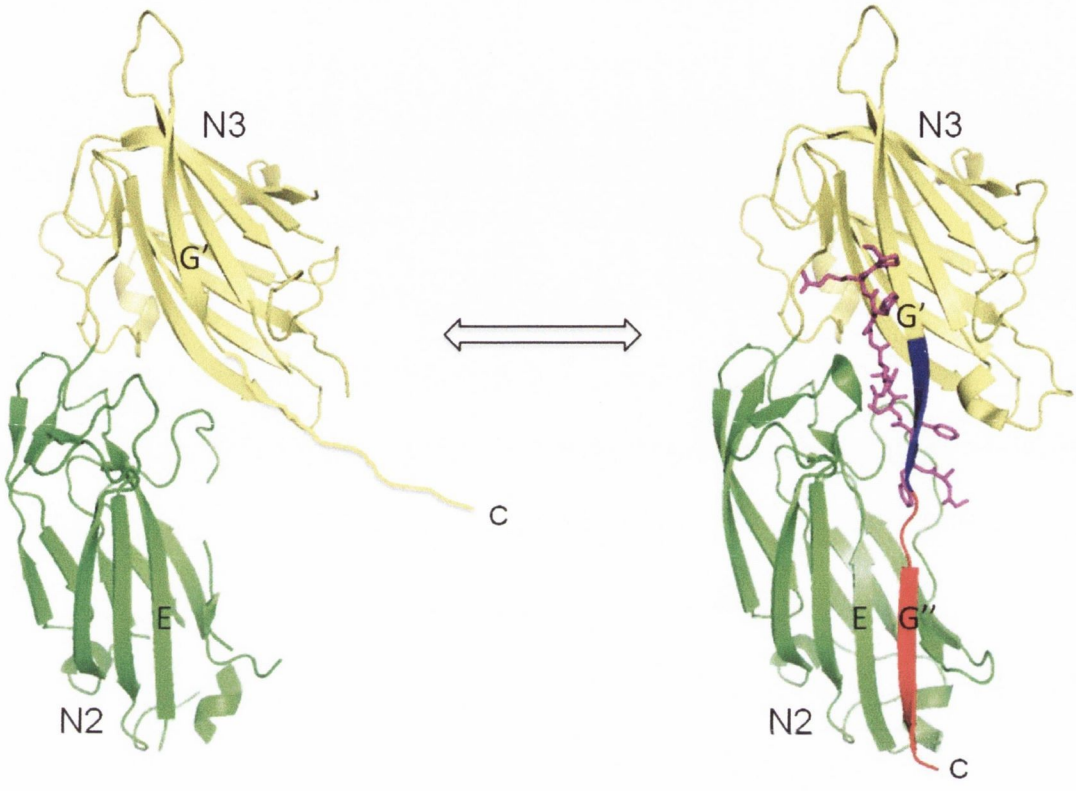


Figure 1.7 Dock, Lock, Latch mechanism.

Ribbon diagram of the 'dock, lock, latch' mechanism of fibrinogen binding by SdrG. Docking of the peptide in the binding trench induces a conformational change in the flexible extension of the C-terminus of the N3 subdomain which is reoriented to cover the ligand peptide and "locks" it in place (blue). Latching is completed by formation of an extra β-strand which is complementary to a β-sheet of the N2 domain (red). The MSCRAMMs ClfB, FnBPA and FnBPB bind to fibrinogen by a similar mechanism. In all cases the ligand binding is confined to subdomains N2N3. Adapted from (Foster et al, 2013).

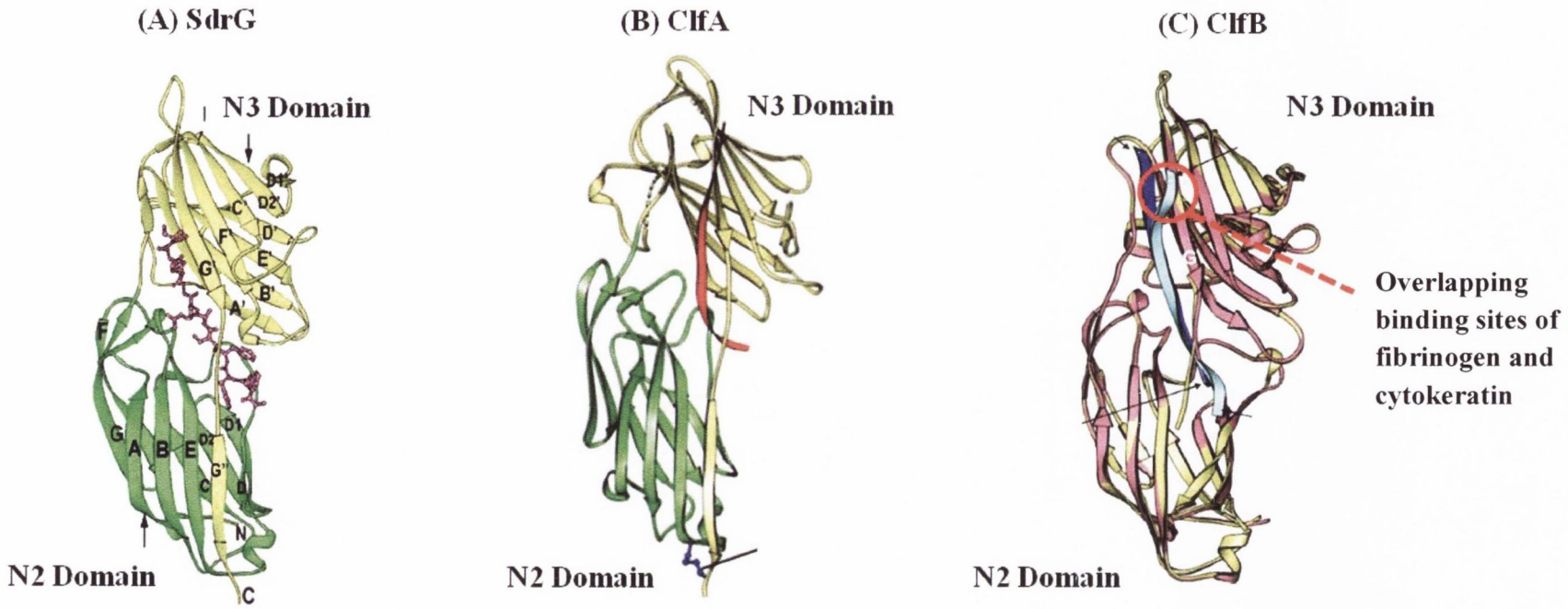


Figure 1.8 Subdomains N23 of SdrG, ClfA and ClfB in complex with their ligands.

Ribbon diagrams N23 domains of SdrG, ClfA and ClfB. **(A)** The fibrinogen β-chain is shown in ball and stick form (purple) in complex with SdrG (adapted from Ponnuraj *et al.*, 2003). **(B)** The fibrinogen γ-chain C-terminal region (red ribbon) in complex with ClfA (adapted from Ganesh et al, 2008). **(C)** Comparison of the overlapping binding sites of the fibrinogen α-chain (cyan) and human cytokeratin 10 (dark blue) in complex with ClfB (adapted from Ganesh *et al*, 2011).

Figure 1.9 The second fibrinogen binding site in ClfA

Ribbon diagram of the N23 subdomains of ClfA in complex with fibrinogen (purple). The γ-chain of fibrinogen binds in the hydrophobic trench at the junction of the N2 and N3 subdomains. A second site at the top of the N3 subdomain forms additional interactions with the D-domain of fibrinogen.

Binding to fibronectin promotes internalization into mammalian cells such as endothelial cells, epithelial cells, osteoblasts and keratinocytes (Dziewanowska *et al.*, 1999, Peacock *et al.*, 1999). Fibronectin acts as a bridging molecule between the FnBPs on the bacterial cell and the host cell integrin α5β1 which binds fibronectin via its RGD motif (Fig 1.6C) (Massey *et al.*, 2002, Fowler *et al.*, 2000, Sinha *et al.*, 1999). This leads to integrin clustering. The interaction triggers phosphorylation at the C-terminal cytoplasmic domain of the integrin leading to cell signalling events, actin rearrangements and subsequent bacterial internalisation by endocytosis (Schwarz-Linek *et al.*, 2004). The biological functions of FnBPs which have been validated in animal models of infection are summarised in Table 1.1.

The fibronectin binding repeat region (FnBR) contains repeats with both high and low affinity for fibronectin (Meenan *et al.*, 2007). A single high affinity FnBR is sufficient to stimulate invasion of bacteria. However, this is less efficient than a variant expressing multiple FnBRs. Furthermore, multiple FnBRs are required for virulence and to promote cellular dissemination. Internalisation of *S. aureus* into mammalian cells is proposed to act as an important survival strategy, simultaneously protecting from antibiotics which poorly penetrate mammalian cells and host defensins to allow further dissemination of the bacteria (Edwards *et al.*, 2010). Upon internalisation bacteria can damage host cells from within by production of cytolytic toxins, triggering necrotic or apoptotic cell death. Alternatively, bacteria can persist in a semi-dormant state as small colony variants which have reduced metabolic rates and are intrinsically resistant to antibiotic treatment (Sendi and Proctor, 2009).

1.2.2.3.3.1 Fibronectin binding by the tandem β-zipper mechanism

Fibronectin is an extracellular matrix glycoprotein found in blood plasma at high concentrations. It contributes to a number of cell processes including adhesion, migration, growth and differentiation (Pankov and Yamada, 2002). The fibronectin molecule is a dimer composed of two covalently linked 250 kDa subunits. The N-terminal domain of fibronectin contains five type 1 modules (F1) which are involved in self association of fibronectin necessary for formation of fibrils. The F1 modules also represent the binding site for FnBPs by a tandem β-zipper mechanism. The structure of two fibronectin binding repeats of FnBPA in complex with F1 has been solved (Bingham *et al.*, 2008, Schwarz-Linek *et al.*, 2003). The fibronectin binding repeats form anti-parallel strands along adjacent type 1 modules of fibronectin. The FnBR of

FnBPA lacks secondary structure. However, upon binding fibronectin it takes on an ordered structure (Fig 1.10). Lacking secondary structure the FnBR of FnBPs are weakly recognised by antibodies in the absence of fibronectin. However, in the presence of fibronectin, the immune response is activated predominantly against neo-epitopes created upon fibronectin binding (Casolini *et al.*, 1998).

1.2.2.3.4 Collagen binding protein

The collagen binding adhesin (CNA) represents the third class of MSCRAMM. Although it contains structural features characteristic of MSCRAMMs, CNA displays a distinct organisation (Fig 1.4). The A region of CNA is linked to its wall spanning region by a B repeat region. The B domain lacks sequence similarity to the B domains of the Sdr family and forms inverse IgG-like folds (Deivanayagam *et al.*, 2000). CNA is a virulence factor in animal models of septic arthritis, endocarditis and osteomyelitis which are summarised in Table 1.1 (Patti *et al.*, 1994, Hienz *et al.*, 1996, Elasri *et al.*, 2002).

1.2.2.3.4.1 The 'Collagen hug' model for collagen binding by CNA

The A region of CNA is composed of three independently folded subdomains, N1, N2 and N3. The crystal structure of the CNA N12 apo-protein and a complex with its ligand has been solved and shows that the N1 and N2 subdomains adopt independent Ig-G like folds responsible for collagen adhesion. The mechanism of collagen binding is similar to the 'dock, lock, latch' model and is known as the 'collagen hug' model (Zong *et al.*, 2005). The collagen triple helix initially interacts with a surface trench in the N2 subdomain. This interaction is of low affinity and is stabilised with polar and hydrophobic interactions. The linker region between N1 and N2 is long allowing subdomain N1 to fold over or 'hug' the collagen triple helix and form multiple hydrophobic contacts with the N2 subdomain. This results in a tube-like structure which locks the ligand between the N1 and N2 subdomains. A C-terminal 'latching' extension of N2 reorientates and inserts into a latching trench within the N1 subdomain by β-strand complementation, stabilising the structure (Fig 1.11).

1.2.2.4 NEAT motif family of surface proteins

The NEAT motif family encompasses cell wall-associated proteins which in addition to secretion and sorting signals contain NEAT (Near Iron Transporter) motifs (Fig 1.4). Cell wall associated proteins in this family help bacteria survive within the

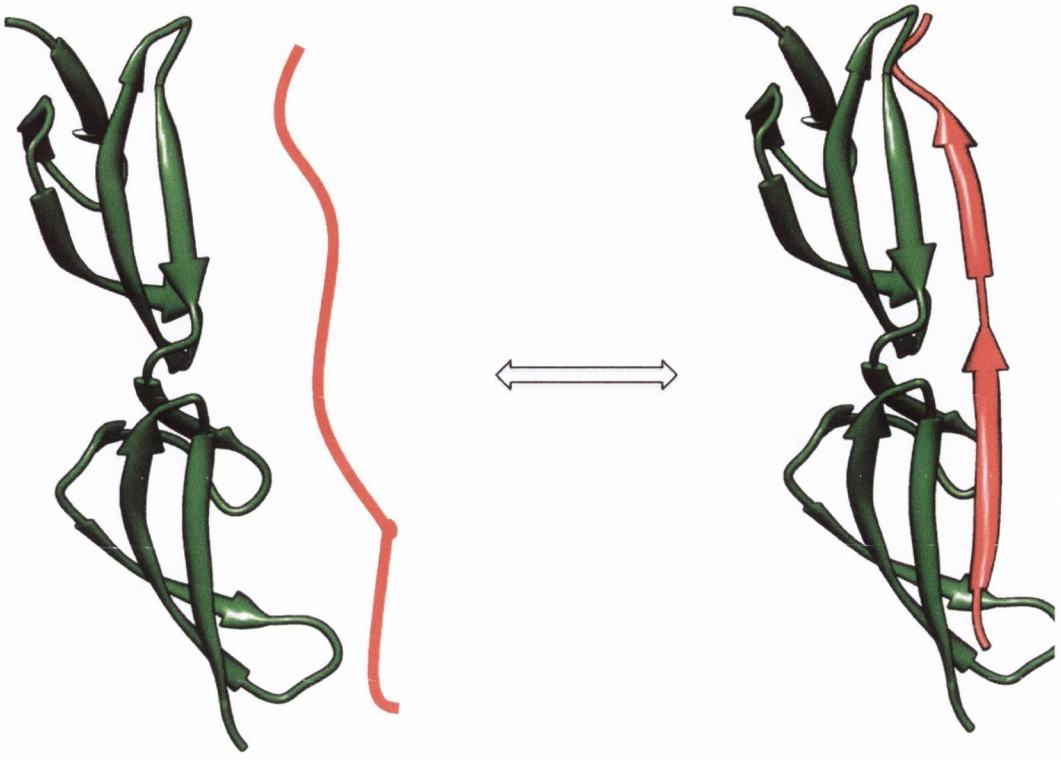


Figure 1.10 The β-tandem zipper mechanism

Ribbon diagram of the β-tandem zipper mechanism. Two fibronectin type 1 modules are shown alone (green) and in complex with one fibronectin binding repeat (red). The fibronectin binding repeats occur as an unstructured linker between the FnBP A region and the wall spanning region. However, upon binding to fibronectin it takes on an ordered structure. Adapted from (Foster *et al*, 2013).

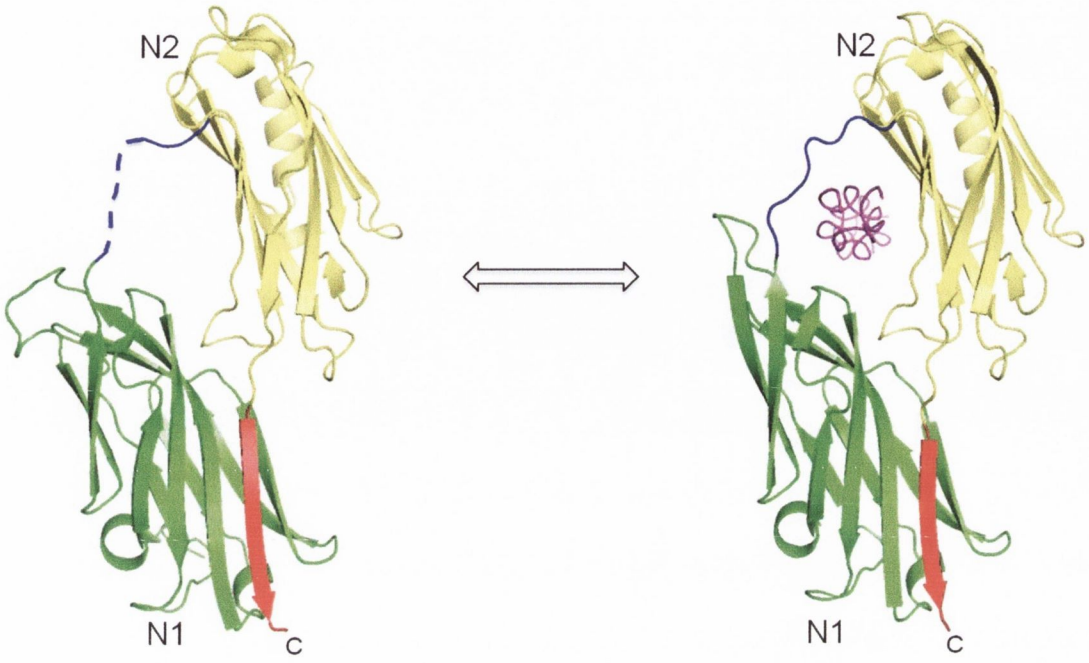


Figure 1.11 The collagen hug

Ribbon diagram of the CNA N12 region as an apo-protein and a complex with its ligand (purple). The linker region between N1 and N2 (blue) allows subdomain N1 to fold over or 'hug' the collagen peptide and form multiple hydrophobic contacts with the N2 subdomain. A C-terminal 'latching' (red β-strand) extension of N2 reorientates and inserts into a latching trench within the N1 subdomain by β-strand complementation, stabilising the structure. Adapted from (Foster *et al*, 2013).

host under iron limited conditions by capturing heme from haemoproteins. The Iron regulated surface determinant (Isd) proteins are cell wall associated proteins characterised by the presence of one or more NEAT motifs (Hammer and Skaar, 2011).

The cell wall associated IsdA, IsdB, IsdC and IsdH proteins contain one to three NEAT motifs. NEAT motifs consist of ~125 residues which adopt a conserved structure despite poor identity in primary sequences. NEAT domains adopt IgG-like beta sandwich folds of 7 or more β-strands in 2 β-sheets (Fig 1.4). Neat domains can be divided into two subclasses, those that bind haemoproteins or those that bind haem. Haem binding requires a conserved tyrosine to capture the porphyrin ring of haem and is utilised by the haem binding domains of IsdA, IsdC, IsdB NEAT2 and IsdH NEAT2. Haemoglobin binding requires aromatic residues to recognise the side chain of haemoglobin and is utilised by IsdB and IsdH (Grigg *et al.*, 2010). In addition, NEAT domains are multifunctional and recognise other host ligands.

Gram-positive bacteria sense iron limited environments via the conserved ferric uptake regulator (Fur). Under iron repleat conditions Fur inhibits transcription of Fur regulated genes by binding to consensus sequences known as Fur boxes. Under conditions of iron starvation Fur repression is alleviated resulting in transcription of numerous genes including the *isd* locus (Xiong *et al.*, 2000).

Expression of the Isd proteins allows coordinated acquisition of iron from the host. Host haemoglobin is initially released when secreted cytolytic toxins such as α-, β-, γ- or δ-toxins lyse erythrocytes. Free haemoglobin is toxic to the host and is collected by haptoglobin, the host haemoglobin carrier molecule. IsdH binds haemoglobin or the haemoglobin-haptoglobin complex, strips it of haem and passes it to IsdB and/or IsdA. Haem is then passed to IsdC and then to the ABC transporter formed by IsdDEF. Alternatively, IsdB can bind directly to haemoglobin release haem and deliver it to IsdA or directly to IsdC. The haem is imported through the plasma membrane into the cytoplasm where it is degraded by haemoxygenases (Grigg *et al.*, 2010, Haley and Skaar, 2012, Liu *et al.*, 2008, Muryoi *et al.*, 2008, Zhu *et al.*, 2008).

In addition to their role in binding haem and haemoproteins other ligands have been identified for the Isd proteins. IsdA binds to fibrinogen and fibronectin. IsdA

promotes bacterial adhesion to desquamated epithelial cells and an IsdA mutant was defective in colonization of the nares of a cotton rat. IsdA mediated adherence to squames may be due to binding components of the cornified envelope such as cytokeratin 10, loricrin and involucrin (Clarke *et al.*, 2006, Clarke *et al.*, 2009). Furthermore vaccination of cotton rats with IsdA or IsdH protected against colonisation (Clarke *et al.*, 2006).

In addition to its roles in ligand binding and nutrient acquisition IsdA promotes survival of *S. aureus* on human skin (Clarke *et al.*, 2007). A mutant of *S. aureus* deficient in *isdA* was more sensitive to killing by bactericidal lipids. Expression of IsdA makes the cell surface more hydrophilic and less susceptible to hydrophobic bactericidal lipids present in sebum. Mutants defective in IsdA expression are also more sensitive to cationic antimicrobial peptides. IsdA binds to and neutralises the anti-bacterial activity of lactoferrin which is thought to promote survival of *S. aureus* at mucosal surfaces (Clarke and Foster, 2008).

Recently IsdB has been shown to promote adhesion and internalisation of *S. aureus* into mammalian cells which express β3–containing integrins. IsdB binds directly to β3–containing integrins and this interaction promotes both adhesion and internalisation (Zapotoczna *et al.*, 2013). IsdH is anti-phagocytic and promotes survival of *S. aureus* in whole human blood. Expression of IsdH induces accelerated degradation of the opsonin C3b to iC3b leading to reduced *S. aureus* uptake by human neutrophils (Visai *et al.*, 2009). The biological functions of Isd proteins which have been validated in animal models of infection are summarised in Table 1.1.

1.2.2.5 Three-helical bundle family of surface proteins

Protein A is the only cell wall-anchored member of the three-helical bundle family. Protein A contains 5 homologous ligand-binding domains (EABCD). Each of these domains comprises a single separately folded three-helical bundle composed of anti-parallel α-helices (Fig 1.4). Distal to the EABCD region are the Xr and Xc region. The Xr is composed of octapeptide repeats which are highly variable in number this contributes to size variation frequently observed in protein A between strains (Moks *et al.*, 1986, Uhlen *et al.*, 1984).

The ligand binding region is localised to the EABCD region and each domain is capable of binding multiple ligands including the Fc region of IgG, the Fab heavy chain

of V_H3 on IgM (Hillson *et al.*, 1993), tumour necrosis factor receptor-1 (TNFR-1) and von Willebrand factor (vWF) (Hartleib *et al.*, 2000, O'Seaghdha *et al.*, 2006).

Protein A binding of the Fc region of IgG coats the bacterial cell with IgG molecules in an incorrect, non-immune orientation. This prevents recognition by neutrophil Fc receptors and inhibits phagocytosis and stimulation of complement fixation by the classical pathway (Foster, 2009). In addition, protein A is a B cell mitogen. It binds to the Fab region of V_H3-class IgM molecules on B-cells causing their activation, proliferation and subsequent apoptotic destruction (Silverman, 1992, Silverman and Goodyear, 2006). This leads to significant depletion of potential antibody-secreting cells and is thought to account for the immunosuppressive activity of protein A.

The crystal structure of domain B of Spa in complex with an Fc fragment has been solved. The Fc binding site comprises 11 residues and spans one face of helices I and II (Deisenhofer, 1981). The binding sites of vWF and TNFR-1 have been localised by site-directed mutagenesis to a region in helices I and II that overlaps the Fc binding site (O'Seaghdha *et al.*, 2006, Gomez *et al.*, 2006). The crystal structure of domain D of Spa in complex with V_H3-Fab demonstrated that the V_H3 binding site spans helices II and III. Distinct residues in helix II bind Fc compared to those that bind V_H3 (Graille *et al.*, 2000) demonstrating that it is possible for a single Spa domain to bind Fc and V_H3-Fab simultaneously. Protein A is an important virulence factor in numerous animal models for human disease summarised in Table 1.1.

Protein A is the only cell wall-anchored protein which contains three-helical bundles. However, the second binding protein of immunoglobulin (Sbi) also contains three-helical bundles (Atkins *et al.*, 2008). Sbi is located in the cell wall by non-covalent attachment to lipoteichoic acid and is also secreted into the extracellular milieu (Smith *et al.*, 2011). Sbi contains two three-helical bundles (D1 and D2) which mediate binding to the Fc region of IgG (Zhang *et al.*, 1998).

1.2.2.6 G5-E domain family of surface proteins

SasG is the prototype of the G5-E domain family in *S. aureus*. In addition to an N-terminal secretion signal and a C-terminal sorting signal this family of cell-wall associated proteins contain G5 domains which are tandemly arrayed and separated by E regions of ~50 residues (Fig 1.4). G5 domains adopt a β-triple helix-β like –fold which

contains 5 conserved glycine residues. The primary sequence of each G5 domain is identical. Typically tandemly arrayed domains with a high level of sequence identity are prone to misfolding. In the G5 domain family alternating G5 and E regions appear to have circumvented this problem (Fig 1.4).

SasG is closely related to the accumulation associated protein (Aap) from *S. epidermidis*. In both SasG and Aap the G5-E region is preceded by an N-terminal region, which is removed to facilitate the biological functions of the protein. In Aap this is mediated by the proteolytic removal of the A region, and in SasG limited autocleavage occurs within the G5-E domains (Rohde *et al.*, 2005, Geoghegan *et al.*, 2010).

Both SasG and Aap promote biofilm formation. Proteolytic removal of the N-terminal A region is necessary to promote biofilm formation. The G5-E region of SasG promotes biofilm accumulation in a zinc-dependent manner. SasG molecules on opposing cells can interact through zinc-dependent self association events between stretches of tandem G5-E domains. This is known as a 'zinc zipper' and leads to extensive cell-cell contact and accumulation (Conrady *et al.*, 2008, Conrady *et al.*, 2013).

The crystal structure of the B domains of SasG has been solved and it revealed that the identical G5 domains form highly extended structures (fibrils) on the surface of the cell (Gruszka *et al.*, 2012). The extended SasG fibrils inhibit adherence to the extracellular matrix proteins fibrinogen, fibronectin and cytokeratin 10 when SasG is expressed at high-level *S. aureus* strains (Corrigan *et al.*, 2007). This observation suggests that SasG may function to mask binding of other adhesins to extracellular matrix ligands, possibly as a method of dissemination into tissue during certain stages of infection.

1.3 Immune evasion mechanisms

1.3.1 Complement

Complement is one of the first lines of defence in the human immune system. The complement system is composed of a family of proteins and their proteolytic fragments that generate a specific and sequential cascade which aids in clearing foreign organisms from the bloodstream. This is achieved either by direct complement killing or targeting the foreign bodies for destruction by white blood cells. Complement fixation

can occur by three distinct but closely liked pathways, the classical, the lectin and the alternative pathways. All three pathways converge upon the activation of the central complement protein C3 (Walport, 2001a, Walport, 2001b).

The classical pathway was the first method of complement activation to be discovered. When circulating antibodies recognize surface antigens of an invading microorganism they opsonise its surface. The classical pathway is activated when the C1q molecule recognizes the antibody-antigen complex. This causes the sequential activation of the serine proteases within the $C1r_2$-$C1s_2$ complex. Activated C1s cleaves the C4 molecule, releasing an N-terminal region of C4, designated C4a. The major portion of C4 remains and is designated C4b. C4b binds to C2 allowing cleavage by C1s to release C2b. C4b retains the catalytically active C2a to generate the classical pathway C3 convertase C4b2a (Fig 1.12i).

The lectin pathway of complement fixation converges with the classical pathway at the formation of the C3 convertase C4b2a. The lectin pathway is activated upon recognition of carbohydrate moieties on the surface of the microorganism by mannose binding protein (MBP) or ficolin. MBP is associated with a serine protease (MBP-associated serine protease or MASP) which adopts a similar structure and role to the $C1r_2$-$C1s_2$complex. MASP cleaves C4 and C2 to generate the C3 convertase C4b2a (Fig 1.12ii).

The C3 convertase activates C3 by proteolytic cleaveage generating C3a and C3b. C3a is an anaphylatoxin and is released upon cleavage of C3. This induces a conformational change within the remainder of the molecule, C3b. In native C3 an internal thioester is buried within the molecule, this becomes surface exposed in C3b where it is free to form covalently linked complexes with any nucleophile. This allows C3b to opsonize the surface of cells (Fig 1.12).

The alternative pathway is distinct from both the classical and lectin pathway because it does not depend upon recognition of specific structures on the surface of a foreign organism. C3 can be activated at a slow rate in the fluid phase by proteolysis generating C3b or nucleophilic attack of its internal thioester by small nucleophiles without the loss of C3a generating $C3(H_20)$ which has a molecular conformation similar to C3b. C3b or $C3(H_20)$ can form the C3 convertase of the alternative pathway by interacting with activated factor B. Factor B is activated by factor D to generate factor

Bb. Bb then interacts with activated C3 to generate C3bBb. In this way the alternative pathway can be initiated spontaneously. However, once it has been activated or C3b has been generated via the classical or lectin pathway the alternative pathway acts as an amplification loop to deposit more C3b on the foreign surface (Fig 1.12iii).

Inappropriate activation of C3 in blood or on non-activating host cell surfaces is tightly controlled by members of the regulators of complement activation (RCA) family. The RCA family fulfills two main functions in the regulation of complement. RCA proteins may either impair the generation of new C3b by accelerating the decay of the C3 convertases or by acting as a cofactor for the serine protease factor I (FI). Members of the RCA family are characterized by the presence of short consensus repeats denoted complement control protein (CCP) repeats. Each CCP repeat comprises ~60 highly conserved residues which adopt a secondary structure composed mainly of β-sheets and turns. Four Cys residues form two disulphide bonds in a 1-3, 2-4 arrangement (Ferreira *et al.*, 2010).

Two important members of the RCA family which are found in the fluid phase are factor H (FH) and C4-binding protein (C4BP). Both proteins are made up almost exclusively of CCP repeats and have both decay accelerating activity and factor I cofactor activity. Factor H regulates complement activation specifically through its interaction with C3b. Factor H can accelerate the decay of all alternative pathways convertases. Furthermore, factor H recognises C3b when it is inappropriately deposited on non-activating host cell surfaces. Activation of complement on host surfaces could cause significant damage to host tissue. Factor H recognises polyanionic host cell structures such as glucosaminoglycans in conjunction with C3b. It is unclear exactly how factor H activates factor I but it is thought that factor H first binds C3b, then factor I binds the C3b-factor H complex (Fig 1.13) (Wu *et al.*, 2009, Soames and Sim, 1997).

Factor H acts as a cofactor for factor I which cleaves the opsonin C3b, generating iC3b to release C3f. iC3b can be further degraded to C3c, C3dg and C3d. This alters both the conformation and binding affinity of C3b (Janssen *et al.*, 2006). While iC3b is still recognized by receptors on the surface of neutrophils it is no longer capable of binding activated factor B to form the alternative pathway C3 convertase. As the alternative pathway acts as a positive amplification loop for all of the complement

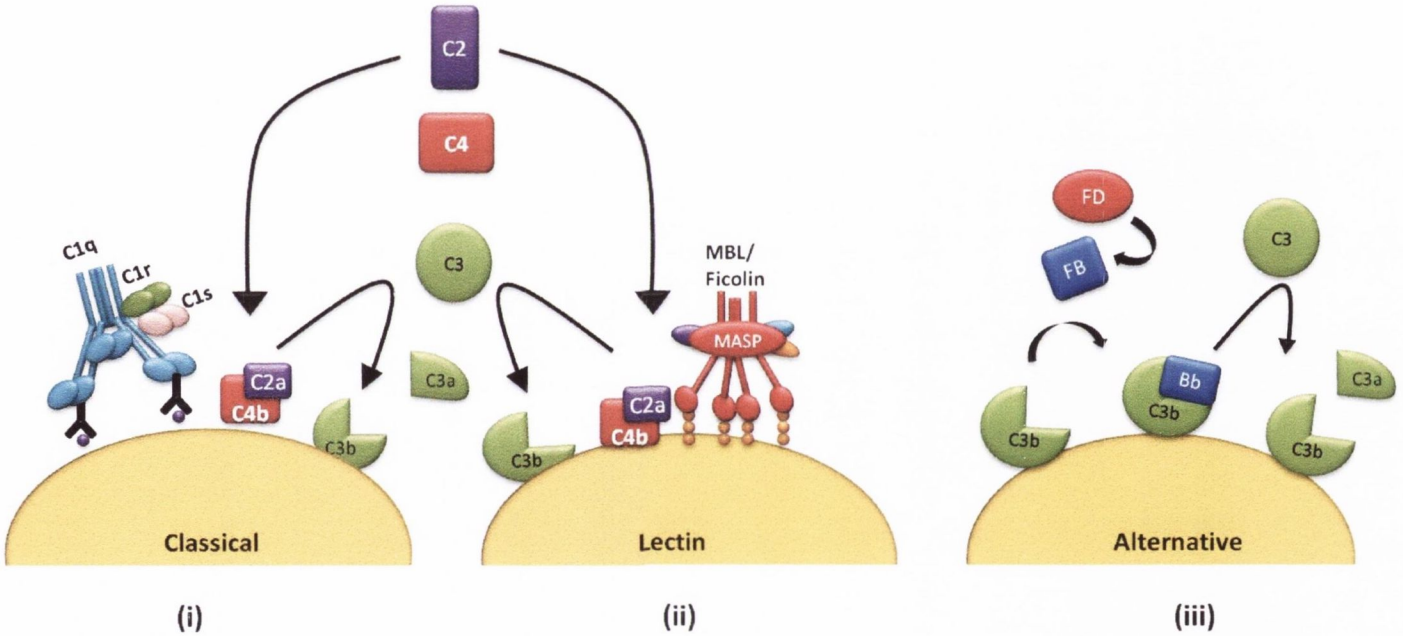


Figure 1.12 Activation of complement

(i) Recognition of antigen-bound IgG by C1q triggers activation of the classical pathway. Activation of the C1r-C1s serine protease complex results in cleavage of C4 and C2 to generate the C3 convertase C4b2a. **(ii)** The lectin pathway of complement activation is triggered by recognition of carbohydrate residues on the microbe surface by mannose binding lectin (MBL). MBL associated serine protease (MASP) cleaves C4 and C2 to generate the C3 convertase C4b2a. **(iii)** The C3 convertase of the alternative pathway is generated when C3b interacts with activated factor B (FB) in the presence of factor D (FD) to generate C3bBb. The cleavage of C3 results in fragments C3a and C3b. The chemoattractant C3a diffuses away while C3b is covalently linked to the cell surface. Accumulation of C3b results in the formation of C5 convertases (C4b2aC3b, C3bBbC3b) that cleave C5 releasing the neutrophil chemoattractant C5a.

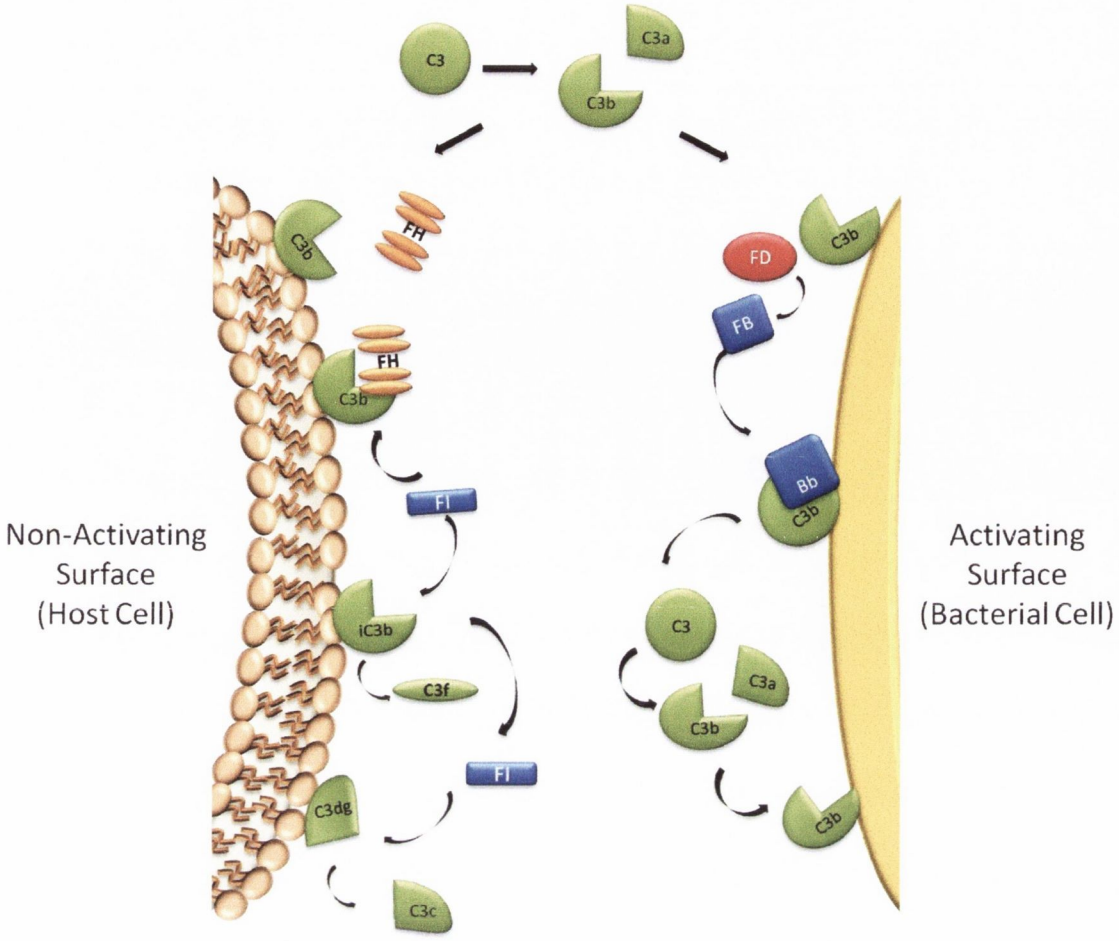


Figure 1.13 Regulation of complement fixation by factor H

Schematic of the regulation of C3b deposition on activating and non-activating surfaces. C3b inappropriately deposited on host cell surfaces is recognized by factor H in coordination with polyanionic host cell surface molecules. Facror I then recognizes the factor H-C3b complex and cleaves C3b to iC3b with the release of C3f. Further degradation of iC3b to C3dg with the release of C3c can occur. C3b deposited on activating bacterial surfaces is bound by activated factor B (Bb) to generate the alternative pathway C3 convertase C3bBb. This readily converts C3 into more C3b to opsonize the bacterial surface generating the positive feed-back loop of the alternative pathway.

pathways, the generation of a dead-end substrate stymies further opsonization of bacteria and subsequent phagocytosis (Walport, 2001a).

C4BP has both decay accelerating and factor I cofactor activities. Its binding specificity is for C4b allowing it to accelerate the decay of the classical and lectin convertases. In its cofactor activity for factor I, C4BP promotes the cleavage of C4b at two sites on either side of its thioester to yield C4c and C4d.

Factor I is a serine protease which regulates activation of the C3/C5 convertases in all three complement cascades. Factor I circulates in blood at a concentration of 35 μg/ml and is produced predominantly by hepatocytes but is also synthesised in monocytes and fibroblasts (Nilsson *et al.*, 2010). Factor I is an 88 kDa multidomain glycoprotein comprising two chains, a heavy chain (50 kDa) and a light chain (38 kDa) which are linked by a disulphide bond. The serine protease activity of factor I is located in the light chain. Little is known about the specific functions of the individual domains in the heavy chain. These have been named based upon their predicted structural similarity to other proteins (Tsiftsoglou *et al.*, 2005). The crystal structure of factor I has not yet been elucidated so the overall structure of the protein is also unknown.

Opsonization of the surface of an invading organism targets it for destruction by the cellular immune response, but complement can mediate direct killing of cells in the absence of phagocytes. This is achieved by formation of the membrane attack complex (MAC). C5 is activated by the C5 convertases C4b2aC3b or C3bBbC3b to form C5a and C5b. C5a is the most potent anaphylatoxin in the complement cascade. Generation of C5b initiates the assembly of the MAC. C5b binds to C6, C7 and C8 in a sequenial order. C5b-8 has membrane binding and weak cytolytic activity, however its main role is to recruit C9 and act as a cayalyst for C9 polymerisation. This yields the C5b-9 MAC which is highly cytolytic. C5b-9 forms a transmembrane channel which is capable of disrupting phospholipid bilayers of target cells resulting in cell swelling and lysis. Due to its thick peptidoglycan cell wall, *S. aureus* is resistant to direct complement killing. The MAC is active mainly against Gram-negative cells and host cells targeted for destruction.

1.3.1.1 Modulation of complement activation by *S. aureus*

1.3.1.1.1 Secreted factors that modulate complement activation

The staphylococcal complement inhibitor (SCIN) protein of *S. aureus* is a 9.8 kDa secreted protein which is expressed during the early exponential growth phase of bacteria (Rooijakkers *et al.*, 2005, Rooijakkers *et al.*, 2006). SCIN is encoded by the *S. aureus* pathogenicity island 5 (SaPI5) (van Wamel *et al.*, 2006). SaPI5 encodes the genes for several human specific secreted immune evasion factors including the chemotaxis inhibitory protein (CHIPS) and staphylokinase (de Haas *et al.*, 2004, Gladysheva *et al.*, 2003) SCIN specifically modulates human complement activation through interactions with the C3 convertases of the classical and alternative pathway. In this way SCIN can inhibit all three complement pathways.

In their normal biological functions the C3 convertases are transiently active. Dissociation of the convertase allows C4b and C3b to act as cofactors for future convertases. SCIN stabilises the C3 convertases preventing the dissociation of C2a and Bb and can form stable C3bBb:SCIN dimers (Rooijakkers *et al.*, 2005, Rooijakkers *et al.*, 2009). By alterning the stability of the convertase SCIN blocks the amplification loop preventing further complement activation. This inhibits deposition of the serum opsonin C3b on the bacterial cell surface, formation of the C5 convertase and generation of the potent chemoattractant C5a ultimately inhibiting phagocytosis of *S. aureus* by human neutrophils (Rooijakkers *et al.*, 2005).

S. aureus encodes two 9.8 kDA secreted SCIN homologs SCIN-B and SCIN-C. Both SCIN-B and SCIN-C inhibit opsonisation, complement-mediated phagocytosis and all three complement pathways (Jongerius *et al.*, 2007). Opsonisation of *S. aureus* in the presence of SCIN-B and SCIN-C results in increased levels of bacterial cell surface-bound C2a and factor Bb. This suggest that SCIN-B and SCIN-C act to stabilise the C3 convertases preventing the release of C2a and factor Bb (Jongerius *et al.*, 2007).

Staphylokinase (SAK) is a secreted protein with potent anti-opsonic activity. Host plasminogen can deposit on the surface of invading bacteria. SAK binds and activates surface-bound plasminogen into plasmin, a potent serine protease. Deposition of plasmin on the bacterial surface results in cleavage of IgG and C3b. Destruction of opsonins inhibits phagocytosis *in vitro* (Rooijakkers *et al.*, 2005).

Extracellular fibrinogen-binding protein (Efb) and extracellular complement-binding protein (Ecb) are small secreted proteins which can modulate the alternative pathway convertase by binding to the C3 molecule directly (Jongerius *et al.*, 2007). Both proteins bind the C3d thioester-containing domain of C3. Binding to C3d results in a conformational change in the C3 molecule that renders it unable to be activated by proteolytic cleavage of C3a (Hammel *et al.*, 2007).

The metalloprotease aureolysin cleaves C3 in the fluid phase generating functional derivatives similar to C3a and C3b blocking all three complement pathways. This inhibits complement-mediated phagocytosis of the bacteria by preventing C3b deposition on the bacterial cell surface. In serum the C3b-like derivative is then degraded by factor H and factor I. This results in inhibition of C5a formation as there is no C3b available to form the C5 convertases. Aureolysin was also shown to inhibit C3a-mediated neutrophil activation by cleaving C3a into an inactive derivative referred to as C3a´ (Laarman *et al.*, 2011).

1.3.1.1.2 Surface proteins that modulate complement

Protein A binds the Fc region of IgG inhibiting its recognition as an opsonin by C1q and inhibiting activation of the classical pathway. Similarly, Sbi binds IgG in a non-immune manner inhibiting classical pathway activation. Although protein A is covalently attached to the cell wall and Sbi is anchored non-covalently to lipoteichoic acid high levels of secreted protein A and Sbi have been reported (Smith *et al.*, 2012). In addition to its role in antibody binding Sbi can also modulate complement activation by binding C3.

Domains D3 and D4 of Sbi bind to the central complement protein C3 (Burman *et al.*, 2008). SbiD3D4 binds to C3 through its thioester-containing C3d domain, which is also present in C3b (Burman *et al.*, 2008). Incubation of recombinant SbiD3D4 in serum results in the formation of a SbiD3D4:C3b adduct (Burman *et al.*, 2008). This is proposed to provide a platform for the assembly of the alternative pathway C3 convertase, C3bBb, that is transiently resistant to decay by factor H. This is thought to lead to the fluid phase consumption of C3 (Isenman *et al.*, 2010). By titrating the C3 convertase of the alternative pathway SbiD3D4 can inhibit activation of all three complement pathways by stymying the crucial amplification loop.

Recently CNA has been shown to inhibit activation of the classical pathway by binding to the C1q molecule. The C1q molecule recognises antibodies on the surface of an invading organism. C1q forms a bouquet structure made up of six identical heterotrimeric proteins which contain collagen-like triple helix structures in the stalk region. CNA can bind to the collagenous domain of C1q and displace the $C1r_2$-$C1s_2$ complex inhibiting activation of the C3 convertase by the classical pathway (Kang *et al.*, 2013).

In recent years, cell wall-associated proteins of *S. aureus* have been identified which directly modulate the activation of complement regulatory proteins. Direct manipulation of members of the RCA family was previously observed in meningococci and group A streptococci. *Neisseria meningitidis* expresses a surface protein capable of binding to complement factor H. This reduces opsonisation of *N. meningitidis* and prevents complement mediated killing (Jongerius *et al.*, 2013). *Streptococcus pyogenes* expresses a multifunctional M protein which is composed of two polypeptides which form an α-helical coiled-coil structure on the surface of the cell. M proteins can bind factor H and C4BP, limiting deposition of C4b and C3b on the surface of the cell and promoting resistance to phagocytosis (Laarman *et al.*, 2010).

In addition to its role in fibrinogen adhesion ClfA has been shown to bind and activate complement factor I. ClfA binding to factor I promotes cleavage of the opsonin C3b to iC3b (Hair *et al.*, 2010, Hair *et al.*, 2008). Although iC3b can still be recognised as an opsonin by phagocyte receptors it can no longer act as a cofactor in the alternative pathway C3 convertase, or the C5 convertase of any pathway. This inhibits further C3b generation and opsonisation. However, the exact location within ClfA responsible for factor I adherence and the mechanism of factor I activation have not yet been elucidated.

SdrE has been shown to bind the RCA protein factor H (Sharp *et al.*, 2012). Bound factor H was capable of activating factor I, promoting cleavage of C3b to iC3b. Expression of SdrE from the surrogate host *Lactococcus lactis* enhanced recruitment of FH with a concomitant increase in iC3b generation. Furthermore, surface expression of SdrE led to a reduction in deposition of C3-fragments, less C5a generation, and reduced killing by polymorphonuclear cells. The RCA protein C4BP has been shown to be recruited by the surface of *S. aureus.* However, its binding partner is not yet known (Hair *et al.*, 2012).

1.3.2 Inhibition of neutrophil migration

In order to exit the blood stream, neutrophils initiate a process known as neutrophil rolling. The P-selectin glycoprotein ligand-1 (PSGL-1) located on the surface of circulating neutrophils binds to P-selectin which is present on the surface of endothelial cells during inflammation. *S. aureus* secretes two proteins which directly inhibit neutrophil rolling, staphylococcal superantigen-like (SSL) 5 and SSL11. Both secreted proteins bind to PSGL-1. This inhibits PSGL-1 binding to P-selectin *in vitro* and prevents neutrophil rolling on activated endothelial cells (Fig 1.14) (Bestebroer *et al.*, 2007).

After neutrophil rolling the next major step in neutrophil migration targeted by *S. aureus* is transmigration across the endothelial cells which line blood vessels. Intercellular adhesion molecule-1 (ICAM-1) on the surface of endothelial cells is bound by the β-integrins Mac-1 and leucocyte function associated antigen-1 (LFA-1) present on the surface of the leucocytes shortly before transmigration. The *S. aureus* extracellular adherence protein (Eap) can bind to ICAM-1 and block the interaction with LAF-1. This reduces neutrophil tight attachment to endothelial cells and subsequent transmigration through the blood vessel (Chavakis *et al.*, 2002).

Neutrophils migrate to the site of infection by detection of a chemoattractants. Invading bacteria generate chemoattractants such as formyl peptides and the anaphylatoxins C3a and C5a (Gasque, 2004), but can inhibit their detection and inhibit migration of neutrophils. The chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a 14.1 kDa secreted protein that is expressed during the early exponential growth phase of the bacteria (de Haas *et al.*, 2004, Rooijakkers *et al.*, 2006). Similar to SCIN, CHIPS is encoded on SaPI5 and is found in over 60% of clinical isolates (van Wamel *et al.*, 2006). CHIPS inhibits chemotaxis by binding directly to the C5a receptor (C5aR) and the formylated peptide receptor (FPR). This blocks the normal binding of C5a and formylated peptide which induce phagocyte activation and chemotaxis (Postma *et al.*, 2004). CHIPS has been shown to reduce neutrophil recruitment towards C5a in a mouse peritonitis model. However, similar to the secreted complement modulator proteins CHIPS is significantly more active against human cells then mouse cells (de Haas *et al.*, 2004). A related protein, FLIPr is secreted by *S. aureus.* FLIPr binds to the formyl peptide receptor like-1 (FPRL-1) receptor on neutrophils inhibiting recognition of formyl peptides (Fig 1.14).

1.3.3 Resistance to phagocytosis

S. aureus utilizes multiple cell wall-associated proteins to avoid phagocytosis by human leucocytes. Protein A and Sbi both bind IgG in a non-immune manner. Sbi, ClfA, SdrE and other as yet unidentified surface proteins modulate complement activation. External to the cell wall secreted proteins modulate complement activation, neutrophil extravasation and chemotaxis.

1.3.3.1 Capsule

Capsular polysaccharide is produced by ~70% of *S. aureus* clinical isolates (Roghmann *et al.*, 2005, O'Riordan and Lee, 2004). There are 11 distinct serotypes of capsule. However, serotypes 5 and 8 are expressed by the majority of isolates. Expression of capsule is associated with increased virulence in animal infection models and reduces phagocytosis *in vitro* (Luong and Lee, 2002, Thakker *et al.*, 1998). Expression of capsule hinders opsonisation by inhibiting antibody binding to the *S. aureus* cell surface (Thakker *et al.*, 1998). Furthermore, expression of capsule prevents recognition of complement components which are assembled beneath the capsule layer by phagocyte complement receptors (Cunnion *et al.*, 2003).

1.3.3.2 Biofilm

S. aureus can form a dense multi-cellular structure or biofilm which protects it from phagocytosis. The first step in biofilm formation is initial attachment. Once inserted, implanted medical devices rapidly become coated in host proteins such as fibrinogen and fibronectin. Surface proteins can mediate attachment to pre-conditioned surfaces. AtlE can mediate initial attachment to an uncoated surface (Heilmann *et al.*, 1997). The second step in biofilm formation is the accumulation step. Proteinacious biofilms can form when cell wall associated proteins form protein-protein interactions between opposing cells. Autolysis of bacterial cells results in release of bacterial DNA. Extracellular DNA (eDNA) is viscous and aids in intercellular adhesion. *S. aureus* can also form a polysaccharide dependent biofilm where cells are held together by the polysaccharide intracellular adhesion, a charged polymer comprising β-1, 6-linked N-acetylglucosamine (PNAG) encoded by the *ica* locus. Expression of PNAG promotes resistance to antimicrobial peptides and reduces uptake and killing of *S. aureus* by neutrophils (Vuong *et al.*, 2004, Kropec *et al.*, 2005).

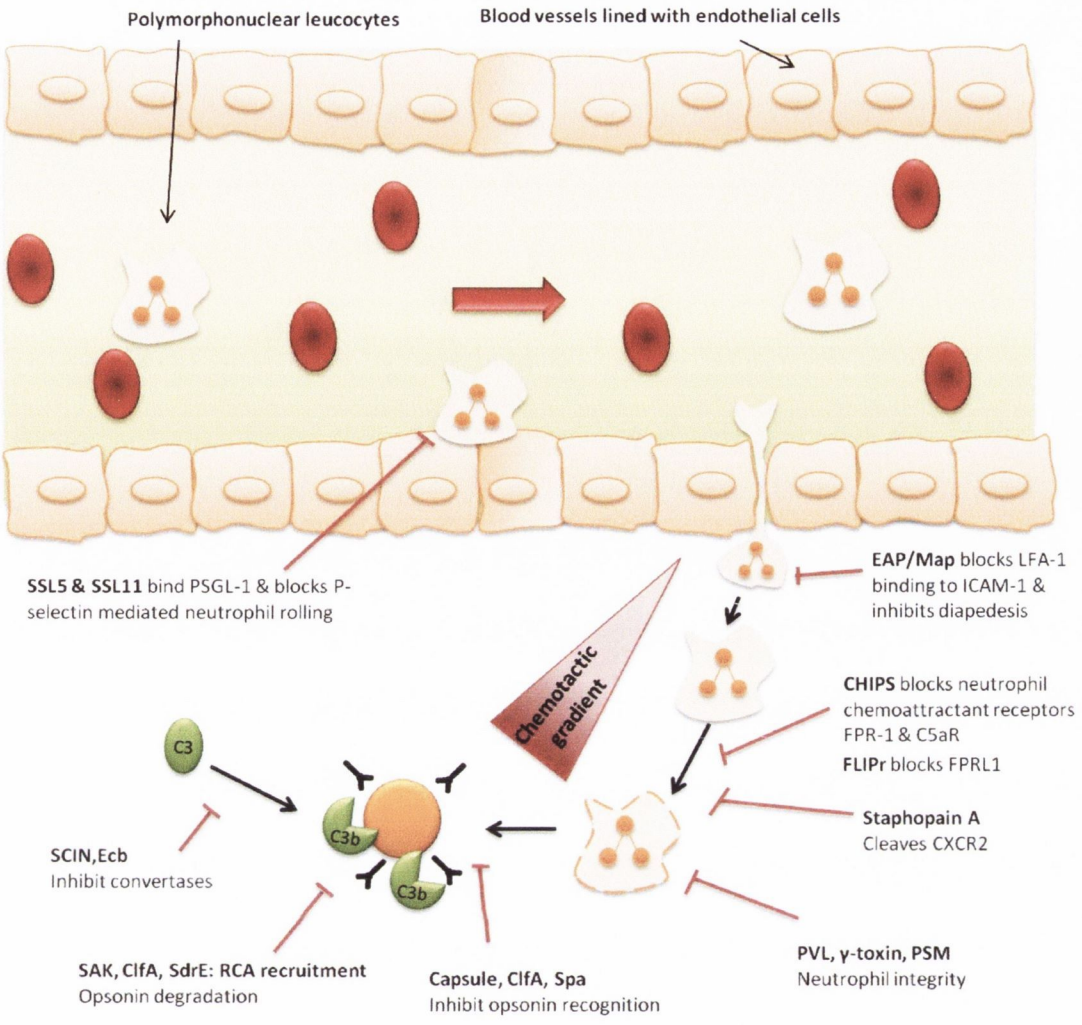


Figure 1.14 Inhibition of neutrophil migration by *S. aureus*

Invading bacteria generate the chemoattractants such as formyl peptides, C3a, and C5a that stimulate the migration of neutrophils to the site of infection. Each chemoattractant diffuses away from the bacteria creating a concentration gradient along which neutrophils migrate, guiding them to the site of infection. To inhibit migration, *S. aureus* secretes proteins that specifically block (i) neutrophil rolling (SSL5 and SSL11) (ii) tight adherence and transmigration (Eap) and (iii) detection of and migration along the chemotactic gradient, (CHIPS, FLIPr and staphopain A). Upon arrival secreted PSMs, γ-toxin and PVL damage neutrophil membrane integrity causing lysis. *S. aureus* is poorly opsonised/opsonins are poorly recognised by neutrophils because of surface elaboration of capsule, ClfA and protein A. Expression of ClfA, SdrE and SAK results in degradation of surface opsonins. Expression of SCIN, Ecb, Ecf and aureolysin inhibit the activity of the complement convertases inhibiting opsonization.

1.3.3.3 Cytolytic pore forming toxins

The elaboration of cytolytic pore forming toxins contributes to the development of abcesses by killing neutrophils which are attempting to engulf and kill bacteria at the site of infection. Cytolytic toxins act by forming β-barrel pores in the cytoplasmic membrane of target cells. This causes leakage and ultimately lysis of the cell.

The archeytypal cytolytic toxin of *S. aureus* is α-haemolysin, encoded by the *hla* gene. α-haemolysin recognizes the membrane associated metalloprotease 10 (ADAM-10) on the surface of human erythrocytes. α-haemolysin is secreted as a monomer but associates into a heptamer, forming a β-barrel. ADAM-10 coordinates formation of the β-barrel in the cytoplasmic membrane (Inoshima *et al.*, 2011).

The second class of cytolytic toxin expressed by *S. aureus* are the bi-component leucotoxins. These are secreted as two distinct subunits which assemble on the leucocyte membrane to form a single heptameric pore. There are four types of bicomponent leukotoxin, γ-haemolysin, Panton-Valentine leucocidin (PVL), leukocidin E/D and leukocidin M/F-PV-like toxins. γ-haemolysin is capable of lysing both erythrocytes and leukocytes while PVL is toxic only for leukocytes (Menestrina *et al.*, 2003).

PVL is encoded by the *lukPV* genes and is associated with CA-MRSA strains. CA-MRSA strains have emerged which cause severe contagious skin infections and severe nectotising pneumonia in young and previously healthy individuals. PVL was initially associated with these cases. However, not all MRSA strains which cause such diseases produce PVL and the role of PVL as a virulence factor has become controversial (Gillet *et al.*, 2002, Lina *et al.*, 1999, Moroney *et al.*, 2007, Voyich *et al.*, 2006, Brown *et al.*, 2009). This controversy stems from the fact that PVL is highly specific for human polymorphonuclear leukocytes and fails to bind to and lyse murine or primate PMNs, although it is active against rabbit PMNs. Expression of PVL enhanced the ability of the epidemic CA-MRSA strain, USA300, to cause lung inflammation and injury in a rabbit model of necrotising pneumonia. PVL contributed to the virulence of USA300 by recruiting and lysing polymorphonuclear leukocytes which damage the lung by releasing their cytotoxic granule contents (Diep *et al.*, 2010).

The third class of cytolytic toxin are the phenol soluble modulins (PSMs). These cytolytic peptides were originally discovered in 1999 but more recently their role in

lysis of red and white blood cells and stimulation of the inflammatory response has been highlighted (Mehlin *et al.*, 1999, Peschel and Otto, 2013). There are three groups of PSM. The smaller α-type (PSMα), the larger β-type (PSMβ) and the δ-type (PSMδ) which is encoded within the coding region for RNAIII, the effector molecule for the accessory gene regulator system (Agr system). PSMs are also found in *S. epidermidis* and related molecules such as slush peptides are found in *S. lugdunensis* (Donvito *et al.*, 1997).

Unlike α-toxin or the bi-component leukotokins PSMs are thought to cause lysis of target cells in a receptor independent process. The receptor for PSMs on neutrophils is formyl peptide receptor 2, although this is not necessary for cytolysis (Kretschmer *et al.*, 2010). Insetead PSMs are thought to bind cell membranes and disrupt their integrity at high peptide cocentrations. This allows them to target almost every eukaryotic membrane. PSMs have differential cytolytic activity. PSMα peptides play an important role in virulence of *S. aureus* skin infections and bacteraemia in animal infection models (Surewaard *et al.*, 2013). PSMα peptides display the highest level of cytolytic activity in animal infection models, PSMδ display moderate cytolytic activity while PSMβ display no cytolytic activity. The receptor for PSMs on neutrophils is formyl peptide receptor 2 (Kretschmer *et al.*, 2010). PSMs are thought to damage neutrophils from within because their toxic activity is neutralized in serum (Surewaard *et al.*, 2013). PSMs have also been shown to facilitate the detachment of cells from biofilm (Wang *et al.*, 2011).

1.3.4 Survival of uptake by neutrophils

When phagocytic cells are activated or lysed they release chromatin and granule contents to form neutrophil extracellular traps (NETs). These provide a net to trap and kill bacteria extracellularly. *S. aureus* expresses a thermostable extracellular DNase which degrades the NETs and allows bacteria to survive in the presence of neutrophils (Berends *et al.*, 2010). In addition to its ability to evade opsonophagocytosis and lyse white blood cells, *S. aureus* had developed mechanisms to survive once phagocytosed.

1.3.4.1 Resistance to antimicrobial peptides and bactericidal proteins

S. aureus modifies its teichoic acids and membrane phospholipids to increase the net positive charge on the surface of the cell. The *S. aureus dlt* operon (*dltABCD*) is is responsible for the addition of D-alanine residues to WTA and LTA (Peschel *et al.*,

1999) while MprF catalyses the modification of membrane phosphatidylglycerol with L-lysine residues (Peschel *et al.*, 2001). This counteracts the negative charge of cationic antimicrobial defensin peptides which are secreted into the phagosome.

Both aureolysin and staphylokinase (SAK) have potent anti-defensin activity. During lung infections, the defensin cathelicidin is present on the airway epithelial surfaces prior to inflammation. One molecule of SAK can bind up to six defensin molecule peptides sequestering the defensins away from the cell (Jin *et al.*, 2004). Aureolysin can cleave and inactivate cathelicidin LL-37 contributing to *in vitro* resistance to this peptide (Sieprawska-Lupa *et al.*, 2004).

Lysozyme and lactoferrin are bactericidal proteins and two important components of the innate defense agains bacterial infection. *S. aureus* encodes a membrane bound O-acetyltransferase which confers resistance to lysozyme by modifying the C6 hydroxyl group of muramic acid (Bera *et al.*, 2005). Lactoferrin is bound and neutralized by IsdA, protecting *S. aureus* from its bactericidal effects (Clarke and Foster, 2008).

1.3.4.2 Resistance to oxidative and nitrosative stress

Engulfment of *S. aureus* triggers an oxidative burst by activating phagosome oxidase. The golden carotenoid pigment of *S. aureus* has anti-oxidant activity by scavenging and forming adducts with oxygen free radicals. Reactive oxygen species damage proteins by oxidizing sulphur in methionine to methione sulphoxide. *S. aureus* encodes two superoxide dismutases *sodA* and *sodM* which remove oxygen free radicals (Karavolos *et al.*, 2003) and three methionine sulphoxide reductases (Singh and Moskovitz, 2003).

$NO^{\cdot}$ radicals are highly toxic because they modify a variety of compounds including haemoglobin and cytochrome C by oxidizing the ferrous haem to its ferric form. *S. aureus* expresses of an $NO^{\cdot}$–inducible lactate dehydrogenase (Ldh) to counteract reactive nitrogen species (Richardson *et al.*, 2008).

The *adsA* gene of *S. aureus* encodes an adenosine synthase (AdsA) which is expressed on the bacterial cell surface. Adenosine regulates innate immune responses and suppresses production of superoxides and nitric oxide. Elaboration of a cell surface adenosine synthase supports bacterial survival within the phagosome (Thammavongsa *et al.*, 2009).

1.4 Vaccination strategies for the prophylactic and therapeutic treatment of *S. aureus*

There is considerable interest in developing an effective vaccine for *S. aureus* due to the fact that it has developed resistance to every antibiotic introduced into clinical practice (Chambers and Deleo, 2009). The ideal vaccine candidate should be present in all strains *in vivo,* either on the surface of the cell or a secreted factor (Spaulding *et al.*, 2014) and be highly immunogenic with no antigenic variation. Furthermore, the ideal candidate vaccine should trigger both opsonising antibodies and immunological memory. Numerous attempts have been made to generate both prophylactic and therapeutic vaccines by passive and active immunization strategies (Table 1.2). No vaccine for *S. aureus* has successfully met its endpoints in clinical trial, despite apparent success in pre-clinical studies. The search for a successful *S. aureus* vaccine is complicated by the pleothora of immunomodulatory factors expressed by *S. aureus* and a dearth of knowledge on the correlates of protection from *S. aureus* in humans.

Colonization or natural infection with *S. aureus* rarely promotes functional opsonophagocytic antibodies in humans and does not induce immunological memory. Furthermore, people with defects associated with antibody production are not at an increased risk of acquiring an *S. aureus* infection (Jansen *et al.*, 2013). Therefore, there is considerable scepticism as to whether antibodies against *S. aureus* will induce protection against infection (Hawkins *et al.*, 2012, Bagnoli *et al.*, 2012). Investigation of the correlates of protection against *S. aureus* infection is complicated by the fact that *S. aureus* is highly adapted to the human host. Host adaptation means that there is no preclinical animal model of infection which fully replicates the natural infection process in humans due to differences in host proteins. In addition, a high challenging dose of bacteria is necessary to produce disease in a non-human host.

Although the most effective way to mount a protective immune response for *S. aureus* is not clear, it is apparent that functional neutrophils are crucial. Individuals who are immunocompimised such as AIDS patients, or those with neutropenia or impaired neutrophil function are at an increased risk of *S. aureus* infection. In recent years it has become evident that an effective *S. aureus* vaccine will likely need to induce both T cell and B cell responses to provide full protection. Antigen specific T cells are crucial for

Table 1.2 Summary of vaccination strategies

Vaccine Name /Manufacturer	Composition	Problems/Limitations	Status	References
StaphVAX,NABI	Capsular polysaccharide 5&8 conjugated to Pseudomonas aeruginosa exotoxoin	• Single antigen approach • Failed to reach primary endpoints	PhaseIII complete	NCT00071214 (Shinefield *et al.*, 2002)
Veronate, Inhibitex(BMS)	Enriched serum from unvaccinated subjects	• Antibodies made through natural exposure are not functional • Neonates do not have a mature immune system • No efficacy seen	PhaseIII complete	NCT00113191 (Vernachio *et al.*, 2003) (Kaufman, 2006)
Pagibaximab, Biosynexus	Anti-LTA	• Lipoteichoic acid may not be accessible to antibody • Neonates do not have a mature immune system • No efficacy seen	PhaseII/III complete	NCT00646399 (Daum and Spellberg, 2012)
V710, Merck/Intercell	IsdB	• Single antigen vaccine • Did not generate opsonophagocytic killing antibodies • No efficacy seen	PhaseI/II/III complete;terminated	NCT00518687 (Kuklin *et al.*, 2006)
PF-06290510, Pfizer	conjugated capsular polysaccharide 5&8, ClfA, and MntC.	• TBD, problem of protein A not addressed	PhaseII	NCT01643941 (Hawkins *et al.*, 2012)
PVL/α-toxin, NABI/GSK	Panton-Valentine Leukocidin toxoid Alpha toxin	• TBD, problem of protein A not addressed	PhaseI complete	NCT01011335

Unnamed, GSK	Tetravalent vaccine; components not disclosed, adjuvanted with GSK2392102	• TBD, problem of protein A not addressed	PhaseI	NCT01160172
Unnamed, Novartis	Tetravalent vaccine; components not disclosed but are all proteins, no adjuvant	• Protein antigens have not been reported to induce robust opsonophagocytic killing antibodies	PhaseI	
SA75, Vaccine International Research	Whole cell vaccine	• Immunisation with whole-cell *S. aureus* vaccines may not induce protective antibodies.	PhaseI	
SAR279356, Sanofi	PNAG	• Single antigen target. • Antibodies and effector cells may not effectively penetrate pre-existing biofilm layer.	PhaseII; terminated	NCT01389700
Altastaph, NABI	Immune serum from subjects vaccinated with StaphVAX	• Single antigen target. • Immune serum from StaphVax	PhaseI; terminated	NCT00066989
Aurexis, Inhibitex	Anti-ClfA mAb	• Single antigen target. • Monoclonal antibodies are not as effective as pAbs. • Prevents binding fibrinogen binding, not bactericidal	PhaseII	NCT00198289 NCT00198302 (Bubeck Wardenburg and Schneewind, 2008)
Medi4893, MedImmune LLC	Anti-α toxin mAb	• Single toxin target.	PhaseI	NCT01769417
KBSA301, Kenta Biotech	Alpha toxin	• Single antigen target	PhaseI/II suspended	NCT01589185

Staphyban, Berne	Whole cell vaccine Alpha toxin toxoid	• Secreted toxin, does not directly kill organism. • Infection already established • Immunization with whole–cell *S. aureus* vaccines may not induce protective antibodies.	Small investigational trial	
Aurograb, Novartis/Neutec Pharma	F(ab) against ABC transporter YkpA Vancomycin	• Infection already established. • Single antigen target. • F(ab) only blocks transporter function. • No Fc portion to facilitate uptake and killing.	PhaseIII; terminated	NCT00217841

Clinical trial references can be accessed at www.ClinicalTrials.gov

generating an optimal antibody response and Th17 cells can enhance neutrophil function potentially increasing bacterial clearance. Adjuvants which can successfully stimulate cell mediated immunity as well as promoting an effective humoral immune response will be needed for an efficacious *S. aureus* vaccine.

Expression of protein A on the surface of *S. aureus* presents a major barrier to its effective opsonisation and subsequent phagocytosis. Recently a variant of protein A containing four key substitutions in each of the binding domains resulting in failure to bind IgG or IgM was generated (SpaKKAA). *S. aureus* expressing the SpaKKAA variant was phagocytosed and elicited B cell responses against important virulence factors protecting mice against a lethal challenge with *S. aureus*. Furthermore, mice vaccinated with *S. aureus* expressing the SpaKKAA variant were protected against subsequent challenge with USA300. This study demonstrates that an effective antibody response can be mounted against *S. aureus* and that the immunomodulatory effects of protein A can be circumvented (Falugi *et al.*, 2013).

A summary of the clinical trials for vaccines against *S. aureus* is presented in Table 1.2. Although previous attempts at vaccine construction have failed important information can be derived from these failures. It is clear the single antigen approach adopted by many vaccines including StaphVAX (capsular polysaccharide) and Mercks V710 (IsdB) may not be sufficient. Strain variation and functional redundancy make single antigens weak targets. Furthermore, immunization with multiple candidate antigens is more protective that vaccination with a single antigen (Stranger-Jones *et al.*, 2006).

Although the efficacy of a humoral response has been questioned in recent years it is perhaps more appropriate to question the type antibody response being elicited. Generation of an antibody response that is not functional and does not elicit opsonophagocytic killing is not sufficient. This is exemplified by Veronate a formulation of enriched serum from unvaccinated subjects. Natural exposure to *S. aureus* does not generate functional antibodies and Veronate was deemed to have no efficacy in Phase III trials. Furthermore, unless the activity of protein A is inhibited passive immunization with antibodies directed against *S. aureus* is unlikely to be successful. This is demonstrated by the failures of Aurexis, Pagibaximab and Altastaph.

In addition to a more critical approach to vaccine design it has been suggested that similar consideration should be put into the cohort to be vaccinated (Jansen *et al.*, 2013). In the past groups at high risk of *S. aureus* infections such as hemodialysis patients and neonates have been selected. Although these cohorts are at high risk of *S. aureus* infection, their underlying morbidity and mortality rates and underdeveloped immune system complicate our further understanding of the correlates of protection against *S. aureus* infection in healthy humans. Hemodialysis patients are immunocomprimised and undergo regular dialysis which may deplete antibodies generated to prevent infection. Passive vaccination with Veronate and Pagibaximab was performed on neonates, but it has been suggested that any protective effect may have been limited by the ability of the neonates immature immune system to use the antibodies supplied to them. It has been suggested that a more appropriate vaccination cohort would be healthy adults undergoing elective surgery (Jansen *et al.*, 2013). Such a study may provide valuable insights into what mediates effective *S. aureus* protection, before vaccination strategies are developed to protect more high risk and immunocompromised individuals.

1.5 Rationale and aims of this study.

The MSCRAMM family of proteins are the most abundant of surface proteins in *S. aureus*. This family encompasses some of the most important adhesins and immune evasion molecules expressed by *S. aureus*. All MSCRAMMs of *S. aureus* contain an A region with an N123 substructure followed by a long, flexible unstructured repeat region, with the exception of CNA. In all such proteins the N1 subdomain lacks predicted secondary structure and is not predicted to be involved in ligand binding. With no discernible structure or ligand binding function it is unclear why the N1 subdomain is retained by the MSCRAMM family.

The aim of this project was to investigate the role of the N1 subdomain. ClfA is the archetypal fibrinogen binding MSCRAMM. For this reason the N1 subdomain of ClfA was investigated as the prototypical N1 subdomain. The first aim of this study was to investigate the role of the N1 subdomain on the surface of *S. aureus* in the biological functions of ClfA. To achieve this ClfA variants lacking most of the N1 subdomain were expressed on the surface of *S. aureus* in a controlled manner. ClfA variants lacking N1 were examined for their ability to promote binding to the two known ligands of ClfA and to promote survival in whole human blood.

The second major aim of this project was to identify why MSCRAMMs retain an N1 subdomain. This study investigated the importance of the N1 subdomain in growth and colony formation of *S. aureus* and identified a new and previously undocumented role for this region in surface expression of ClfA. Through the controlled expression of ClfA variants lacking most of N1 on the surface of *S. aureus* this study identified the specific region required for surface expression. Furthermore this study investigated why MSCRAMMs require subdomain N1 in addition to the signal sequence for appropriate surface elaboration. The study was expanded to encompass a second MSCRAMM, FnBPB allowing the proposal of a universal function for subdomain N1.

The third aspect of this study investigated the interaction between staphylococcal surface proteins and complement regulatory proteins. Initially the interaction between ClfA and human complement factor I was investigated. Recombinant ClfA A regions, individual subdomains and variants with reduced affinity for fibrinogen were examined in an attempt to identify the binding site for factor I. ClfA-factor I binding assays were performed to determine the nature of the ClfA-factor I interaction. Furthermore, attempts to identify potential co-factors for the ClfA-factor I interaction were made. The study was then expanded to demonstrate that several staphylococcal surface proteins are capable of binding complement regulatory proteins including factor H and C4BP.

Chapter 2
Materials and Methods

2.1 Bacterial culture conditions and reagents

Strains used in this study are listed in Table 2.1. *S. aureus* was grown on trypticase soy agar or broth (TSA, TSB, Oxoid) or RPMI 1640 with shaking (200 r.p.m) at 37 °C. For expression studies stationary phase cultures were diluted 1:100 in brain heart infusion broth (BHI, Difco), grown to an OD_{600} of 0.5 and induced with anhydrotetracycline (ATc) for 2 h at 37 °C. In order to mimic *in vivo* growth conditions, *S. aureus* strains were grown to stationary phase in RPMI 1640 medium (Sigma). RPMI medium is an iron deficient medium which was originally designed for the culture of human leukocytes (Moore *et al.*, 1967). Starter cultures were grown overnight at 37 °C in RPMI and diluted in fresh RPMI containing ATc to an OD_{600} 0.05. Strains were then grown at 37 °C with shaking (200 rpm). *E. coli* strains were cultured on Luria agar or broth (Difco) at 37°C. *L. lactis* strain MG1363 (pKS80) were cultured in M17 medium (Difco) containing 0.5% (v/v) glucose at 28 °C. Antibiotics were incorporated into media where appropriate at the following concentrations: ampicillin (Ap) 100 μg/ml, chloramphenicol (Cm) 10 μg/ml, erythromycin (Em) 10 μg/ml, kanamycin (Ka) 50 μg/ml, anhydrotetracycline (ATc) 100 – 1200 ng/ml. Unless otherwise stated all reagents were obtained from Sigma.

2.2 DNA Manipulation

DNA manipulations were performed using standard methods (Sambrook, 1989). Restriction endonucleases were purchased from New England Biolabs and Finnzyems and were used according to the manufacturers' instructions. DNA ligase was purchased from Promega. DNA sequencing was carried out by MSC Operon or GATC-Biotech.

2.2.1 Isolation of plasmid and genomic DNA

The plasmids used in this study are listed in Table 2.2. Plasmid DNA was isolated using the Wizard® Plus SV Miniprep kit (Promega) according to the manufacturer's instructions. DNA purification was carried out using the Wizard® SV Gel and PCR Clean-up System (Promega). Genomic DNA was prepared using Bacterial Genomic DNA purification kit (Edge BioSystems) according to the manufacturer's instructions. 200 μg/ml of lysostaphin was added to digest cell wall peptidoglycan to facilitate extraction of plasmid and genomic DNA from *S. aureus.*

2.2.2 Polymerase chain reaction

Polymerase Chain Reaction (PCR) amplification was carried out in a DNA thermal cycler (Techne or PIKO). Reactions were typically carried out in 50 µl volumes using 1 U Phusion™ Hot Start DNA polymerase in Phusion HF buffer or Phire Hot Start DNA polymerase in Phire buffer (Finnzymes). Plasmid DNA (10 ng) or genomic DNA (25 ng) were used as templates for PCR. Primers and dNTPs (Bioline) were used at final concentrations of 2 µM and 200 µM, respectively. Primers were purchased from Integrated DNA Technologies and are listed in Table 2.3. Initial denaturation was carried out at 98 °C (30 s) followed by 30-35 cycles of denaturation (10 s) at 98 °C, 20 s annealing (temperature dependent on primer used) and extension at 72 °C. When amplifying plasmid DNA, an extension time of 15 s/1 kb DNA was used. For high complexity genomic DNA a longer extension time was used (30 s/kb). A final extension step was carried out at 72 °C for 5 min. PCR products were purified using Wizard SV gel and PCR clean-up system (Promega).

2.2.3 Agarose gel electrophoresis

1-1.5 % agarose was dissolved by boiling in TAE buffer (Invitrogen), cooled to 65 °C and cast in mini trays (Life Technologies). DNA samples in loading buffer containing an elecrophoretic dye were pipetted into wells and DNA size markers (Bioline) were loaded to confirm the size in base pairs. Electrophoresis of samples was routinely performed at 90 V. Gels were bathed in ethidium bromide for 10 min, washed and viewed under UV light. Gel images were analysed using Alpha Imager™ software.

2.2.4 Strain construction

Bacterial strains used in this study are listed in Table 2.1. Chromosomal resistance markers were moved between *S. aureus* strains by generalized bacteriophage ϕ85-mediated transduction. Bacteriophage were propagated on the donor strain and used to infect the recipient strain with antibiotic selection for the required marker. Putative transductant colonies were single colony purified three times on TSA plates containing the appropriate antibiotic and 0.05% (w/v) sodium citrate to eliminate contaminating phage particles.

Strain NM1 was constructed by transduction of *spa*::Ka^R into strain Newman *clfA*5 *clfB*::Em^R. Strain BH1CC Δ*fnbAfnbB spa*:: Ka^R was constructed by transduction of *spa*:: Ka^R into strain BH1CC Δ*fnbAfnbB*. Strain NM3 was constructed by

Table 2.1 Bacterial strains.

Strain	Relevant Features	Reference
S. aureus		
Newman	Human clinical isolate, NCTC 8178	(Duthie and Lorenz, 1952)
Newman *clfA*5 *clfB*::Em^R	Derivative of strain Newman deficient in ClfA, ClfB. Frameshift mutation in the *clfA* gene, *clfB*::Em^R	(Fitzgerald *et al.*, 2006)
Newman *spa*	Derivative of strain Newman deficient in protein A. *spa*::Ka^R	(Higgins *et al.*, 2006a)
SH1000 *sdgAB*	Derivative of SH1000 deficient in *sdgA* and *sdgB*. *sdgAB*::Ka^R	A kind gift from Simon Foster.
NM1	Derivative of strain Newman deficient in ClfA, ClfB and protein A. Frameshift mutation in the *clfA* gene, *clfB*::Em^R *spa*::Ka^R	This study
NM2	Derivative of NM1. Replacement of *clfB*::Em^R with a deletion of the *clfB* gene by allele exchange.	This study
NM3	Derivative of Newman *clfA*5 *clfB*::Em^R deficient in *sdgA* and *sdgB*. *sdgAB*::Ka^R	This study
BH1CCΔ*fnbAfnbB*	Derivative of MRSA strain BH1CC deficient in FnBPA and FnBPB	(Geoghegan *et al.*, 2013)

Table 2.1 Bacterial strains continued

Strain	Relevant Features	Reference
BH1CCΔ*fnbAfnbB spa*::Ka^R	Derivative of BH1CC Δ*fnbAfnbB* deficient in protein A. *spa*::Ka^R	This study
L. lactis		
MG1363	Derivative of NCD 0712, plasmid-free	(Gasson, 1983)
E. coli		
DC10B	*dam*$^+$ Δ*dcm* Δ*hsdRMS endA1 recA1*	(Monk *et al.*, 2012)
XL1-Blue	Host for cloning. *recA1 endA1 gyrA96 thi-1 hsdR17 suppE44 relA1 lac* (F′*pro*AB *lac*I^{q}ZΔM15 Tn*10*[Tet^R])	Stratagene
Topp3	Protease deficient strain used for recombinant protein expression. RifR (F′*pro*AB*lac*I^{q}ZΔM15Tn*10*[Tet^R][Ka^R])	Stratagene

Table 2.2 Plasmids

Plasmids	Relevant Features	Reference
pALC2073	Vector allowing high level gene expression in the absence of inducer. Ap^R Cm^R	(Bateman *et al.*, 2001)
pALC2073*clfA*	pALC2073 containing full-length *clfA* gene.	A. Loughman, Trinity College Dublin.
pALC2073*clfA*ΔN1$_{40\text{-}220}$	pALC2073*clfA* lacking region encoding residues 40-220 of ClfA	This study
pRMC2	Tetracycline-inducible expression vector with tight repression in the absence of inducer. Ap^R Cm^R	(Corrigan and Foster, 2009)
pRMC2*clfA*	pRMC2 containing full-length *clfA* gene from strain Newman	This study
pRMC2*clfA*ΔN1$_{40\text{-}220}$	pRMC2*clfA* lacking DNA encoding residues 40-220 of ClfA	This study
pRMC2*clfA*ΔN1$_{40\text{-}228}$	pRMC2*clfA* lacking DNA encoding residues 40-228 of ClfA	This study
pRMC2*clfA*ΔN1$_{40\text{-}210}$	pRMC2*clfA* lacking DNA encoding residues 40-210 of ClfA	This study
pRMC2*clfA*Δ$_{211\text{-}220}$	pRMC2*clfA* lacking region encoding residues 211-220 of ClfA	This study
pRMC2*clfA*Δ$_{211\text{-}228}$	pRMC2*clfA* lacking region encoding residues 211-228 of ClfA	This study

Table 2.2 Plasmids continued

Plasmids	Relevant Features	Reference
pRMC2*clfA*ΔN1$_{40\text{-}220}$ +10Ala	pRMC2*clfA* lacking DNA encoding residues 40-220 and inserting DNA encoding 10 alanine codons.	This study
pRMC2*clfA*ΔSD$_{559\text{-}875}$	pRMC2*clfA* lacking DNA encoding residues 559-875	This study
pRMC2*clfA*ΔN1$_{40\text{-}220}$ ΔSD$_{559\text{-}875}$	pRMC2*clfA* lacking DNA encoding residues 40-220 and 559-875	This study
pRMC2*clfA*ΔN123$_{40\text{-}559}$ FLAG	pRMC2*clfA* lacking DNA encoding residues 40-559 of ClfA and incorporating a FLAG epitope	This study
pRMC2*clfA*ΔN23$_{221\text{-}559}$ FLAG	pRMC2*clfA* lacking DNA encoding residues 221-559 of ClfA and incorporating DNA encoding a FLAG epitope	This study
pRMC2*fnbB*	pRMC2 containing full-length *fnbB* gene from strain 8325-4	This study
pRMC2*fnbB*ΔN1$_{38\text{-}162}$	pRMC2*fnbB* lacking DNA encoding residues 38-162	This study
pRMC2*fnbB*ΔN1$_{38\text{-}162}$+ClfA211-220	pRMC2*fnbB* lacking DNA encoding residues 38-162 and incorporating DNA encoding residues 211-220 N-terminal to the N2 subdomain	This study
pRMC2*fnbB*ΔN1$_{38\text{-}138}$	pRMC2*fnbB* lacking DNA encoding residues 38-138	This study
pRMC2*fnbB*ΔN1$_{38\text{-}134}$	pRMC2*fnbB* lacking DNA encoding residues 38-134	This study

Table 2.2 Plasmids continued

Plasmids	Relevant Features	Reference
pRMC2*fnbB*ΔN1$_{38\text{-}162}$ ΔFnBR$_{481\text{-}811\ FLAG}$	pRMC2*fnbB* lacking DNA encoding residues 38-167 and 481-811 and incorporating DNA encoding a FLAG epitope	This study
pKS80	*L. lactis* expression plasmid. Erm^R	(Hartford *et al.*, 2001)
pKS80*clfA*	pKS80 containing full length *clfA* gene.	(Hartford *et al.*, 2001)
pKS80*clfB*	pKS80 containing full length *clfB* gene.	(Hartford *et al.*, 2001)
pKS80*fbl*	pKS80 containing full length *fbl* gene of *S. lugdunensis* strain N920143.	(Mitchell *et al.*, 2004)
pKS80*isdH*	pKS80 containing full length *isdH* gene.	(Visai *et al.*, 2009)
pKS80*sdrC*	pKS80 containing full length *sdrC* gene.	(O'Brien *et al.*, 2002)
pKS80*sdrD*	pKS80 containing full length *sdrD* gene.	(O'Brien *et al.*, 2002)
pKS80*sdrE*	pKS80 containing full length *sdrE* gene.	(O'Brien *et al.*, 2002)
pKS80*sdrG*	pKS80 containing full length *sdrG* gene.	(Hartford *et al.*, 2001)
pQE30	*E. coli* expression vector, polyhistidine-tagged. Amp^R	Qiagen

Table 2.2 Plasmids continued

Plasmids	Relevant Features	Reference
pCF40	pQE30 derivative encoding the full length A region (subdomains N123) of ClfA (residues 40-559)	(O'Connell *et al.*, 1998)
pCF41	pQE30 derivative encoding the N23 subdomains of ClfA (residues 221-559)	(O'Connell *et al.*, 1998)
pGEX-4T2	*E. coli* vector for the expression of glutathione S-transferase-tagged recombinant proteins	GE Lifesciences
pGEX*clfA*N123	pGEX4T-2 derivaive encoding the full length A region (subdomains N123) of ClfA (residues 40-559)	This study
pGEX*clfA*N23	pGEX4T-2 derivative encoding the N23 subdomains of ClfA (residues 221-559)	This study
pGEX*clfA*$N1_{40-220}$	pGEX4T-2 derivative encoding the N1 subdomain of ClfA (residues 40-220)	This study
pGEX*clfA*$N1_{40-228}$	pGEX4T-2 derivative encoding the N1 subdomain of ClfA (residues 40-228)	This study
pGEX*clfA*N2	pGEX4T-2 derivative encoding the N2 subdomain of ClfA	This study
pGEX*clfA*N3	pGEX4T-2 derivative encoding the N3 subdomain of ClfA	This study
pIMAY Δ*clfB*	pIMAY containing 500 bp of DNA from upstream and downstream from *clfB*. Cm^R	(Mulcahy *et al.*, 2012)

Table 2.3 Primers

Primers	Sequence 5′-3′	Modifications
pRMC2*clfA*F	CCGAGATCTAAAGAGGGAATAA AATGAATATG	BglII
pRMC2*clfA*R	CCGGAATTCCTTATTTCTTATCTT TATTTTC	EcoRI
pRMC2*clfA*ΔN1$_{40\text{-}220}$F	GTAGCTGCAGATGCACCGGC	5′Phos
pRMC2*clfA*ΔN1$_{40\text{-}220}$R	TGCATCTGCTTCTTTACTGCT	5′Phos
pRMC2*clfA*ΔN1$_{42\text{-}220}$R	CATATTCATTTTATTCCCTCT	5′Phos
pRMC2*clfA*ΔN1$_{40\text{-}228}$F	GGCACAGATATTACGAATCAG	5′Phos
pRMC2*clfA*ΔN1$_{40\text{-}210}$F	CCTAGAATGAGAGCAATTTAG	5′Phos
pRMC2*clfA*ΔN1$_{40\text{-}210+\ 10Ala}$ F	GCTGCGGCTGCGGCTGTAGCTGC AATATGCAC	5′Phos
pRMC2*clfA*ΔN1$_{40\text{-}210+\ 10Ala}$ R	CGCTGCCGCTGCCGCTGCATCTG CTTCTTAACT	5′Phos
pRMC2*clfA*Δ$_{211\text{-}220}$ R	CGCACTTGTATTAACCGTTGA	5′Phos
pRMC2*clfA*Δ$_{211\text{-}228}$ R	TGCCGCTAAACTAAATGCTCTCA TTC	5′Phos
pRMC2*clfA*ΔSD$_{559\text{-}879}$ F	TCACCTAAAAATGGTACTAATGC	5′Phos
pRMC2*clfA*ΔSD$_{559\text{-}879}$ R	TGGAATTGGTTCAATTTCACCAG	5′Phos
pRMC2*clfA*ΔN123$_{40\text{-}559}$ F	GACGATGACAAACCTGGTGAAA TTGGACCAATTCC	5′Phos
pRMC2*clfA*ΔN23$_{220\text{-}559}$ R	ATCTTTATAATCTGCCGCTAAACT AAATGCTCTC	5′Phos
pRMC2*clfB* F	GGGAGATCTAATGGAGTAATATT TTTGAAAAAAAGA	BglII
pRMC2*clfB* R	CCCGAATTCTTACGCTTTTTCTTT ATGATCTTGC	EcoRI
pRMC2*clfB*ΔN1$_{48\text{-}200}$ F	GGTACAAATGTAAATGATAAAGT TACG	5′Phos
pRMC2*clfB*ΔN1R	AGCTTGTGCTTGATGATTGCCTA	5′Phos
pRMC2*fnbB*F	GGGAGATCTAGGGAGAATATTAT AGTGAAAAGC	BglII
pRMC2*fnbB*R	GCGGAATTCTTATGCTTTGTGATT CTTTTTATTTC	EcoR1
pRMC2*fnbB*ΔN1$_{38\text{-}162}$ F	GGTACAGATGTAACAAATAAAGT G	5′Phos
pRMC2*fnbB*ΔN1$_{38\text{-}162}$ R	TGCAGCTTCTTTTTCTTGTCCC	5′Phos

Table 2.3 Primers continued

Primers	Sequence 5′-3′	Modifications
pRMC2*fnbB*ΔN1$_{38\text{-}162+ClfA}$ F	CCTAGAATGAGAGCATTTAGTTT AGCGGCAGGTACAGATGTAACA AATAAAGTG	5′Phos
pRMC2*fnbB*ΔN1$_{38\text{-}138}$ F	CCAAGAATGAAAAGATCAACTG A	5′Phos
pRMC2*fnbB*ΔN1$_{38\text{-}134}$ F	TCAGAAATTAAACCAAGAATGA AAAG	5′Phos
pRMC2*fnbB*ΔFnBR$_{481\text{-}811}$ F	GACGATGACAAACCAATCGTGCC ACCAACGCC	5′Phos
pRMC2*fnbB*ΔFnBR$_{481\text{-}811}$ R	ATCTTTATAATCCTTTAGTTTATCT TTGCCGTCGC	5′Phos
fnbA u/s400	GCAGAAAATCGTCTGAAATAC	
fnbB u/s400	TGACTATCAAAATGATCTTCAA	
pRMC2 MCS F	CAGTCACGACGTTGTAAAAC	
pRMC2 MCS R	CTCGAGTTCATGAAAAACTAAAA	
pGEX*clfA*N123F	CGCGGATCCAGTGAAAATAGTGT TACGCAATCT	BamHI
pGEX*clfA*N123R	CGCGAATTCCTCTGGAATTGGTT CAATTTC	EcoRI
pGEX*clfA*N1$_{40\text{-}220}$R	CGCGAATTCTGCCGCTAAACTAA ATGCTCTC	EcoRI
pGEX*clfA*N1$_{40\text{-}228}$R	CGCGAATTCAGCTGCCGGTGCAT CTGC	EcoRI
pGEX*clfA*N2F	TATGGATCCGTAGCTGCAGATGC ACCG	BamHI
pGEX*clfA*N2R	GCGGAATTCTTAATATTTTTCATA ATCTACTAATACTGT	EcoRI
pGEX*clfA*N3F	TATGGATCCGATTATGAAAAATAT GGTAAGTTTTA	BamHI

transduction of *sdgAsdgB*::Ka^R from strain SH1000 *sdgAsdgB* into strain Newman *clfA*5 *clfB*::Em^R. Transductants were screened by Western immunoblotting, antibiotic selection and tested for δ-toxin production on sheep blood agar plates. Deletion of *clfB*::Em^R from strain NM1 to yield NM2 was achieved by allelic replacement using pIMAYΔ*clfB* (Table 2.2) as previously described (Monk *et al.*, 2012).

2.2.5 Plasmid construction

Primers used in this study are listed in Table 2.3. The complete *clfA* gene from strain Newman was amplified from genomic DNA by PCR. Recognition sequences for EcoRI and BglII were incorporated into the ends of the amplicon by primer extensions during PCR to facilitate directional cloning. The *clfA* gene was digested with EcoRI and BglII overnight and purified using Wizard SV gel and PCR clean-up system (Promega). Purified digested products were ligated to pRMC2 digested with EcoRI and BglII using T4 DNA ligase (Promega) to generate pRMC2*clfA*. The *fnbB* gene from strain 8325-4 was amplified from genomic DNA by PCR and cloned between the EcoRI and BglII sites of pRMC2 to generate pRMC2*fnbB* as described above. Positive clones were identified by restriction digest with EcoRI and BglII and confirmed by DNA sequencing. Inverse PCR was used to generate derivatives of pRMC2*clfA*, pALC2073*clfA*, pRMC2*clfB* and pRMC2*fnbB*. Alanine substitutions and FLAG epitopes were incorporated by primer extension during inverse PCR. PCR products were treated with DpnI to eliminate parental template DNA and following blunt-end ligation plasmids were transformed into *E. coli* DC10B (see Section 2.3). Plasmids were purified from DC10B and transformed into *S. aureus* made electrocompetent as described in Section 2.4.

2.2.6 Construction of GST-tagged recombinant proteins

DNA encoding the A region of ClfA (*clfA*$_{40\text{-}559}$ or *clfA*$_{221\text{-}559}$) was amplified from pCF40. Recognition sequences for EcoRI and BamHI were incorporated into the amplicon during PCR to facilitate directional cloning. The *clfA* fragments were digested with EcoRI and BamHI overnight and purified using Wizard SV gel and PCR clean-up system (Promega). Purified digested products were ligated to pGEX-4T-2 which had been digested with EcoRI and BamHI using T4 DNA ligase (Promega). DNA encoding each of the individual subdomains of *clfA* (N1$_{40\text{-}220}$,N1$_{40\text{-}228}$, N2 and N3) was amplified from pCF40 and cloned between the EcoRI and BamHI sites of pGEX-4-T2 as

described above. Positive clones were identified by restriction digest with EcoRI and BamHI and confirmed by commercial sequencing.

2.3 Transformation of *Escherichia coli* with plasmid DNA

Chemically competent *E. coli* were prepared using $CaCl_2$ treatment. An overnight culture of *E. coli* was used to inoculate 1L fresh L-broth in a 2L flask and grown to an OD_{600} of 4.0-5.0. Cells were chilled on ice for 1 h and then harvested. Cells were resuspended in $MgCl_2$ (100 mM) and were harvested again. Cells were washed twice in ice cold buffer (60 mM $CaCl_2$, 10 mM PIPES, 15% (v/v) glycerol, pH 8) before being aliquoted. Cells were snap-frozen in liquid nitrogen and were stored at -70°C. Thawed aliquots were incubated with 50 ng of plasmid for 10 min on ice. Samples were heated to 42 °C for 2 min and then chilled on ice for 2 min. 1 ml of L-broth was added and the mixture was incubated for 1 h at 37°C with shaking before plating onto L-agar containing the appropriate antibiotic. Plates were incubated for 16 h. *E. coli* transformants were screened for the presence of recombinant plasmids using a rapid colony screening procedure (Le Gouill and Dery, 1991).

2.4 Transformation of *Staphylococcus aureus* with plasmid DNA

Plasmids isolated from *E. coli* DC10B were introduced into *S. aureus* by electroporation. Electrocompetent *S. aureus* cells were prepared by diluting an overnight culture of *S. aureus* to an OD_{578} of 0.5 in 50 ml pre-warmed TSB. The culture was reincubated at 37 °C for 30 min, until an OD_{578} of 0.8-0.9 was reached. Cells were cooled in an ice-water slurry for 10 min. Cells were harvested at 3,900 x *g* for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 50 ml sterile filtered ice-cold water. Cells were harvested again and the pellet was washed in decreasing volumes of sterile ice-cold 10% (v/v) glycerol (5 ml, 1 ml, 250 µl). Aliquots (50 µl) were snap-frozen in liquid nitrogen and stored at -70 °C.

Aliquots were thawed on ice for 5 min and then incubated at room temperature for 5 min. Cells were harvested at 5,000 x *g* for 1 min and were re-suspended in sterile electroporation buffer (50 µl, 10% (v/v) glycerol / 500 mM sucrose). Up to 5 µg of plasmid was added to the cells in a volume of 5 µl with gentle mixing and was then transferred to a 0.1 cm electroporation cuvette (Biorad). The cells were pulsed at 21 kV/cm, 100 Ω and 25 uF. A time constant of 2.2-2.4 ms was achieved. The cells were

immediately resuspended in sterile BHI supplemented with 500 mM sucrose (1 ml) and were incubated for 1-2 h at 37 °C with shaking. Cells were then plated out on TSA containing the appropriate antibiotic. Transformants were screened by PCR, restriction mapping and Western immunoblotting.

2.5 Isolation of extracellular proteins

S. aureus cultures were harvested by centrifugation at 2,000 x g for 10 min. The culture supernatant was filtered through a 0.45 µm filter and proteins were precipitated by addition of a 1:20 volume of ice-cold 100% (w/v) trichloroacetic acid (TCA). Precipitated proteins were recovered by centrifugation at 17,000 x g for 15 min. The pellet was washed once in ice-cold acetone and finally the pellet containing the precipitated extracellular proteins was resuspended in final sample buffer.

2.6 Isolation of cell wall-associated proteins

S. aureus cultures were harvested, washed in phosphate buffered saline (PBS) and adjusted to an OD_{600} of 10. Cell wall-associated proteins were solubilised by resuspending bacteria in 250 µl digestion buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, 30 % (w/v) raffinose, pH 7.5) containing protease inhibitors (Roche) and lysostaphin (AMBI Products LLC, 200 µg/ml) at 37 °C for 10 min. Protoplasts were removed by centrifugation at 12,000 x *g* for 10 min and the supernatant containing solubilised cell-wall proteins was aspirated and boiled in an equal volume of Laemmli sample buffer for 5 min.

2.7 Isolation of cell membrane and cytoplasmic proteins

Protoplast pellets were washed with digestion buffer and resuspended in ice-cold Tris-HCl (50 mM, pH 7.5) containing protease inhibitors (Roche) and DNase (80 µg/ml). Protoplasts were lysed on ice by vortexing. Complete lysis was confirmed by phase contrast microscopy. Lysed cell fractions were separated by centrifugation at 44,500 x *g* for 1 h at 4 °C. The supernatant was retained as the cytoplasmic fraction. The pellet was washed with ice-cold Tris-HCl (50 mM, pH 7.5) and resuspended in Tris-HCl (50 mM, pH 7.5). Cytoplasmic and cell membrane fractions were mixed in an equal volume of Laemmli sample buffer and boiled for 5 min.

2.8 Protein Electrophoresis and Western immunoblotting

2.8.1 SDS-PAGE

Proteins for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were boiled for 5 min in an equal volume of final sample buffer before a brief centrifugation to pellet debris. In general, 20 µl of each sample was separated using 4.5% stacking and 7.5-12 % acrylamide separating gel at 40 mA for 2 hr. Gels were either stained in Coomassie Brilliant Blue and destained in 45 % (v/v) methanol, 10% (v/v) acetic acid or transferred by wet transfer onto polyvinylidene difluoride (PVDF) membranes (Roche) in 20 mM Tris, 150 mM glycine and 20% (v/v) methanol.

2.8.2 Western immunoblotting

Non-specific binding to membranes was inhibited by incubation for 1-16 hrs in 10% (w/v) skimmed milk powder (Marvel) in TS buffer (10 mM Tris-HCl, 0.9% (w/v) NaCl pH7.4). Blots were probed with the appropriate primary antibody for 1 h, washed three times with gentle agitation for 10 min in TS buffer. Bound antibody was detected using a horseradish peroxidise (HRP)-conjugated antibody where appropriate. Primary and secondary antibodies used are listed in Table 2.4. Reactive bands were visualised using the LumiGLO reagent and peroxide detection system (Cell Signalling Technology). Antibodies were removed from blots by incubation at 70 °C for 30 min in stripping buffer (2 % SDS, 100 mM β-mercaptoethanol and 50 mM Tris). Stripped blots were washed twice in PBS, blocked in 10 % (w/v) skimmed milk proteins and reprobed as above.

2.8.3 Ligand affinity blotting

2.8.3.1 Ligand affinity blotting with biotinylated fibronectin

Cell wall extracts from *S. aureus* expressing FnBPB were probed with biotinylated fibronectin. Human fibronectin (0.5 mg/ml in PBS, Calbiochem) was incubated with *N*-hydroxysuccinimidobiotin (NHS-biotin, 2 mg/ml) for 20 min at room temperature. The reaction was stopped by addition of 10 mM NH_4Cl. Biotinylated fibronectin was dialysed overnight at 4 °C to remove excess biotin. PVDF membranes were probed with biotinylated fibronectin (30 µg/ml) in 10% (w/v) skimmed milk for 1 hr at room temperature. Bound biotin was detected using horseradish peroxidise (HRP)-conjugated streptavadin (1:5000, Roche).

Table 2.4 Antibodies used in this study

Antibody	Working dilution	Reference
Rabbit anti-ClfA A region IgG	1:10,000	(Higgins *et al.*, 2006b)
Mouse monoclonal anti-ClfA (ClfA mAb)	1:1000	Inhibitex
Polyclonal rabbit anti-SdrE	1:2000	(Josefsson *et al.*, 1998)
Polyclonal rabbit anti- EbpS	1:500	(Downer *et al.*, 2002)
Rabbit anti-ClfB A region	1:2000	(Walsh et al., 2004)
Polyclonal rabbit anti-IsdH	1:5000	P. Speziale
Polyclonal anti-Sdr B repeats	1:1000	E. Josefsson
Polyclonal goat anti-factor I	1:2000	Quidel
Monoclonal mouse anti-factor I	1:1000	Quidel
Monoclonal mouse anti-factor H	1:1000	Quidel
Monoclonal mouse anti-C4BP	1:1000	Quidel
Polyclonal anti-C3	1:2000	Calbiochem
HRP-conjugated anti-His IgG	1:500	Roche
HRP-conjugated anti-GST IgG	1:1000	GE Healthcare
Protein A-peroxidase	1:500	Sigma
HRP-conjugated anti-fibrinogen IgG	1:3000	Dako
HRP-conjugated rabbit anti-mouse IgG	1:3000	Dako
HRP-conjugated rabbit anti-human IgG	1:3000	Dako
HRP-conjugated rabbit anti-goat IgG	1:3000	Dako
HRP-conjugated goat anti-rabbit IgG	1:2000	Dako

2.8.3.2 Ligand affinity blotting with GST-tagged recombinant protein

PVDF membranes were probed with GST-tagged ClfA (8 μM) in 10% (w/v) skimmed milk for 1 hr at room temperature. Bound protein was detected using (HRP)-conjugated anti-GST IgG (1:1000, GE Healthcare).

2.8.3.3 Ligand affinity blotting with normal human serum

Membranes were probed with 10 % (v/v) normal human serum (Complement Technologies) in 10% (w/v) skimmed milk for 1 hr at room temperature. Bound complement components were detected using specific monoclonal antibodies (Table 2.4).

2.8.4 Dot immunoblotting

Bacteria were washed twice in PBS and adjusted to an OD_{600} of 1.0. Doubling dilutions (5 μl) were spotted onto a nitrocellulose membrane (Protran) and allowed to dry. Membranes were blocked for 1 h in 10 % (w/v) skimmed milk powder and immunoblotting was performed as above.

2.9 Aureolysin treatment of *S. aureus*

S. aureus NM1 pRMC2 *clfA* was grown with ATc (800 ng/ml) as described section 2.1. Bacteria were harvested, washed in PBS and adjusted to an OD_{600} of 10. Aureolysin (8 μg/ml, BioCentrum) was added and bacteria were incubated at 37 °C for 1 h. Cells were incubated briefly on ice, harvested by centrifugation and washed with PBS.

2.10 Bacterial adherence to fibrinogen

Microtitre plates (Sarstedt) were coated with doubling dilutions of fibrinogen or fibronectin (Calbiochem) overnight at 4 °C. Wells were blocked with 5 % (w/v) bovine serum albumin (BSA) for 2 h at 37 °C. Washed bacteria were adjusted to an OD_{600} of 1.0 in PBS and 100 μl was added to each well and incubated for 2 h at 37 °C. Wells were washed with PBS and adherent cells fixed with formaldehyde 25 % (v/v), stained with crystal violet and the A_{570} measured.

2.11 Bacterial survival in whole human blood

Bacteria were grown overnight in RPMI containing Cm, diluted to an OD_{600} of 0.05 in RPMI containing Cm and ATc (800 or 1200 ng/ml) and grown overnight at 37

°C. Bacteria were washed twice in RPMI. The number of input cfu was determined by plating on TSA incorporating Cm. For blood survival experiments fresh human blood (0.5 ml) containing the anticoagulant hirudin (50 μg/ml, Refludan, Pharmion) was inoculated with *S. aureus* (2 x 10^4 cfu/ml). Tubes were incubated for 3 h at 37 °C with shaking at 70 r.p.m. The number of cfu surviving after incubation in blood was determined by diluting the culture 1:10 in sterile endotoxin free water and plating on TSA incorporating Cm.

2.12 Competitive growth of bacteria

For competitive growth experiments stationary phase cultures were adjusted to an OD_{600} of 0.025 in BHI containing Cm. Equal volumes of two *S. aureus* strains were mixed and grown in BHI incorporating Cm to an OD_{600} of 0.5. Where appropriate ATc (1200 ng/ml) was added and cultures were incubated at 37 °C for a further 2 h before being plated on TSA incorporating Cm. Colonies which were resistant to Cm were replica plated onto TSA containing Em and the percentage of Em resistant bacteria was calculated.

2.13 Electron microscopy analysis of bacteria

2.13.1 Scanning electron microscopy

Stationary phase cultures of *S. aureus* were adjusted to an OD_{600} of 0.05 in BHI containing Cm and grown to an OD_{600} of 0.5. ATc (1200 ng/ml) was added and cultures were incubated at 37 °C for a further 2 h. Cells were washed, harvested and fixed in 3 % gluteraldehyde for 1 h with gentle rocking.

Samples were dehydrated on an ethanol gradient by washing in increasing concentrations of ethanol (10-100 % (v/v)) followed by critical point drying. Dried samples were mounted onto SEM stubs, sputter coated in 20 nm of gold and imaged by SEM. Critical point drying and gold coating of samples was performed by the Trinity Centre for Microscopy Analysis (CMA).

2.13.2 Transmission electron microscopy

Stationary phase cultures of *S. aureus* carrying pRMC2*clfA* or pRMC2*clfA*ΔN1$_{40\text{-}220}$ were adjusted to an OD_{600} of 0.05 in BHI containing Cm and grown to an OD_{600} of 0.5. ATc (1200 ng/ml) was added and cultures were incubated at 37 °C for a further 2 h. Cells were washed, harvested and fixed in 3 % gluteraldehyde for 1 h with gentle

rocking. Gluteraldehyde fixed cells were washed for 1 h six times in PBS to remove unreacted gluteraldehyde. A second fixation step was preformed for 30 min in 2% osmium tetroxide with gentle agitation. Fixed cells were dehydrated on an alcohol gradient (10- 100% (v/v)). Dehydrated cells were incubated with 100 % propylene oxide for 30 min at room temperature twice. Cells were incubated in a 50 % resin solution in propylene oxide for 2- 3 h followed by a 100 % resin solution for 2-3 h to embed in resin and then placed under vacuum to remove air bubbles. Samples embedded in resin were cut into thin sections, stained with 0.5 % aqueous uranyl acetate and Reynold's lead citrate and imaged using TEM. Preparation of samples following gluteraldehyde fixation was performed by the Trinity CMA.

2.14 Expression, purification and modification of recombinant proteins

2.14.1 Expression and purification of recombinant His-tagged proteins

Recombinant His-tagged proteins were purified by immobilised nickel chelate affinity chromatography according to the manufacturer's instructions using a peristaltic pump (Amersham). DNA encoding *clfA*N123$_{40\text{-}559}$ and *clfA*N23$_{221\text{-}559}$ was previously cloned into pQE30 to generate recombinant proteins with an N-terminal hexahistidine tag (O'Connell *et al.*, 1998). Full-length recombinant ClfA A domain constructs rClfA $_{40\text{-}559}$ and recombinant ClfA lacking the N1 domain rClfA $_{221\text{-}559}$ were purified from *E. coli* Topp3 (Stratagene) carrying pCF40 and pCF41 respectively. Overnight cultures (20 ml) were inoculated into fresh medium (1:100) and grown to an OD_{600nm} of 0.6 at 37 °C. IPTG was added to a final concentration of 1 mM and the culture was grown for a further 3 h. Cells were harvested by centrifugation at 7,000 rpm for 10 min at 4°C in a Sorvall GS-3 rotor. The pellet was resuspended in 30 ml binding buffer (0.5 M NaCl, 20 mM Tris-Hcl, 20 mM imidazol, pH 7.9) containing protease inhibitors (Complete EDTA-free Protease inhibitor, Roche) and frozen overnight. Resuspended cells were thawed on ice and lysed by repeated passage through a French Pressure Cell. Cell debris was removed by centrifugation at 17,000 rpm for 20 min at 4 °C in a Sorvall SS-34 rotor and the supernatant was filtered through a 0.45 μm filter. The cleared lysate was applied to a HisTrap HF chelating column (GE Healthcare) and washed with binding buffer. Bound protein was eluted in fractions with a continuous linear gradient of imidazole (5-100 mM) in binding buffer. Eluted fractions were analysed by SDS-PAGE and positive fractions were pooled. To prevent excessive breakdown of rClfA 1 mM 1, 10-phenanthroline was added to pooled factions and to the dialysis buffer. rClfA was

further purified by anion affinity exchange with a Hi-Trap MonoQ column (GE Healthcare) using a NaCl gradient for elution on the Akta PRIME FPLC. Protein concentrations were determined by measuring the absorbance at 280 nm according to the Beer-Lambert Law (A_{280}=E.c.l).

2.14.2Expression and purification of recombinant GST-tagged proteins

DNA encoding *clfA* $N123_{40-559}$, *clfA*$N23_{221-559}$, $N1_{40-220}$, $N1_{40-228}$, N2, N3 was cloned into pGEX-4T-2 to generate recombinant proteins with an N-terminal GST tag as described in Section 2.2.6. Protein expression was induced with 1 mM IPTG and cells were harvested and lysed as described in Section 2.14.1 with the exception that harvested cells were resuspended in PBS. The GST-fusion proteins were purified using a GSTrap™ column (GE Healthcare) according to the manufacturer's instructions using a peristaltic pump (Amersham). Briefly, the column was equilibrated with 10 column volumes of PBS. The cleared lysate was loaded onto the column and washed with 10 column volumes of PBS. Bound protein was eluted with 10 mM glutathione in 50 mM Tris-HCL, pH 8.0 in 2ml fractions and samples were analysed by SDS-PAGE. Positive fractions were pooled and dialysed against phosphate-buffered saline (PBS) overnight. To prevent excessive breakdown of rClfA 1mM 1, 10-phenanthroline was added to eluted factions and to the dialysis buffer. rClfA was further purified by anion affinity exchange with a Hi-Trap MonoQ column (GE Healthcare) using a NaCl gradient for elution on the Akta PRIME FPLC.

2.14.3 Aureolysin cleavage of recombinant proteins

Recombinant $ClfA_{40-559}$ was expressed with an N-terminal hexahistidine tag and purified from *E. coli* Topp3 by Ni^{2+} affinity chromatography as described in Section 2.14.1. Recombinant $ClfA_{40-559}$ was incubated with aureolysin (4 µg/ml, BioCentrum) for 1 h at 37 °C. Samples were analysied by SDS-PAGE. For N-terminal sequencing, samples were transferred to a PVDF membrane and sequencing was performed by Alta Biosiences, Birmingham, UK.

2.15 Solid phase binding assays

2.15.1 Fibrinogen binding assays

A solution of fibrinogen in PBS (10 µg/ml) was used to coat microtitre plates (96-well, Sarstedt). Wells were washed three times with PBS and were incubated for 2 h at 37 °C in 5% (w/v) bovine serum albumin (BSA) in PBS to block non-specific

binding. Wells were washed again and serial dilutions of recombinant protein in PBS were added. Following a 2 h incubation at 37 °C, wells were washed 3 times with PBS to remove unbound protein. Bound protein was detected with HRP-conjugated rabbit anti-His IgG or HRP-conjugated rabbit anti-GST IgG in 10 % (w/v) Marvel in PBS buffer. Plates were incubated for 1 h at room temperature with shaking. After washing, 100 µl of a chromogenic substrate solution (1 mg/ml tetramethylbenzidine and 0.006% H_2O_2 in 0.05 M phosphate citrate buffer pH 5.0) was added, and plates were developed for 10-30 min in the dark. The reaction was stopped by the addition of 2 M H_2SO_4 (50 µl/well), and the A_{450} was measured in a plate reader (Labsystems). Data was plotted and analysed using Prism Graphpad 5 software.

2.15.2 Fibrinogen and complement protein capture assays

A range of concentrations of recombinant ClfA proteins and variants were coated onto a microtitre plate (Nunc, maxisorb) in sodium carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and were incubated for 16 h at 4 °C. Wells were washed three times with PBS and were incubated for 2 h at 37 °C in 5% (w/v) bovine serum albumin (BSA) in PBS to block non-specific binding. Wells were washed again and a solution of fibrinogen (10 µg/ml) or 10 % normal human serum in PBS was added. Following a 2 h incubation at 37 °C, wells were washed 3 times with PBS to remove unbound protein. Bound protein was detected with HRP-conjugated anti-fibrinogen IgG or monoclonal anti-factor I IgG, monoclonal anti-factor H IgG or monoclonal anti-C4BP IgG and HRP-conjugated rabbit anti-mouse IgG in 10 % (w/v) Marvel in PBS buffer. Plates were incubated for 1 h at room temperature with shaking. After washing, 100 µl of a chromogenic substrate solution (1 mg/ml tetramethylbenzidine and 0.006% H_2O_2 in 0.05 M phosphate citrate buffer pH 5.0) was added, and plates were developed for 10-30 min in the dark. The reaction was stopped by the addition of 2 M H_2SO_4 (50 µl/well), and the A_{450} was measured in a plate reader (Labsystems). Data was plotted and analysed using Prism Graphpad 5 software.

2.15.3 Serum recognition of recombinant protein assays

2.15.3.1 Preparation of mouse serum

Challenged mice received a single intra-peritoneal injection of *S. aureus* Newman (5×10^8 CFU) and were allowed to recover for 35 days before serum was isolated. Vaccinated mice were vaccinated intranasally with His-tagged ClfA $N123_{40\text{-}559}$

(10 μg) and an experimental adjuvant (Karen Misstear and Ed Lavelle) or were mock immunised with PBS. Boosters were administered on day 14 and day 28 before mice were sacrificed at day 35 and serum was obtained. Serum samples were a kind gift from Kate O'Keefe and Rachel McLoughlin

2.15.3.2 ELISA with human and mouse sera

Recombinant GST-tagged ClfA variants were coated onto a microtitre plate (Nunc, maxisorb) in sodium carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and were incubated for 16 h at 4 °C. Wells were washed three times with PBS and were incubated for 2 h at 37 °C in 5% (w/v) bovine serum albumin (BSA) in PBS to block non-specific binding. Wells were washed again and dilutions of serum from mice or humans in PBS were added. Following a 2 h incubation at 37 °C, wells were washed 3 times with PBS to remove unbound antibody. Bound human antibodies were detected with HRP-conjugated rabbit anti-human IgG in 10 % (w/v) Marvel in PBS buffer. Plates were incubated for 1 h at room temperature with shaking. After washing, 100 μl of a chromogenic substrate solution (1 mg/ml tetramethylbenzidine and 0.006% H_2O_2 in 0.05 M phosphate citrate buffer pH 5.0) was added, and plates were developed for 10-30 min in the dark. The reaction was stopped by the addition of 2 M H_2SO_4 (50 μl/well), and the A_{450} was measured in a plate reader (Labsystems). Data was plotted and analysed using Prism Graphpad 5 software.

2.16 Prediction of secondary structure of the N1 subdomains of MSCRAMMs

Predictions of the secondary structure of the N1 subdomains was generated by submitting the amino acid sequence to the Protein Homology/analogy Recognition Engine (Phyre) service website (http://www.sbg.bio.ic.ac.uk/phyre2/)

2.17 Statistical analysis

Statistical analysis was performed using Student's *t*-test in Prism Graphpad 5 software. P values were considered significant if they were less than 0.05.

Chapter 3

Investigating the role of the N1 subdomain in the biological functions of ClfA

3.1 Introduction

The MSCRAMM family comprises proteins with an N-terminal ligand-binding A domain. This contains two tandemly arrayed subdomains N2 and N3 that are composed of IgG-like folds (Deivanayagam *et al.*, 2002) and the N-terminal N1 which, in the case of the related protein ClfB, is elongated and is not compact (Perkins *et al.*, 2001). The A domains are linked to the cell wall by an extended, unstructured flexible region comprising repeats of the dipeptide Ser-Asp (ClfA, ClfB, SdrC, SdrD, SdrE, Bbp) or 10/11 fibronectin binding domains (FnBPA, FnBPB) (Hartford *et al.*, 1997, Schwarz-Linek *et al.*, 2003).

The A domain of ClfA binds to a peptide sequence at the extreme C-terminus of the γ-chain of fibrinogen (McDevitt *et al.*, 1997). The X-ray crystal structure of ClfA N23 has been solved both as an apo-protein and with the fibrinogen peptide ligand bound (Ganesh *et al.*, 2008). The fibrinogen γ-chain peptide binds in a hydrophobic trench formed between the separately folded N2 and N3 subdomains by the 'dock, lock, and latch' mechanism (Ponnuraj *et al.*, 2003). Docking of the peptide in the binding trench induces a conformational change in the flexible extension of the C-terminus of the N3 subdomain which is reoriented to cover the ligand peptide and "locks" it in place. Latching is completed by formation of an extra β-strand which is complementary to a β-sheet of the N2 domain. The MSCRAMMs ClfB, Bbp, FnBPA and FnBPB bind to fibrinogen by a similar mechanism (Ganesh *et al.*, 2011, Keane *et al.*, 2007, Vazquez *et al.*, 2011, Burke *et al.*, 2011). In all cases the ligand binding site appears to be confined to subdomains N2N3 (Keane *et al.*, 2007, Burke *et al.*, 2011, Vazquez *et al.*, 2011, Ganesh *et al.*, 2011).

The boundaries of the N1 and N2 subdomains were originally defined for ClfB before structures of any of the proteins were solved. The *S. aureus* zinc-metalloprotease aureolysin was found to cleave ClfB at a 'SLAVA' motif during bacterial growth. Since ClfA contains a similar 'SLAAVA' motif this was designated as the boundary between N1 and N2 (McAleese *et al.*, 2001). Virtually nothing is known about the structure or function of subdomain N1 of the MSCRAMM family of surface proteins and to date has been considered dispensable for function.

To facilitate the study of the N1 subdomain of ClfA it was necessary to manipulate *S. aureus* genetically. Genetic manipulation of staphylococci has long been

complicated by the strong restriction barrier found in *S. aureus* and *S. epidermidis*. To circumvent this difficulty it was necessary to utilise an intermediate host, the restriction defective RN4220, when transforming *S. aureus* with DNA from *E. coli* (Kreiswirth *et al.*, 1983). In *S. aureus* a conserved type IV restriction system which specifically recognises and restricts uptake of foreign cytosine methylated DNA was recently identified as the major barrier for transformation (Monk *et al.*, 2012). A DNA cytosine methyltransferase mutant of *E. coli* (DC10B, Table 2.1) derived from the *E. coli* cloning strain DH10B has been constructed (Monk *et al.*, 2012). DC10B does not cytosine methylate DNA allowing direct transformation of target *S. aureus* strains with a high transformation efficiency without the need for RN4220.

Direct genetic manipulation has proven crucial in defining the role of virulence factors in pathogenic bacteria. To identify the potential virulence factors it is necessary to first disrupt the gene of interest and to perform complementation. Strategies for gene disruption in staphylococci are transposon mutagenesis, directed plasmid integration and allelic replacement.

Transposon mutagenesis is a powerful tool involving the random insertion of transposable elements throughout the target chromosome. It is particularly useful as no knowledge of the target gene is required as long as a phenotypic screen is available to identify mutants. Recently the Nebraska Center for Staphylococcal Research has generated a sequence-defined transposon mutant library consisting of 1,952 strains, each containing a single mutation within a nonessential gene of the community-associated methicillin-resistant (CA-MRSA) *S. aureus* isolate USA300 (Fey *et al.*, 2013).

Directed plasmid integration is commonly achieved through suicide vectors which contain a site of shared homology with the gene of interest (Hartford *et al.*, 2001). After a single cross-over event the plasmid becomes integrated into the chromosome resulting in target gene disruption. Unlike transposon mutagenesis prior knowledge of the target genes sequence is required. One disadvantage of directed plasmid integration is that the mutations are often polar and can have deleterious downstream effects when such mutations are made within an operon.

A more precise method of gene inactivation than transposon mutagenesis is allelic exchange. This exploits homologous recombination to replace precisely a section

of DNA with a defined mutation. With allelic exchange precise control over the mutation is achieved allowing the deletion of entire genes, introduction of point mutations or hybrid genes, and gene restoration eliminating the possibility of polar effects. However unlike transposon mutagenesis, prior knowledge of the sequence of the target gene and its surroundings is necessary.

Allelic exchange is a multi-step process. In order to construct a deletion mutation regions 5′ and 3′ to the target gene are cloned into a multi-copy plasmid. A drug resistance determinant can be inserted between the 5′ and 3′ fragments through specific restriction sites. The plasmid is then used to transform *S. aureus* and can integrate into the chromosome by homologous recombination. Once confirmed mutations can be moved into different genetic backgrounds by generalised transduction. For a complete in-frame chromosomal deletion without drug resistance makers, primer pairs can be designed to construct a deletion cassette which encodes the immediate 5′ and 3′ regions of the target gene. These deletion constructs are conventionally used with temperature sensitive plasmids in *S. aureus*.

Temperature sensitive plasmids contain a temperature sensitive replicon. For this reason they can replicate and are stable within the cell at their permissive temperature (typically 28-30 °C), but growth at the restrictive temperature (typically 37-42 °C) under selective pressure inhibits replication and selects for single cross-over integrants. A further shift back to the permissive temperature without selective pressure allows excision and loss of the plasmid. Excision occurs through a second single cross-over event. A proportion of the population will retain the mutation while the remainder will contain the wild-type gene.

Several temperature sensitive plasmids have been developed for use in *S. aureus* including pMAD, pKOR1 and pIMAY. Each system has its own merits. Plasmid pMAD carries the *bgaB* gene encoding β-galactosidase. This allows staphylococci to cleave the chromogenic substrate X-gal resulting in blue colonies (Arnaud *et al.*, 2004). This facilitates colony screening based on blue/white colony colour to detect excision and loss of the plasmid. Both pKOR1and pIMAY employ antisense *secY* RNA expression for counter-selection (Bae and Schneewind, 2006). SecY is an essential component of the SecYEG translocase that transports signal peptide-bearing proteins across the cytoplasmic membrane (Manting and Driessen, 2000). SecY expression is essential for bacterial growth and expression of *secY* antisense RNA inhibits colony formation on

agar plates (Ji *et al.*, 2001). Anhydrotetracycline (ATc) induced expression of antisense *secY* RNA suppresses growth of bacterial cells containing pKOR1 or pIMAY resulting in a mixture of large and small colonies on agar plates, with large colonies having lost the plasmid. Plasmid pKOR1 also contains a lambda recombination cassette for gateway cloning, which facilities rapid cloning of mutant alleles without the use of restriction enzymes and ligase (Bae and Schneewind, 2006). In both the pMAD and pKOR1 systems the restrictive temperature for plasmid replication is greater than 37 °C (42°C). Plasmid pIMAY replicates normally in *E. coli* at 28 °C but is highly temperature sensitive in *S. aureus* with a low restrictive temperature of 37 °C. At lower restrictive temperature, secondary mutations are less frequent than the more stressful restrictive temperatures used by pMAD and pKOR1. A schematic of allelic replacement by pIMAY is shown in Fig 3.1.

Inducible expression vectors are widespread and powerful tools used in molecular biology to facilitate the study of proteins and elucidate gene function. A variety of regulatable expression systems have been developed for Gram-negative bacteria. However, fewer have been adapted for use in *S. aureus*. Of note the $P_{xyl/xylR}$ inducible promoter, the P_{BAD}, the $P_{spac/lacI}$ and the $P_{xyl/tetO}$ systems have been used in *S. aureus* with varying degrees of success. The xylose promoter is repressed by the presence of glucose rendering it useless for in vivo work. The P_{BAD} promoter is induced by arabinose, however expression is weak in staphylococci. This is likely due to poor penetration of arabinose through the staphylococcal cell wall (Bateman *et al.*, 2001). The $P_{xyl/tetO}$ promoter is induced by tetracycline which is suitable in animal models because it can diffuse through biological membranes. It is very active and achieves a high level of expression but is poorly repressed in plasmid pALC2073 (Bateman *et al.*, 2001, Corrigan and Foster, 2009).

The plasmid pRMC2 was developed as an ATc-inducible expression vector for *S. aureus* which can achieve a high level of expression and is tightly repressed in the absence of its inducer (Corrigan and Foster, 2009). Plasmid pRMC2 was derived from the parental plasmid pALC2073, a high copy number shuttle vector (Fig 3.2). Although a high level of expression could be achieved from pALC2073, an undesirably high level of leaky expression occurs from the $P_{xyl/tetO}$ promoter in the absence of inducer due to poor repression by TetR. Both plasmids allow gene expression through a classical tetON system. Expression of the gene of interest is controlled by TetR expression from

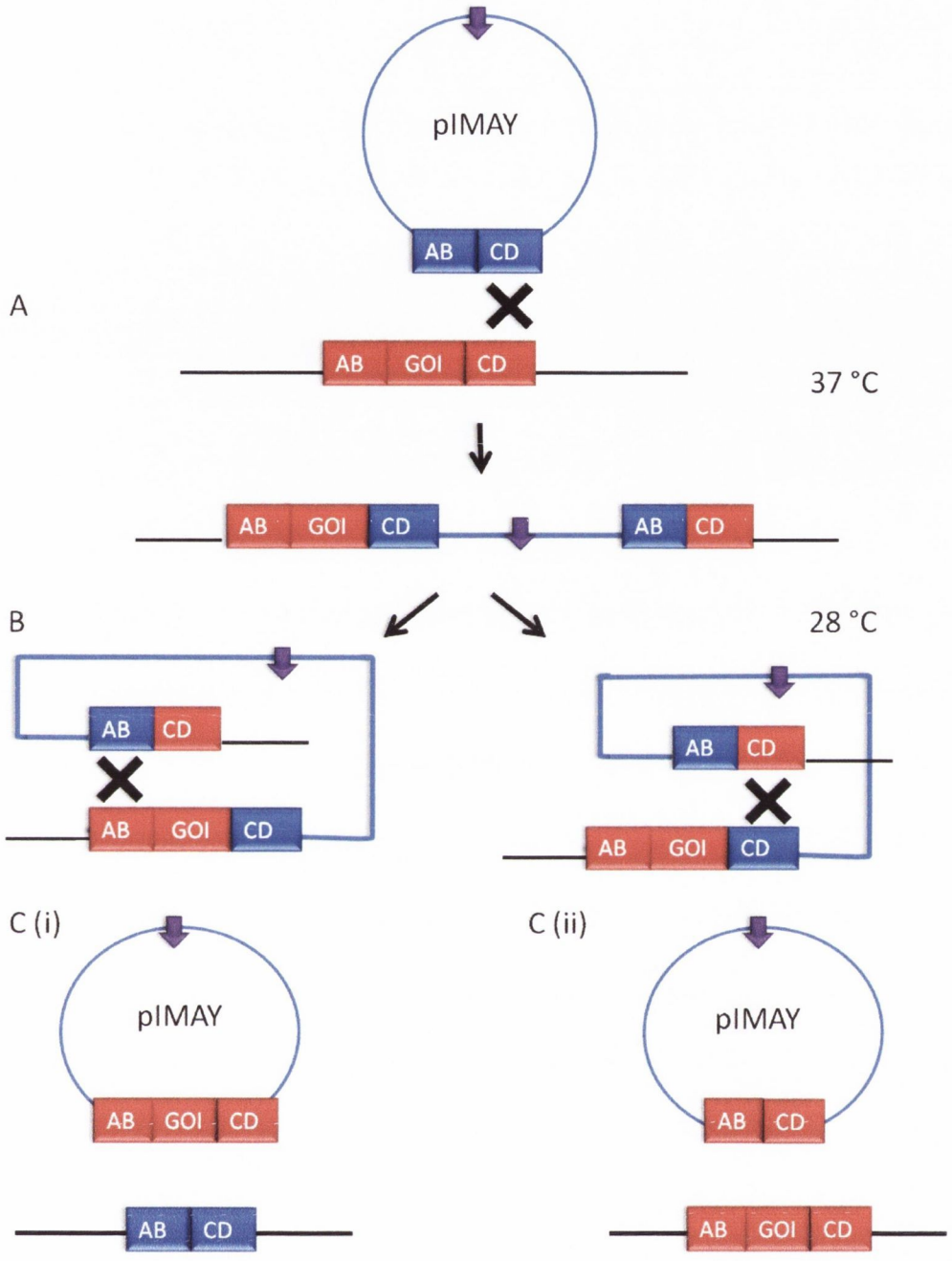


Figure 3.1 Schematic representation of allelic replacement by the temperature sensitive plasmid pIMAY.

(A) pIMAY integrates at the chromosomal locus by a single cross-over event on one side of the gene of interest (GOI) via either the AB or CD sides of homology. **(B)** A second recombination event can occur either at the AB or CD sides of the GOI. **(C)** (i) A second cross-over event on the opposite side to the first causes excision and removal of the wild-type gene. (ii) a second cross-over event on the same side of the gene of interest causes excision of the plasmid without allelic replacement.

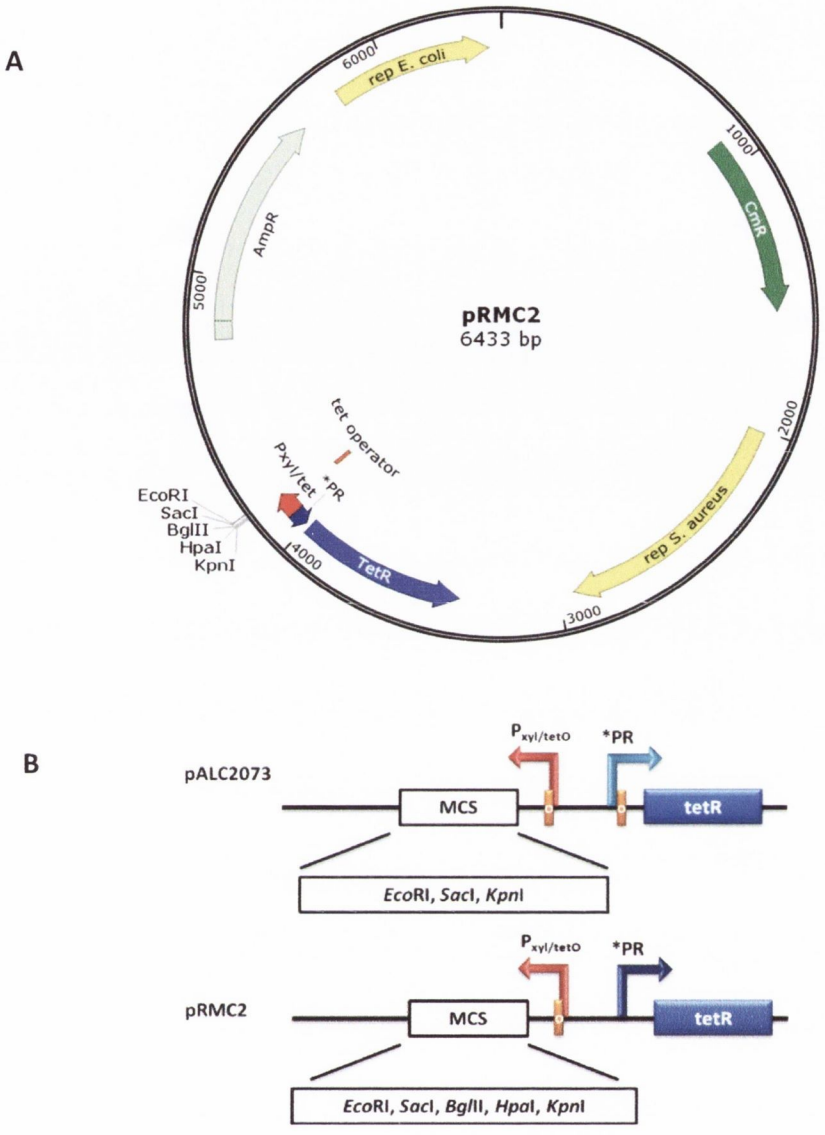


Figure 3.2 Schematic of plasmid pRMC2 and the MCS of pALC2073 and pRMC2.

Plasmid pRMC2 is a multicopy shuttle vector containing an ampicillin resistance determinant which encodes a β-lactamase for selection in *E. coli* and a chloramphenicol resistance determinant which encodes chloramphenicol acetyltransferase for selection in *S. aureus*. **(B)** Schematic of the $P_{xyl/tetO}$ and *PR promoter regions and multiple cloning sites (MCS) of pALC2073 and pRMC2. Expression of target genes is controlled by TetR expression from the *PR promoter. TetR bind to the *tetO* site within the $P_{xyl/tetO}$ promoter. The -10 box of the *PR promoter has been mutated in pRMC2 improving the sequence consensus and simultaneously disrupting its *tetO* site and relieving autorepression. In the presence of ATc TetR binding to *tetO* is disrupted at the $P_{xyl/tetO}$ promoter and high level expression achieved.

the *PR promoter. TetR bind to the *tetO* site within the $P_{xyl/tetO}$ promoter which represses expression of the gene of interest. In the presence of ATc TetR binding to *tetO* is disrupted and high level expression can be achieved.

Corrigan *et al.* identified the sequence of the -10 box of *PR promoter in pALC2073 as 'tagagt' which differs by two bases from the *B. subtilis* consensus of 'tataat'. Hypothesizing that leaky expression in pALC2073 was due to poor TetR expression the -10 box of the *PR promoter was mutated to bring it to the *B. subtilis* consensus sequence 'tataat'. The *PR promoter contains a *tetO* site allowing autoregulation of tetR. Manipulation of the -10 box of *PR simultaneously disrupted the *tetO* site within the *PR promoter removing autoregulation and allowed constitutive expression of TetR. To improve functionality of the plasmid the multiple cloning site was expanded to include the restriction sites HpaI and BglII. The resulting plasmid pRMC2 allows a high level of expression of target genes without leaky expression in the absence of inducer (Corrigan and Foster, 2009).

In this Chapter the role of the N1 subdomain in the biological functions of ClfA is explored. To facilitate the study of the role of N1 on the surface of *S. aureus* it was necessary to carry out genetic manipulations to create a suitable host for expression studies. Plasmid pRMC2 carrying the full length *clfA* gene was constructed and variants lacking subdomain N1 were constructed and utilised to study the role of the N1 subdomain in ClfA function and surface display. Constitutive expression of a ClfA variant lacking subdomain N1 was attempted in pALC2073 and the effect of expression of a ClfA variant lacking subdomain N1 on growth was examined.

3.2 Results

3.2.1 Subdomain N1 is not required for fibrinogen binding by recombinant ClfA A domains.

To confirm that subdomain N1 is not required for fibrinogen binding recombinant His-tagged ClfAN123$_{40\text{-}220}$ and ClfAN23$_{221\text{-}559}$ were expressed from plasmids pCF40 and pCF41, respectively, and purified to homogeneity. The ExPASY protparam tool predicts that His-tagged ClfAN123$_{40\text{-}220}$ and ClfAN23$_{221\text{-}559}$ would have molecular weights of 56 kDa and 37 kDa respectively. Interestingly although ClfAN23$_{221\text{-}559}$ migrates at its predicted molecular weight under denaturing and reducing SDS-PAGE conditions ClfAN123$_{40\text{-}220}$ has an apparent molecular weight of ~85 kDa (Fig 3.3A). This is consistent with observations made for ClfB which suggest that the N1 subdomain migrates on SDS-PAGE with an aberrantly high apparent molecular mass. This retarded migration was predicted to be due to the elongated structure of the N1 subdomain of ClfB as determined by gel permeation chromatography. Intriguingly the N1 subdomain of ClfB was shown to be capable of conferring this elongated structure on the N2 or N23 subdomains when expressed in conjunction with these subdomains (Perkins *et al.*, 2001).

His-tagged ClfAN123$_{40\text{-}220}$ and ClfAN23$_{221\text{-}559}$ were tested for their ability to bind to immobilized fibrinogen to confirm accepted dogma that subdomain N1 is not involved in fibrinogen binding. No difference in affinity at half maximal binding was observed indicating that the loss of subdomain N1 is not detrimental to fibrinogen binding (Fig 3.3B). In a modified version of this fibrinogen binding assay, His-tagged A regions were immobilized on the surface of a microtitre plate and their ability to capture fibrinogen from solution was measured. To ensure both His-tagged constructs coated the plate with equal efficiency and ELISA was performed with monoclonal anti-ClfA IgG. Monoclonal IgG raised against the N3 region of ClfA was used to ensure equal recognition of both ClfA constructs. Intriguingly although both His-tagged ClfAN123$_{40\text{-}220}$ and ClfAN23$_{221\text{-}559}$ coated the surface of a microtitre plate with equal efficiency (Fig 3.3 C) they were not capable of capturing fibrinogen with equal efficiency when immobilized in this fashion. Although this scenario is somewhat artificial it suggests that the N1 subdomain may promote additional flexibility required for the dynamic process of dock, lock and latch.

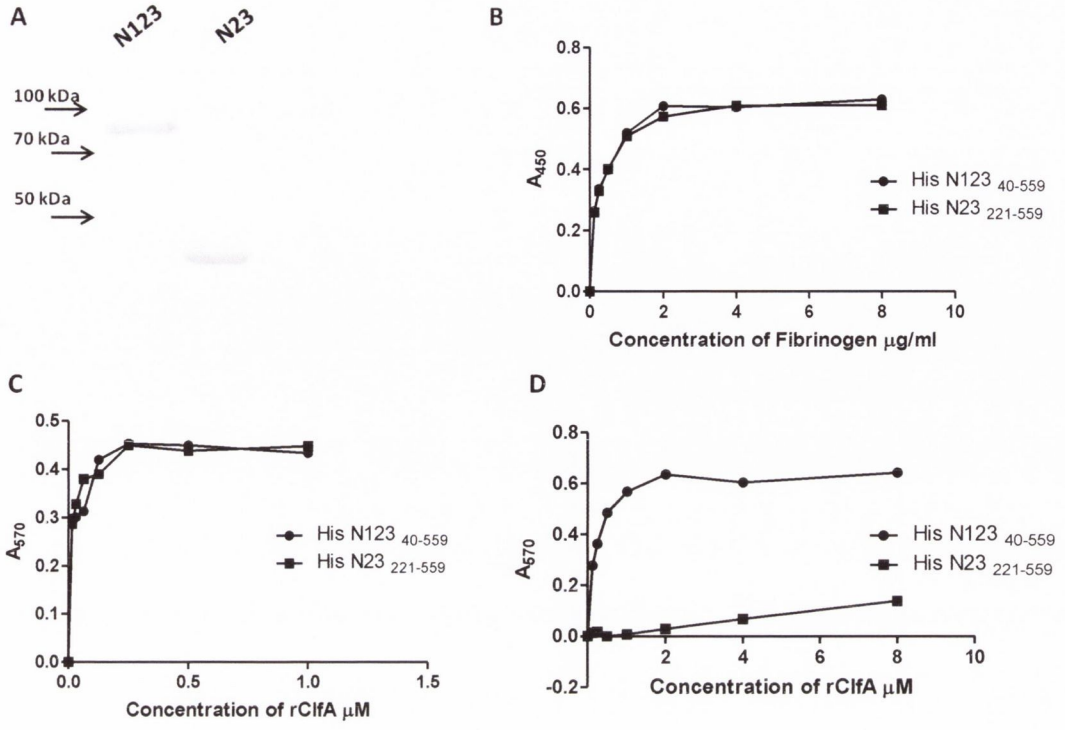


Figure 3.3 Recombinant His-tagged ClfA A regions binding to immobilised fibrinogen.

(A) Recombinant His-tagged ClfAN123$_{40\text{-}559}$ and ClfAN23$_{221\text{-}559}$ separated on a 12.5 % SDS-PAGE gel and stained with Commassie brilliant blue. Molecular weight markers are indicated with black arrows. **(B)** Recombinant His-tagged ClfAN123$_{41\text{-}559}$ and N23$_{221\text{-}559}$ were tested for binding to immobilised human fibrinogen (10 µg/ml). Bound ClfA was recognised by ClfA mAb and HRP-conjugated rabbit anti-mouse IgG. **(C)** His ClfAN123$_{40\text{-}559}$ and ClfAN23$_{221\text{-}559}$ were tested to ensure equal immobilisation on the surface of a microtitre plate. Wells were incubated overnight with 0-1 µM of recombinant ClfA. Bound protein was detected as in (B) **(D)** Capture of fibrinogen (10 µg/ml) by immobilized ClfA A domains. Wells were coated as in with 0-8 µM of recombinant ClfA (rClfA). Bound fibrinogen was detected using HRP-conjugated anti-fibrinogen IgG. ELISAs were developed by incubation with a chromogenic substrate. The absorbance at A_{450} was determined. Data points represent the mean of triplicate wells. The data shown is representative of three individual experiments.

3.2.2 Construction of strain Newman *clfA5clfBspa* (NM1)

We hypothesized that subdomain N1 must have a function and may be important when ClfA is expressed on the surface of *S. aureus*. *S. aureus* expressed four different fibrinogen binding surface proteins, ClfA, ClfB, and FnBPA and FnBPB. To facilitate the investigation into the role of subdomain N1 on the surface of *S. aureus* a derivative of strain Newman which does not bind to fibrinogen was required. In addition, binding of IgG by protein A complicates surface protein detection by Western immunoblotting. In Newman the *fnbA* and *fnbB* genes contain premature stop codons that cause truncated FnBP proteins to be secreted (Grundmeier *et al.*, 2004). A derivative of strain Newman which lacks expression of ClfA and ClfB due to a frame-shift mutation within the *clfA* gene and an erythromycin resistance cassette insertion within the *clfB* gene had previously been constructed (Table 2.1). Strain NM1 was constructed by transducing *spa*::Ka^r from the strain Newman *spa* into Newman *clfA5clfB* using phage 85. The strain was validated by Western immunoblotting to ensure that it lacked the cell wall proteins ClfA, ClfB and protein A (Fig. 3.4 A, B, C). The cell wall fraction of NM1 was also probed with anti-SdrE IgG as a positive control (Fig 3.4 D). Strain Newman was used as a positve contol for surface protein expression.

Spontaneous mutations in the global regulatory system *agr* can occur at high frequency (Tegmark *et al.*, 2000, Traber and Novick, 2006). Mutations within the *agr* locus can seriously affect expression of virulence factors and impact on fitness through growth rate. To insure *agr* function strain NM1 was tested for δ-haemolysin production. δ-haemolysin is a short cytolytic peptide that is translated from RNAIII, the effector molecule of the Agr two-component system. δ-haemolysin has weak haemolytic activity on sheep blood agar but can act in synergy with β-haemolysin potentiating the zone of haemolytic clearing where they interact. Strain NM1 and its parental strain Newman *clfA5clfB* were cross-streaked against strain RN4220, a strain which produces only β-haemolysin (Tegmark *et al.*, 2000)(Traber *et al.*, 2008). Similar zones of clear haemolysis were observed for both strains where the production of β-haemolysin and δ-haemolysin overlapped indicating normal agr function (Fig. 3.4E).

A growth curve was performed to insure no loss of fitness was aquired during transduction of *spa*::Ka^R. No difference in growth was observed when strain NM1 was compared to its parental strain Newman *clfA5clfB* (Fig. 3.4F).

3.2.3 Construction and validation of pRMC2*clfA*

This study set out to identify the role of subdomain N1 of ClfA. To facilitate this, the *clfA* gene of strain Newman was cloned between the BglII and EcoRI sites of pRMC2. A schematic of plasmid pRMC2 is depicted in Fig. 3.2A. Briefly the full length *clfA* gene of strain Newman including its ribosome binding site was amplified from genomic DNA isolated from strain Newman. BglII and EcoRI sites were incorporated into the 5′ ends of the forward and reverse primers, respectively, to facilitate directional cloning. PCR products were digested with EcoRI and BglII, purified and ligated to pRMC2 digested with EcoRI and BglII and transformed into *E. coli* strain DC10B.

Positive pRMC2*clfA* clones were confirmed first by PCR colony screening using primers directed against the *clfA* gene to detect replicating pRMC2*clfA* plasmid (Fig 3.5A). Plasmid was isolated from putative positive clones and insertion of *clfA* was confirmed by restriction digest with EcoRI and BglII (Fig 3.5B). Plasmid pRMC2*clfA* was sequenced to confirm the fidelity of the *clfA* sequence and promoter region. Plasmids were transformed into strain NM1 (Table 2.1) for expression analysis.

Plasmid pRMC2 was chosen as the expression vector for ClfA as it allows high level expression of proteins in the presence of ATc and tight repression in its absence. To confirm that pRMC2*clfA* fulfilled these criteria whole cell dot immunoblotting using monoclonal anti-ClfA IgG of NM1 cells carrying pRMC2 or pRMC2*clfA* was performed to measure surface expression of ClfA. A stationary phase culture of strain Newman *spa* was used as a positive control for ClfA expression from a single chromosomal copy of *clfA*. In the absence of inducing agent no ClfA expression was observed on the surface of *S. aureus* highlighting the tight level of repression achieved by pRMC2. High level dose-responsive expression of ClfA above the level of Newman *spa* was achieved when increasing concentrations of ATc were used (Fig 3.6A). Adherence of cells to immobilised fibrinogen was performed with the same cells as in Fig 3.6A. Dose-responsive binding to fibrinogen was observed with increasing concentrations of ATc and higher expression of ClfA (Fig 3.6B) demonstrating that functional ClfA is expressed from pRMC2 and can be induced in an ATc concentration-dependent manner.

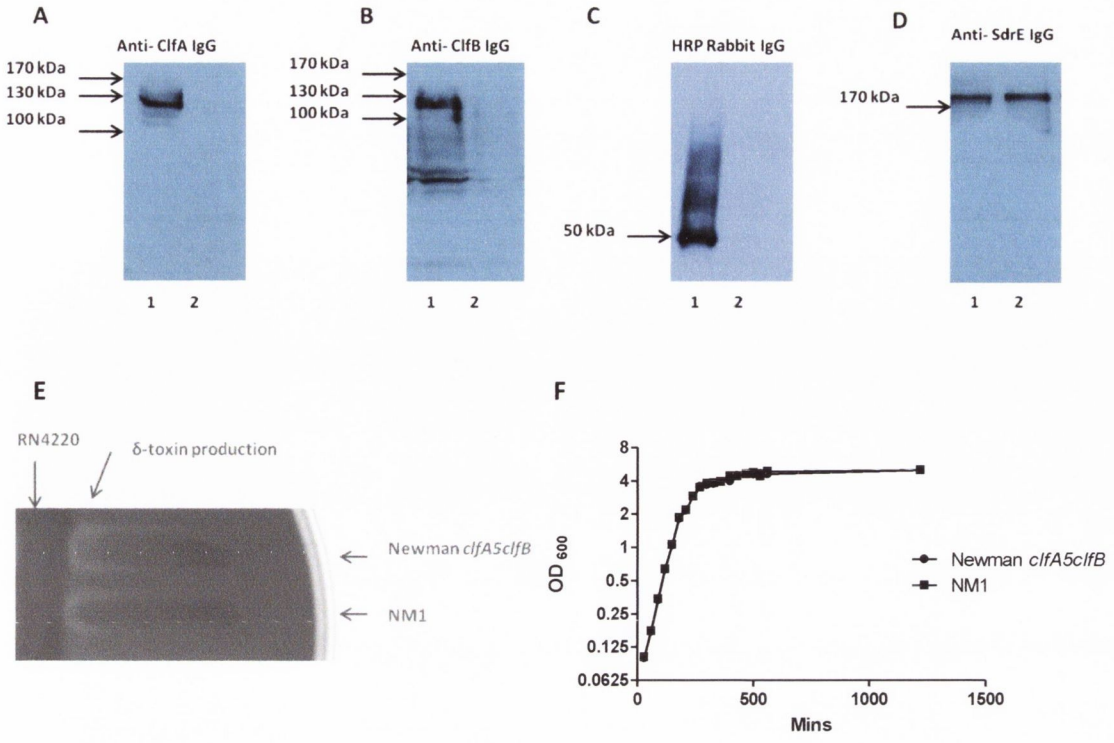


Figure 3.4 Validation of strain NM1.

Western immunoblotting of cell wall fractions. **(A-D)** Lane 1: Newman wild type, Lane 2: NM1. Newman was used as a positive control for MSCRAMM expression. Blots were probed with **(A)** polyclonal anti-ClfA IgG, **(B)** polyclonal anti-ClfB IgG, **(C)** HRP-conjugated rabbit IgG for detection of protein A. **(D)** polyclonal anti-SdrE IgG and detected with protein A peroxidase. SdrE was used as a positive control for the cell wall of strain NM1 **(E)** *S. aureus* NM1 and its parental strain Newman *clfA5clfB* were cross-streaked against RN4220 on sheep blood agar plates in order to compare the haemolytic pattern. Enhanced haemolysis due to δ-toxin is indicated with black arrows. **(F)** Growth curve comparing strain NM1 and its parental strain Newman *clfA5clfB*.

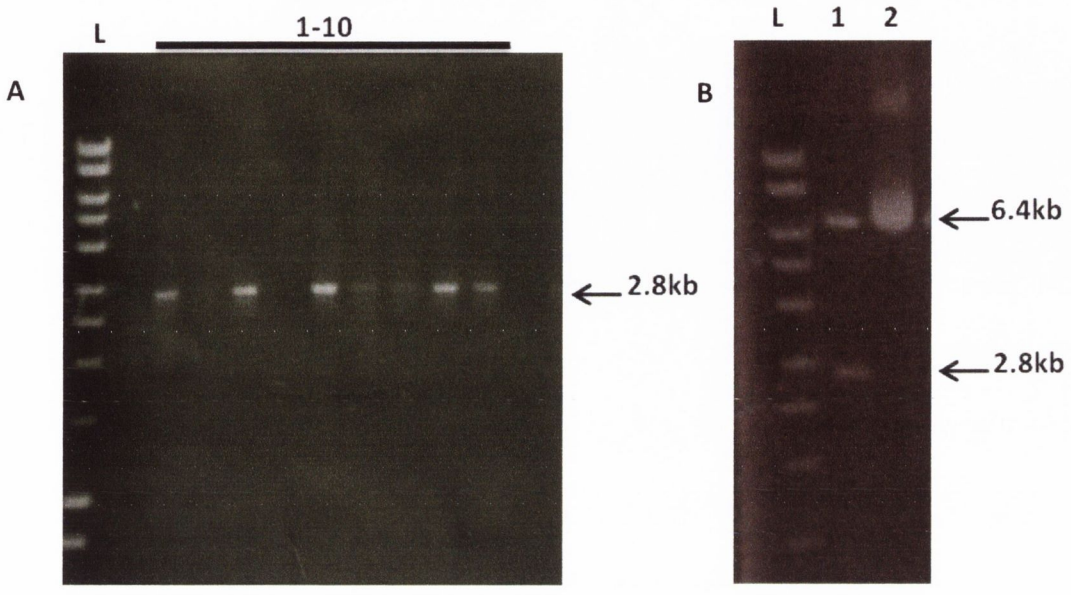


Figure 3.5 Agarose gel electrophoresis of PCR colony screens for and restriction digest of pRMC2*clfA*

(A) Lanes 1-10 PCR colony screen performed on *E. coli* transformed with pRMC2*clfA*. Primers directed against the *clfA* gene were used to detect replicating plasmid containing the *clfA* gene. Positive clones contain a 2.8 kb fragment. **(B)**Lane 1, pRMC2*clfA* digested with EcoRI and BglII. Fragments of 2.8 kb and 6.4 kb correspond to the size of the full length *clfA* gene and pRMC2 vector backbone, respectively. Lane 2, uncut pRMC2.

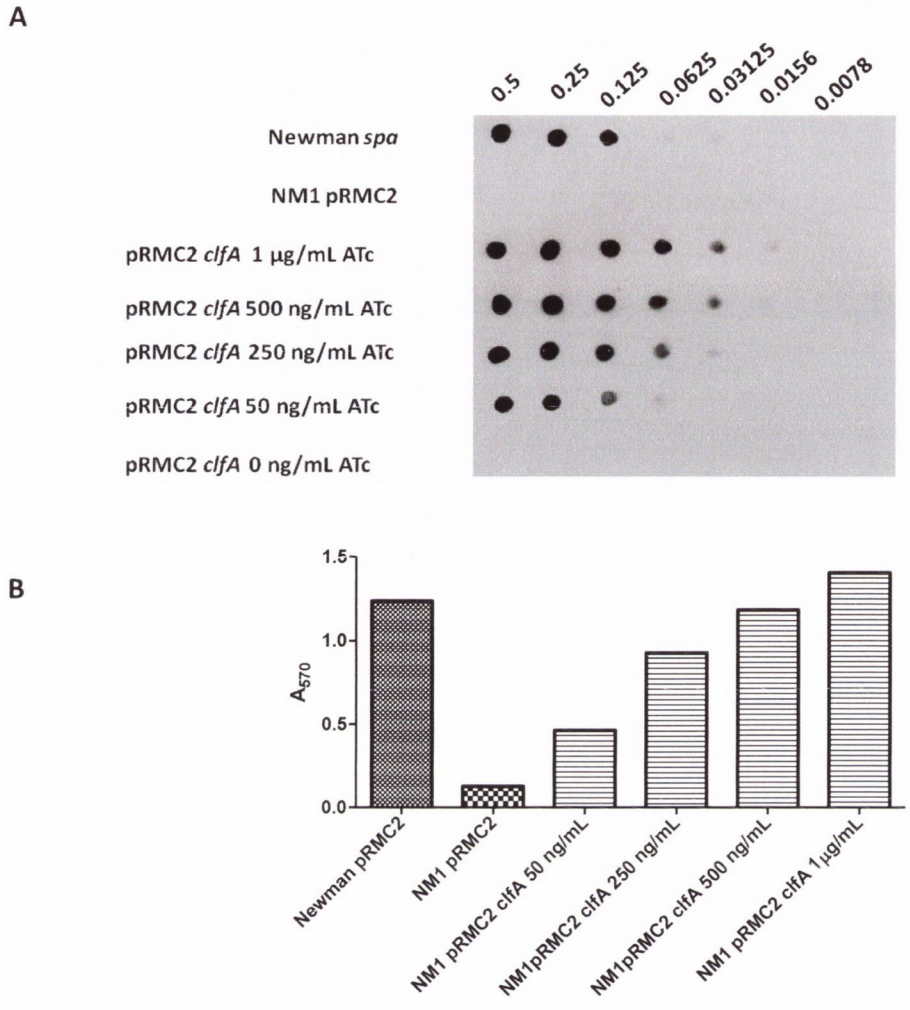


Figure 3.6 Expression of ClfA from pRMC2*clfA*

(A) Whole cell dot immunoblot of NM1 cells carrying pRMC2 or pRMC2*clfA*. Strain Newman *spa* was used as a positive control for ClfA expression in stationary phase from a single chromosomal copy. The strain and level of inducing agent are listed and the OD_{600} of the cells is indicated across the top of the blot. 5µl of each concentration was spotted onto the membrane and probed with ClfA mAb and HRP-conjugated rabbit anti-mouse IgG. **(B)** Adhesion to immobilized fibrinogen by *S. aureus* expressing pRMC2*clfA* induced with ATc (50 ng/ml- 1 µg/ml). Stationary phase Newman pRMC2 was used as a positive control for fibrinogen binding and NM1 pRMC2 as a negative control. Bacterial adherence to fibrinogen was measured by staining with crystal violet and measuring the A_{570}. Data points represent the mean of triplicate wells, the graph is representative of three independent experiments.

3.2.4 Construction and validation of pRMC2*clfA* ΔN1 variants

The subdomain boundaries of the A region of ClfA were originally defined based on amino acid sequence similarity with ClfB by the position of a cleavage site (SLAVA) for the *S. aureus* metalloprotease aureolysin (Perkins *et al.*, 2001). ClfA contains a similar sequence (SLAAVA). Based on this motif the amino acid coordinate of the beginning of the N2 subdomain of ClfA was originally defined as residue 221 (McDevitt *et al.*, 1995, O'Connell *et al.*, 1998). More recently the N2N3 subdomains of ClfA have been crystallized (Ganesh *et al.*, 2008) allowing the precise definition of boundaries of the N2N3 subdomains as comprising residues 229-545 (Fig. 3.7).

To determine the role of the N1 subdomain on the surface of *S. aureus* plasmid pRMC2*clfA* was manipulated by inverse PCR to create derivatives lacking DNA encoding the N1 subdomain of ClfA. A schematic of inverse PCR is depicted in Fig 3.8. Two pRMC2*clfA* ΔN1 constructs were generated to reflect the different definitions of the boundary of subdomain N1 yielding plasmids pRMC2*clfA*ΔN1$_{40\text{-}220}$ and pRMC2*clfA*ΔN1$_{40\text{-}228}$, respectively (Fig. 3.7, Table 2.2). ClfAΔN1$_{40\text{-}220}$ represents a deletion up to and including A_{220} of the SLAAVA motif while ClfAΔN1$_{40\text{-}228}$ removes the entire N1 sequence defined by the X-ray crystal structure of N23 (Fig. 3.6A). Following ligation of inverse PCR amplimers and transformation of *E. coli* strain DC10B, plasmids were electroporated into strain NM1 for expression studies. The fidelity of all constructs was confirmed by DNA sequencing.

Whole cell dot immunoblotting was performed to measure the amount of ClfA on the bacterial surface. Serial dilutions of bacteria were applied to a nitrocellulose membrane and a monoclonal antibody that recognizes an epitope in subdomains N23 of ClfA (ClfA mAb) was used to detect the protein. Expression of ClfA by NM1 (pRMC2*clfA*) could not be detected in the absence of inducer (Fig. 3.9) in agreement with previous work showing that gene expression from pRMC2 is tightly repressed. Strain NM1 carrying the pRMC2 empty vector was used as a negative control. Addition of increasing concentrations of ATc resulted in increasing amounts of ClfA on the surface of *S. aureus* (Fig. 3.9). A high level of expression similar to that achieved by a single chromosomal gene in strain Newman *spa* was achieved at inducer concentrations of 800 ng/ml and 1.2 μg/ml. In contrast neither ClfAΔN1$_{40\text{-}220}$ nor ClfAΔN1$_{40\text{-}228}$ could be detected even at the highest concentration of inducer tested (Fig. 3.9). This was surprising as sequencing revealed that the promoter region if pRMC2 was intact. This

result suggested that N1 might have a role to play in protein export. However, with no evidence of protein expression obtained it cannot be ruled out that the construct was non-functional.

Variants of pRMC2*clfA* were constructed which retained differing amounts of N1 in an attempt to determine the minimum sequence that supported surface expression. A mutant lacking residues 40-210 but retaining C-terminal residues of N1 (ClfAΔN1$_{40-210}$) was expressed on the surface of *S. aureus*, albeit at a slightly lower level than full-length ClfA expressed from pRMC2*clfA* (Fig. 3.9). However, when cultures of NM1 (pRMC2*clfA*) and NM1 (pRMC2*clfA*ΔN1$_{40-210}$) were induced with 800 ng/ml and 1.2 μg/ml of ATc, respectively, the amount of ClfA and ClfAΔN1$_{40-210}$ on the surface of *S. aureus* was found to be the same.

3.2.5 Aureolysin treatment of *S. aureus* and recombinant ClfA

A putative metalloprotease cleavage site (SLAA$_{220}$V$_{221}$A) is located close to the junction between N1 and N2. Bacteria expressing full length ClfA from pRMC2*clfA* were treated with aureolysin to proteolytically remove the N1 subdomain and examined by Western immunoblotting. Aureolysin-treated cell wall fractions revealed a truncated form of ClfA which migrated similarly to ClfAΔN1$_{40-210}$ at a molecular weight of ~100 kDa (Fig 3.10A). This indicates that aureolysin can cleave ClfA on the surface of *S. aureus* presumably at the SLAAVA motif (Fig. 3.7). Proteolytic removal of N1 did not reduce ClfA-mediated adherence of bacteria to immobilized fibrinogen (Fig. 3.10B). This shows that the N1 subdomain up to residue V221 is not required for ClfA expressed on the bacterial cell surface to promote adherence to fibrinogen.

In order to determine if aureolysin cleaves ClfA at the SLAA$_{220}$V$_{221}$A motif, recombinant His-tagged ClfAN123$_{40-559}$ was treated with aureolysin (Fig. 3.11A). SDS-PAGE analysis of aureolysin-treated recombinant ClfA revealed a band of ~40 kDa similar to the molecular weight of recombinant His-tagged ClfAN23$_{220-559}$ (Fig 3.3A). The N-terminal sequence of aureolysin-treated recombinant ClfA was determined. The N-terminal sequence of the truncate was determined as 'VAADA' showing that cleavage occurred between residues A$_{220}$ and V$_{221}$ of the SLAA$_{220}$V$_{221}$A motif as predicted (Fig. 3.11B).

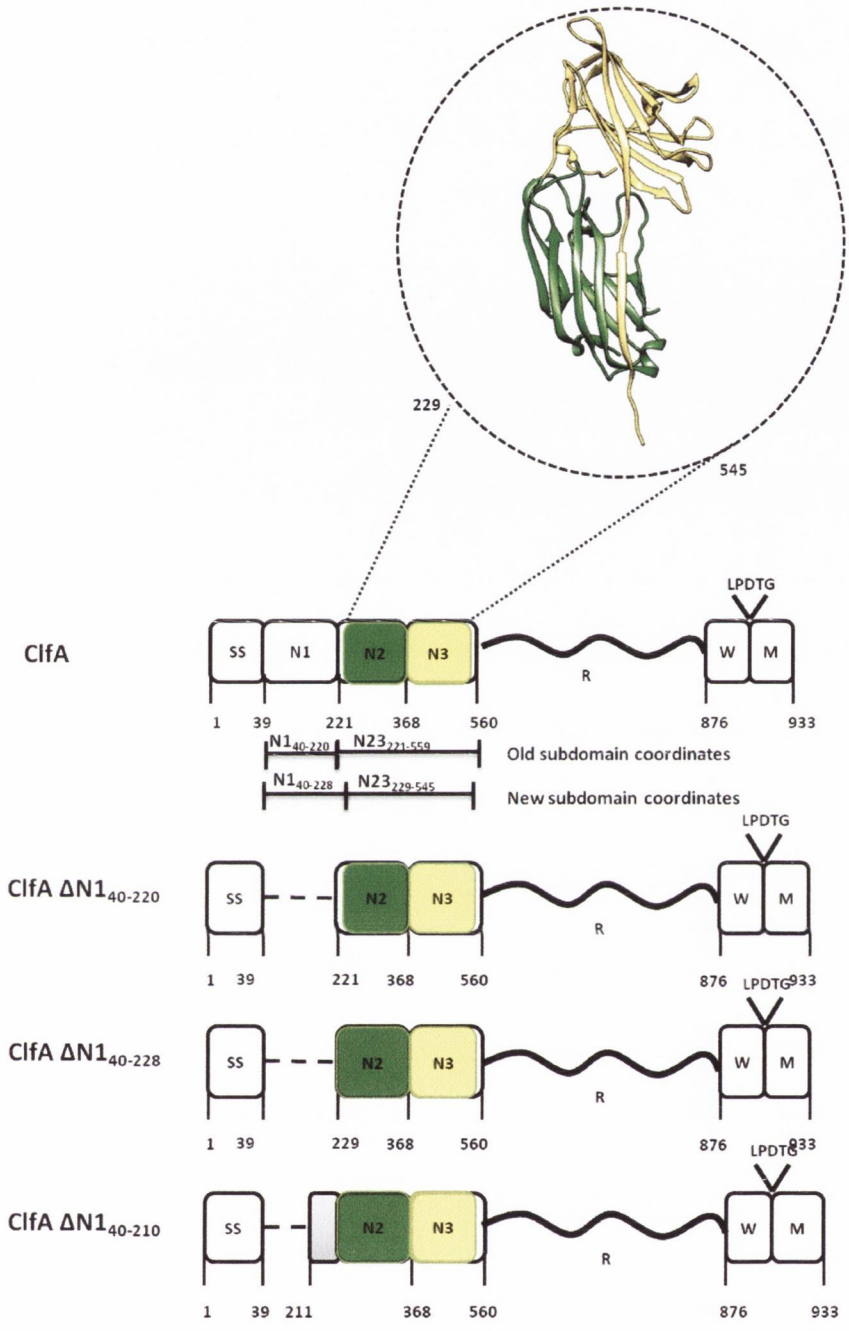


Figure 3.7 Schematic representation of ClfA derivatives.

The amino acid coordinates of the signal sequence (SS), N1, N2 and N3 subdomains, SD repeat region (R) and cell wall (W) and membrane spanning (M) regions are indicated. The original and revised definition for the minimum ligand binding domain of subdomains N23 (colour coded green and yellow, residues 229-545) and its crystal structure of is shown.

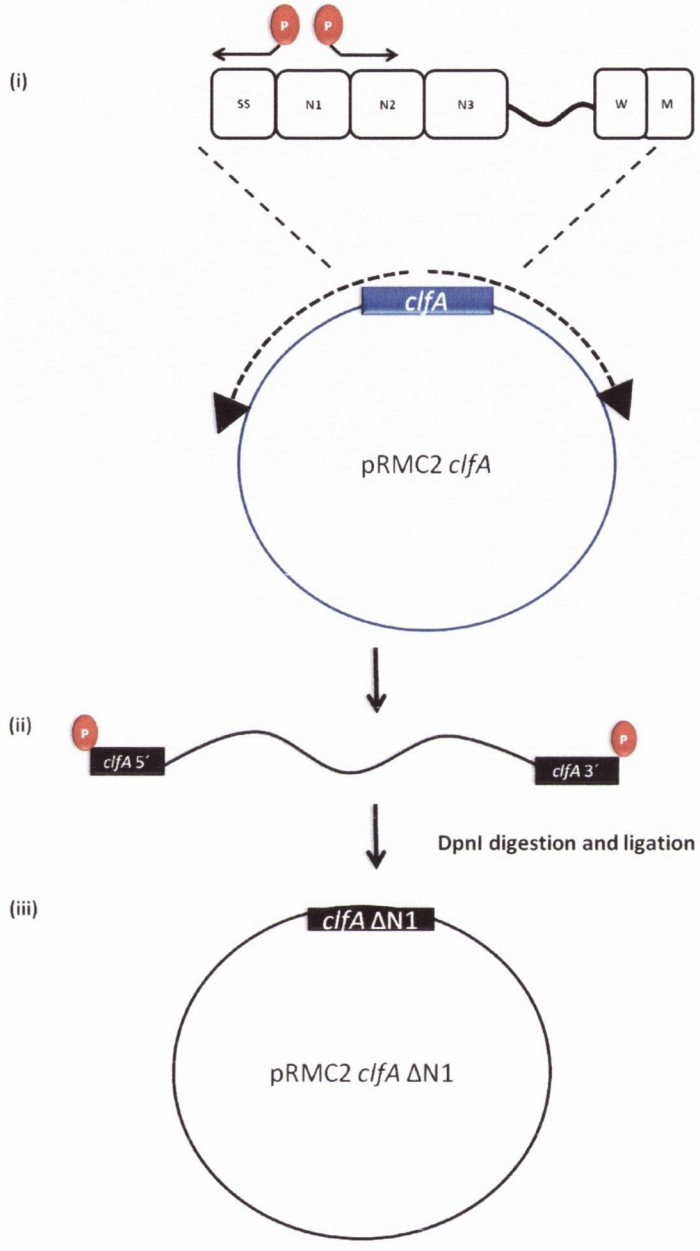


Fig 3.8 Schematic of inverse PCR for deletion of subdomain N1 of ClfA

(i) 5′ Phosphorylated (red ovals) primers were designed in an inverted orientation to regions 5′ and 3′ to the N1 subdomain. **(ii)** PCR amplification using pRMC2*clfA* as template generated a linear fragment of DNA with phosphorylated ends (red ovals) which contains the 5′ and 3′ fragments of the *clfA* gene lacking the region encoding subdomain N1. **(iii)** Following DpnI digestion to remove hemimethylated template DNA the linear amplimer is ligated to generate circular pRMC2*clfA*ΔN1.

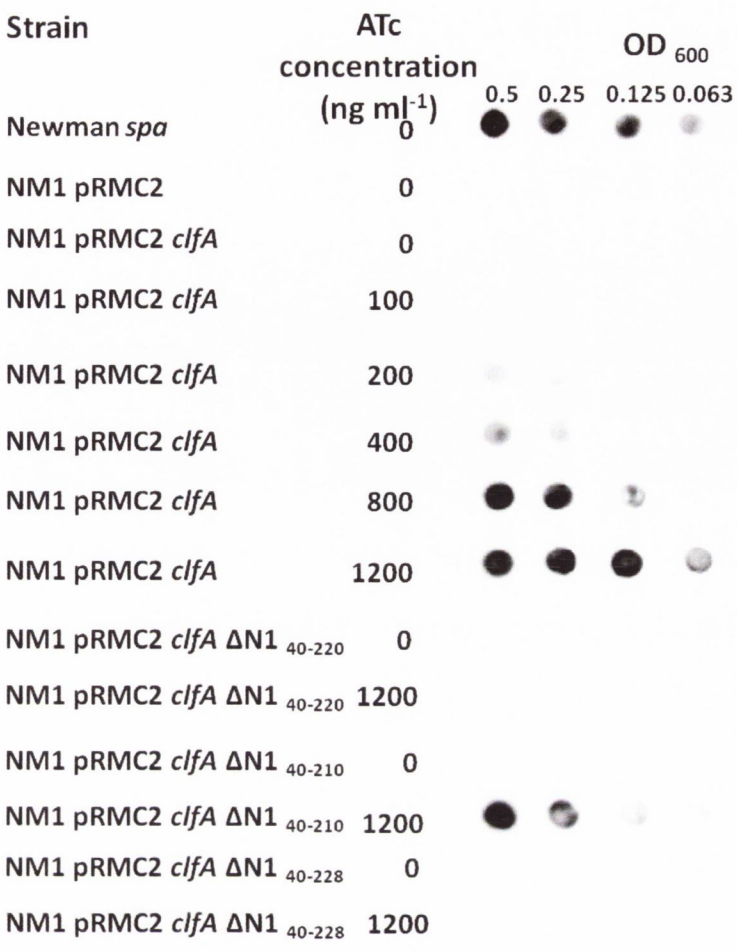


Figure 3.9 Surface expression of ClfA and ClfA derivatives.

Dot immunoblot of *S. aureus* NM1 expressing ClfA, ClfAΔN1$_{40\text{-}220}$, ClfAΔN1$_{40\text{-}228}$ or ClfAΔN1$_{40\text{-}210}$ from pRMC2. Newman *spa* has been included as a positive control for ClfA expression and NM1 carrying pRMC2 empty vector serves as a negative control. The concentration of inducer used and the OD_{600} of the cells are indicated. 5 µl of each dilution of cells was spotted onto the membrane and probed with ClfA mAb and HRP-conjugated rabbit anti-mouse IgG.

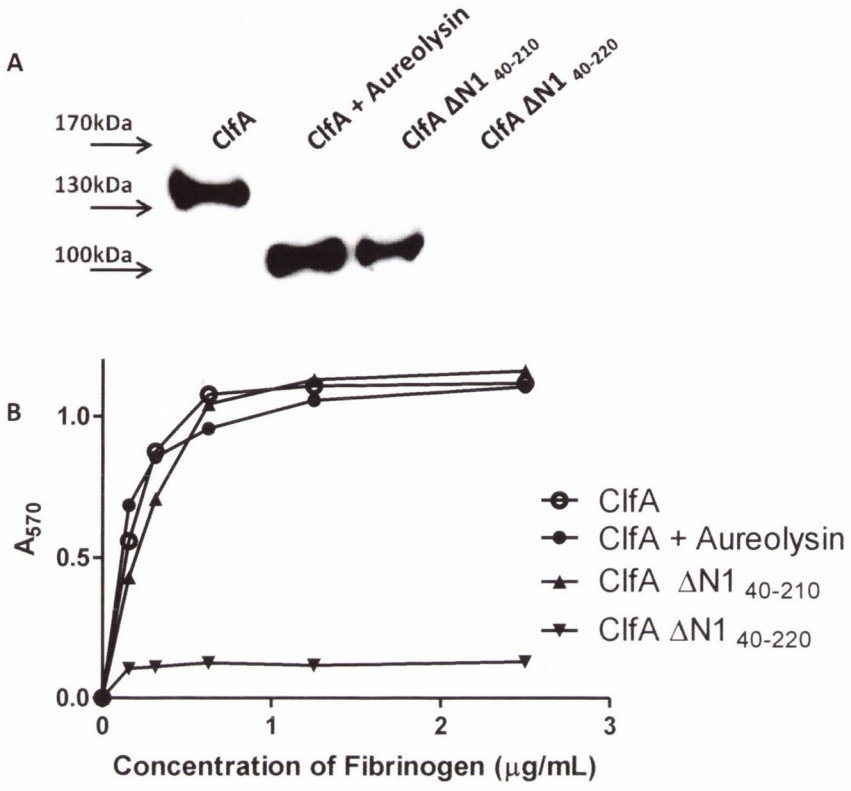
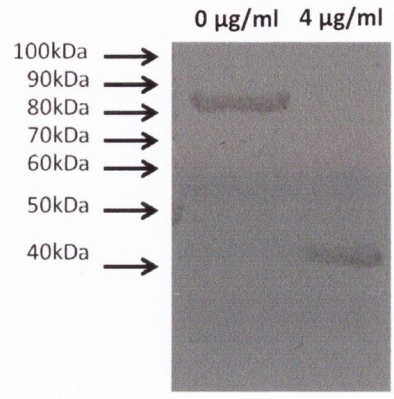


Figure 3.10 Bacterial adherence to fibrinogen by strains lacking subdomain N1 of ClfA

(A) Western immunoblot of cell wall extracts from bacteria expressing ClfA. *S. aureus* NM1 (pRMC2*clfA*) was grown in broth containing ATc (800 ng/ml) and treated with aureolysin where indicated as described above. NM1 (pRMC2*clfA*ΔN1$_{40\text{-}210}$ or pRMC2*clfA*ΔN1$_{40\text{-}220}$) were grown in broth containing ATc (1200 ng/ml). Cell wall extracts were separated on 7.5 % acrylamide gels, blotted onto PVDF membranes and probed with ClfA mAb and HRP-conjugated rabbit anti-mouse IgG. **(B)** *S. aureus* NM1 (pRMC2*clfA*) was grown in broth containing ATc (800 ng/ml). NM1 (pRMC2*clfA*ΔN1$_{40\text{-}210}$ or pRMC2*clfA*ΔN1$_{40\text{-}220}$) were grown in broth containing ATc (1200 ng/ml). Bacteria were incubated in a solution of aureolysin (8 μg/ml) prior to addition to fibrinogen-coated wells where indicated. Bacterial adherence to fibrinogen was measured by staining with crystal violet and measuring the A_{570}. Data points represent the mean of triplicate wells. The graph shown is representative of three independent experiments

A



B

MNMKKKEKHAIRKKSIGVASVLVGTLIGFGLLSSKEADASENSVTQSDSASNESKSNDSS 60

SVSAAPKTDDTNVSDTKTSSNTNNGETSVAQNPAQQETTQSSSTNATTEETPVTGEATTT 120

TTNQANTPATTQSSNTNAEELVNQTSNETTFNDTNTVSSVNSPQNSTNAENVSTTQDTST 180

Aureolysin cleavage site

EATPSNNESAPQSTDASNKDVVNQAVNTSAPRMRAFSLAAVAADAPAAGTDITNQLTNVT 240

VGIDSGTTVYPHQAGYVKLNYGFSVPNSAVKGDTFKITVPKELNLNGVTSTAKVPPIMAG 300

DQVLANGVIDSDGNVIYTFTDYVNTKDDVKATLTMPAYIDPENVKKTGNVTLATGIGSTT 360

ANKTVLVDYEKYGKFYNLSIKGTIDQIDKTNNTYRQTIYVNPSGDNVIAPVLTGNLKPNT 420

DSNALIDQQNTSIKVYKVDNAADLSESYFVNPENFEDVTNSVNITFPNPNQYKVEFNTPD 480

DQITTPYIVVVNGHIDPNSKGDLALRSTLYGYNSNIIWRSMSWDNEVAFNNGSGSGDGID 540

KPVVPEQPDEPGEIEPIPEDSDSDPGSDSGSDSNSDSGSDSGSDSTSDSGSDSASDSDSA 600

Figure 3.11 Degradation of recombinant His-tagged ClfAN123$_{40\text{-}559}$ by aureolysin.

(A) Coomassie stained 12.5% SDS-PAGE gel showing recombinant His-tagged ClfAN123$_{40\text{-}559}$ treated with 4 µg/ml aureolysin. Molecular weight markers are listed at the side of the gel and concentration of aureolysin used at the top of the blot. **(B)** Amino acid sequence of the ClfA A region from strain Newman. The N-terminal sequence of aureolysin treated recombinant ClfA is highlighted in yellow. Aureolysin cleavage site is indicated by black arrow.

3.2.6 Construction of GST-tagged ClfA A region and variants.

As subdomain N1 does not appear to play a role in fibrinogen binding it is unclear why it is retained by *S. aureus*. When expressed on the surface of *S. aureus* subdomain N1 can be cleaved by the *S. aureus* metalloprotease aureolysin. Cleavage of subdomain N1 from surface-expressed mature ClfA protein suggests that the N1 subdomain is either dispensable once it is displayed on the cell surface or its loss is beneficial to the organism. One plausible benefit could be that loss of the N1 subdomain affects antibody recognition and opsonisation of *S. aureus*. To facilitate the study of the N1 subdomain of ClfA the A region comprising residues 40-559 and the N23 subdomains comprising residues 221-559 were cloned into pGEX-4T-2 to allow expression and purification of GST-tagged proteins. Plasmid pCF40 was used as template for PCR amplification of the A domain fragments using primers incorporating EcoRI and BamHI restriction sites. Positive clones were confirmed by restriction digestion with EcoRI and BamHI yielding bands of 1.6 kB and 1 kB for ClfAN123$_{40\text{-}559}$ and ClfAN23$_{221\text{-}559}$ respectively (Fig 3.12A). Protein expression was induced with IPTG in *E. coli* Topp3 cells. Recombinant proteins were purified to homogeneity firstly by affinity chromotography on a GST Hi-Trap column followed by anion exchange. Proteins were examined by SDS-PAGE to confirm size and purity (Fig 3.12B). ExPASy's ProtParm tool predicts the molecular weights for GST tagged ClfAN123$_{40\text{-}559}$ and ClfAN23$_{221\text{-}559}$ to be aproximatly 78 kDa and 60 kDa respectively. As with the His-tagged recombinant ClfA A regions, the GST-tagged ClfAN23$_{221\text{-}559}$ migrates at its predicted molecular weight while the GST-tagged ClfAN123$_{40\text{-}559}$ construct migrates at a higher apparent molecular weight of 100 kDa (Fig 3.12B).

Doubling dilutions of GST-tagged recombinant ClfA were immobilized on the surface of a microtitre plate. To confirm that both constructs coated the plate with equal efficiency bound protein was detected using polyclonal anti-GST IgG. Both constructs coated the plate in a dose-responsive and saturable manner. No difference in the level of immobilized protein was detected (Fig 3.12C). To determine if the immobilized GST-tagged ClfA A regions were fully functional immobilized proteins were incubated with 10 µg/ml human fibrinogen. Both constructs bound fibrinogen in a dose-responsive and saturable manner with similar affinities confirming previous observations that subdomain N1 is not necessary for fibrinogen binding (Fig 3.12 D).

As a surface-expressed protein, ClfA can be recognized by circulating antibodies in the blood stream. To determine which of the individual subdomains of ClfA is most readily recognized by the antibodies the N1, N2 and N3 subdomains of ClfA were cloned individually into pGEX-4T-2. To reflect the two different definitions of subdomain N1 two distinct GST-tagged recombinant protein constructs were generated. $N1_{SHORT}$ comprises residues 40-220 and reflects the N1 subdomain as defined by its aureolysin cleavage site. $N1_{LONG}$ comprises residues 40-228 and reflects the N1 subdomain as defined by the crystal structure of the minimum ligand binding domain of ClfAN23.

At the junction of the N2 and N3 subdomains is a six residue linker region which joins the two individually folded subdomains. The boundary between N2 and N3 was defined as beginning of this linker region. To facilitate folding of the individual subdomains the entire linker region was added as a tail region to both the N2 and N3 subdomains (Fig 3.13A). Each of the individual subdomains was expressed and affinity-purified as described for $ClfAN123_{40-559}$ and $ClfAN23_{220-559}$. ExPASy's ProtParm tool predicted the following molecular weights for each of the GST-tagged subdomains. $N1_{SHORT}$: 42 kDA, $N1_{LONG}$: 42 kDa, N2: 39 kDa, N3: 44 kDa. Intriguingly both $N1_{SHORT}$ and $N1_{LONG}$ migrated at an apparent molecular weight of ~70 kDa while the N2 and N3 subdomains migrated at their predicted molecular weights (Fig. 3.13B). This confirms that it is the N1 subdomain alone which migrates at a higher than predicted molecular weight and that it is capable of conferring this aberrant migration phenotype on subdomains to which it is attached. Doubling dilutions of GST-tagged recombinant ClfA subdomains were immobilized on the surface of microtitre plates. To confirm all ClfA constructs coated the plate with equal efficiency bound protein was detected with polyclonal anti-GST IgG. All individual subdomains bound to the surface of the microtitre plate in dose-responsive and saturable manner with equal efficiency to the full length $ClfAN123_{40-559}$ construct and the $ClfAN23_{220-559}$ construct (Fig 3.13C).

3.2.7 Quantification of the antibody response against individual GST-tagged ClfA subdomains in serum obtained from immunized mice and healthy human donors.

To determine whether the N1 subdomain plays a role in the ability of antibodies in serum to recognise recombinant ClfA, pooled serum was obtained from healthy human donors, mice which had been vaccinated with recombinant His-tagged $ClfAN123_{40-559}$ or mice which had been challenged with *S. aureus* strain PS80. Pooled

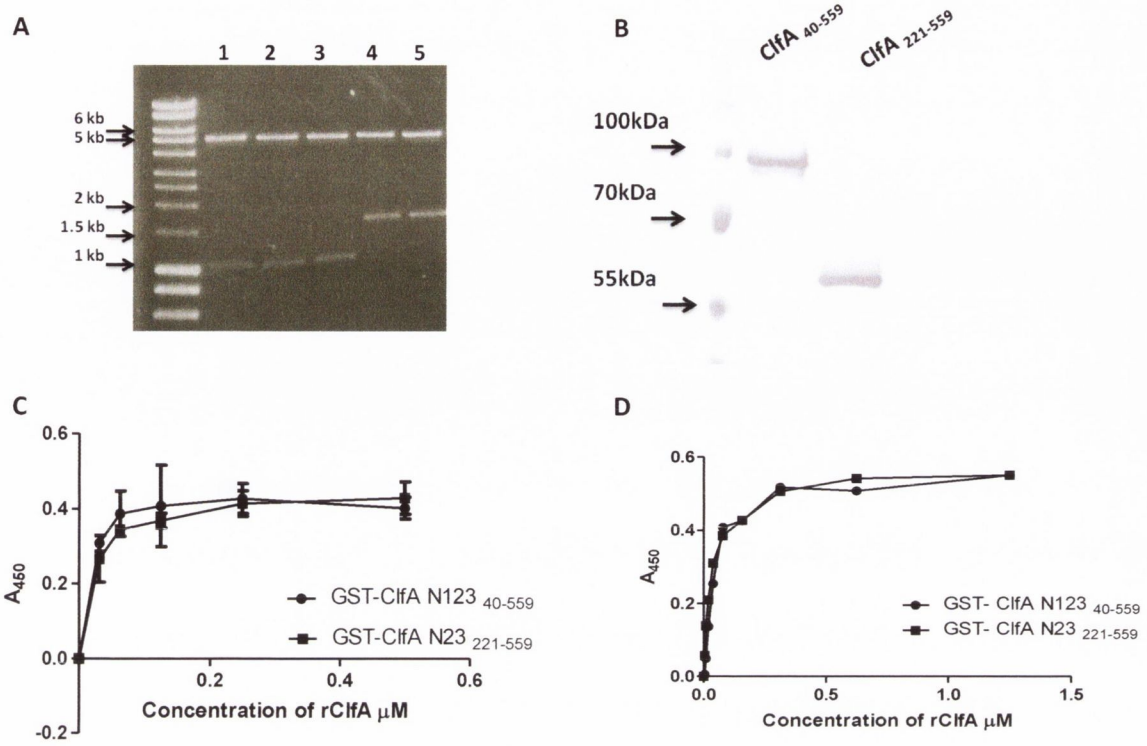


Figure 3.12 Construction of recombinant GST-tagged ClfA A domains

(A) Agarose gel electrophoresis of pGEX-4T-2*clfA*$_{221\text{-}559}$ (Lanes 1-3) and pGEX-4T-2*clfA*$_{40\text{-}559}$ (Lanes 4 and 5) digested with EcoR1 and BamH1. Lanes 1-3 contain fragments of 1 kb corresponding to an insertion in 3 distinct clones of pGEX-4T-2*clfA*$_{221\text{-}559}$. Lanes 5 and 6 contain fragments of 1.6 kb corresponding to an insertion in 2 distinct clones of pGEX-4T-2*clfA*$_{40\text{-}559}$. Cut pGEX-4T-2 vector appears as a fragment of 4.9 kb. **(B)** Recombinant GST-tagged ClfA A domains N123$_{40\text{-}559}$ (Lane 1) and N23$_{221\text{-}559}$ (Lane 2) separated on a 12.5% SDS-PAGE gel and stained with Commassie Brilliant Blue. Molecular weight markers are indicated with black arrows. **(C)** GST ClfAN123$_{40\text{-}559}$ and ClfAN23$_{221\text{-}559}$ were tested to ensure equal immobilisation on the surface of a microtitre plate. Wells were incubated overnight with 0-1.5 μM of recombinant ClfA (rClfA). Bound protein was detected with HRP-conjugated anti-GST IgG **(D)** Capture of fibrinogen (10 μg/ml) by immobilized ClfA A regions. Wells were coated as in C. Bound fibrinogen was detected using HRP-conjugated anti- fibrinogen IgG. Assays were developed by incubation with a chromogenic substrate. The A_{450} was determined. Data points represent the mean of triplicate wells. The data shown is representative of three individual experiments.

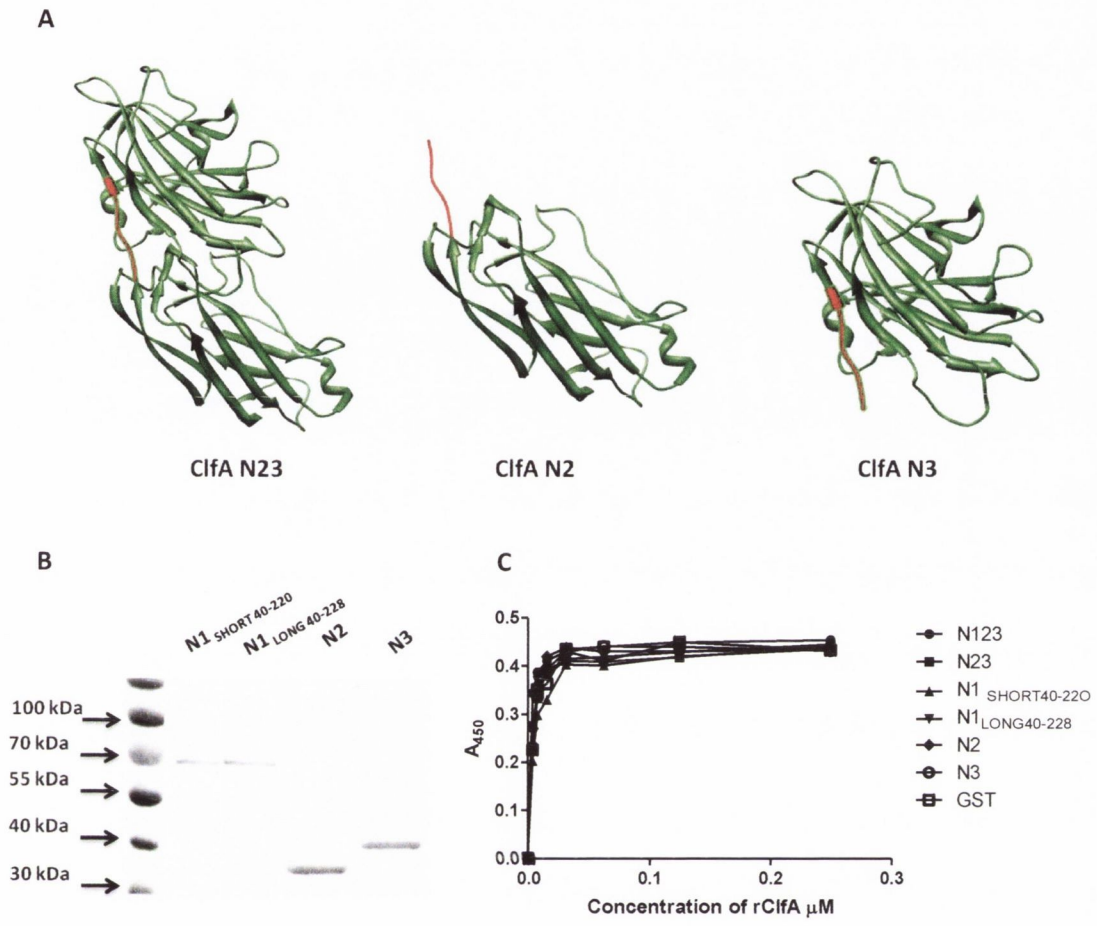


Figure 3.13 Construction and validation of recombinant GST-tagged ClfA subdomains

(A) Ribbon models of the crystal structure of ClfAN23 and the N2 and N3 subdomain. The linker region which attaches the N2 and N3 subdomains is highlighted in red and has been included in both constructs to facilitate proper folding of the individual domains. **(B)** Recombinant GST-tagged ClfA A subdomains separated on a 10% SDS PAGE gel and stained with Commassie Brilliant Blue. Molecular weight markers are indicated with black arrows. **(C)** GST-tagged ClfA A subdomains were tested to ensure equal immobilisation on the surface of a microtitre plate. Wells were incubated overnight with 0-250 nM of rClfA. Bound protein was detected with HRP-conjugated anti-GST IgG. Assays were developed by incubation with a chromogenic substrate. The A_{450} was determined. Data points represent the mean of triplicate wells. The data shown is representative of three individual experiments.

normal human serum contains antibodies against *S. aureus* as approx 20% of the human population demonstrate persistent nasal colonisation with *S. aureus* whilst the remainder are transiently colonised. Serum samples were analysed for ClfA antibodies directed against each of the individual domains. 1 μM of each recombinant GST-tagged ClfA subdomain was immobilized on the surface of a microtitre plate and incubated with doubling dilutions of serum. ELISA results show that the presence of the N1subdomain does not increase the level of antibody bound to the N123 compared to the N23 construct with all three types of serum (Fig 3.14A, B and D). Antibodies recognizing GST-tagged ClfAN123$_{40\text{-}559}$ were compared in untreated mouse serum and serum from mice vaccinated with recombinant His-tagged ClfAN123$_{40\text{-}559}$ or mice challenged with PS80. No cross-reactive antibodies were detected. In all test serum samples the N1$_{SHORT}$ subdomain was not recognised by serum antibodies unless it was expressed in conjunction with a small portion of the N2 domain (N1$_{LONG40\text{-}228}$). The N2 domain is poorly immunogenic and the majority of the antibody response was directed against the N3 subdomain.

These results demonstrate *in vitro* that the presence or absence of the N1 subdomain does not affect recognition of recombinant ClfAN23$_{221\text{-}559}$ by antibodies in human or mouse sera. The N1 and N2 constructs were recognised poorly by antibodies from both human and mouse sera. However, it is possible that the N1 and N2 constructs were not correctly folded in the absence of the remainder of the protein. This seems unlikely as work involving the individual subdomains of recombinant ClfB showed that the individual subdomains were capable of folding independently of each other. However CD spectroscopy would need to be performed on these constructs to show that this is also the case for recombinant ClfA.

3.2.8 Subdomain N1 is not required for ClfA to promote survival of *S. aureus* in human blood.

Both ClfA and protein A promote survival of *S. aureus* in human blood (Smith *et al.*, 2011) by protecting the bacteria from phagocytosis by neutrophils and macrophages (Higgins *et al.*, 2006, Palmqvist *et al.*, 2004). In order to determine if the N1 subdomain of ClfA is required for ClfA-promoted survival of *S. aureus* in human blood, *S. aureus* NM1 (pRMC2*clfA*) and NM1 (pRMC2*clfA*ΔN1$_{40\text{-}210}$) were tested for their ability to persist in whole human blood compared to NM1 carrying the empty vector pRMC2.

Bacteria were grown and induced in RPMI to mimic the iron-limited environment of blood. Equal levels of ClfA and ClfAΔN1$_{40-210}$ were expressed on the surface of NM1 growing in rich media to exponential phase when induced with 800 ng/ml and 1 μg/ml ATc, respectively (Fig 3.9). In order to determine whether equal levels of expression could be obtained at stationary phase in iron-limited conditions induction was performed overnight in RPMI. NM1 (pRMC2*clfA*) and NM1 (pRMC2*clfA*ΔN1$_{40-210}$) were induced overnight in RPMI with 800 ng/ml or 1.2 μg/ml ATc, respectively. Whole cell dot immunoblotting using ClfA mAb demonstrated that equal amounts of ClfA and ClfAΔN1$_{40-210}$ were present on the surface of the cell (Fig 3.15A). Adherence of bacteria expressing ClfA and ClfAΔN1$_{40-210}$ to fibrinogen was dose-responsive and saturable. No difference in fibrinogen binding was observed when equal levels of ClfA and ClfAΔN1$_{40-210}$ were expressed on the surface indicating that under iron-limited conditions subdomain N1 is not required for adherence to fibrinogen and equal levels of ClfA and ClfAΔN1$_{40-210}$ can be surface expressed (Fig 3.15B).

Whole blood survival assays were performed for three hours in fresh hirudin-treated human blood. The anti-coagulant hirudin was used as it does not inhibit the complement pathway. Strain NM1 lacks both ClfA and protein A, two important surface-expressed immune evasion proteins (Smith *et al.*, 2011). Only 9% of the input inocula of NM1 (pRMC2) survived compared to ~70% of the parental strain Newman (Fig. 3.16). NM1 expressing full-length ClfA (NM1 (pRMC2*clfA*) showed an increased level of survival (42% of input inoculum, Fig. 3.16) confirming previous reports that ClfA promotes survival of *S. aureus* in blood (Smith *et al.*, 2011). Bacteria expressing ClfAΔN1$_{40-210}$ survived similarly to those expressing full-length ClfA (43% of input inoculum, Fig. 3.16). These data indicate that residues 40-210 of N1 are not required for ClfA to inhibit killing of *S. aureus* in human blood. Donor blood was screened to ensure it did not contain a high level of anti-ClfA IgG. Aureolysin-treated cells could not be used in whole blood survival assays as aureolysin cleaves the complement protein C3 which would interfer with complement-mediated opsonisation (Laarman *et al.*, 2011).

3.2.9 Construction of a constitutively expressed ClfAΔN1 variant.

Expression of ClfAΔN1$_{40-220}$ or ClfAΔN1$_{40-228}$ could not be detected on the surface of *S. aureus* when NM1 (pRMC2*clfA*ΔN1$_{40-220}$) and NM1 (pRMC2*clfA*ΔN1$_{40-228}$) were grown in the presence of a high concentration of inducer (1.2 μg/ml ATc).

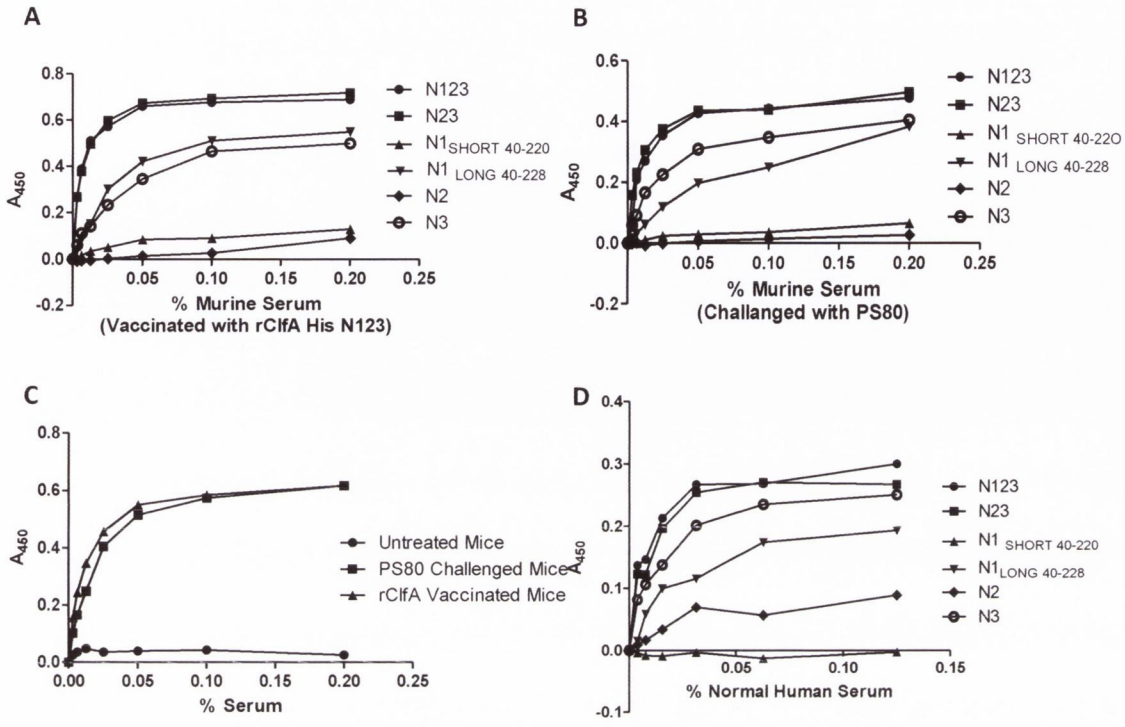


Figure 3.14 Quantification of antibody response against individual ClfA subdomains in sera obtained from mice and healthy human donors.

GST ClfAN123$_{40\text{-}559}$, GSTN23$_{220\text{-}559}$, GST N1$_{40\text{-}220}$, GST N1$_{40\text{-}228}$, GST N2 and GST N3 were tested to determine the antibody response directed against individual ClfA subdomains in sera from mice and humans. Wells were coated with 1 μM recombinant ClfA and incubated with doubling dilutions of serum obtained from **(A)** mice vaccinated with His-tagged recombinant ClfA$_{40\text{-}559}$, **(B)** Mice challenged with *S. aureus* strain PS80, **(C)** healthy untreated mice, and **(D)** healthy human volunteers. Bound antibody was detected with HRP-conjugated rabbit anti-mouse IgG (A, B and C) or HRP-conjugated rabbit anti-human IgG. Assays were developed by incubation with a chromogenic substrate. The A_{450} was determined. Data points represent the mean of triplicate wells. The data shown is representative of three individual experiments.

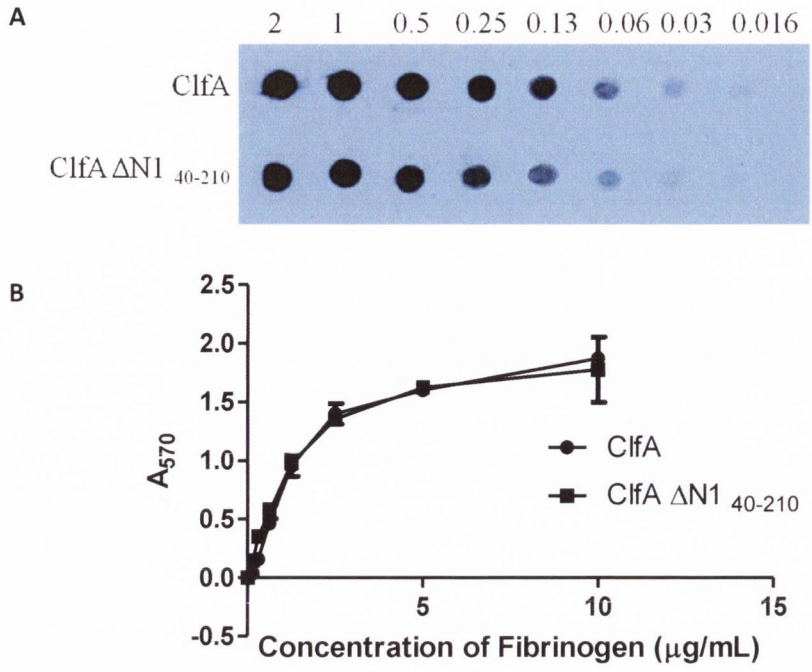


Figure 3.15 Optimizing expression levels of ClfA and ClfAΔN1$_{40\text{-}210}$ under iron limited conditions.

(A) Dot immunoblot performed with *S. aureus* NM1 (pRMC2*clfA*) or (pRMC2*clfA*ΔN1$_{40\text{-}210}$) induced with 800 ng/ml and 1.1 μg/mL Atc, respectively in RPMI.The OD_{600} of cells is indicated at the top of the blot. 5μl of each dilution of cells was spotted onto the membrane and probed with ClfA mAb and HRP-conjugated rabbit anti-mouse IgG. **(B)** Solid phase adhesion assay performed with the same cells as panel (A) binding to wells coated with Fg ranging from 0-10 μg/ml.Adherent cells were stained with crystal violet and A_{570} was measured. Data points represent the mean of triplicate wells. Graphs shown are representative of three different experiments.

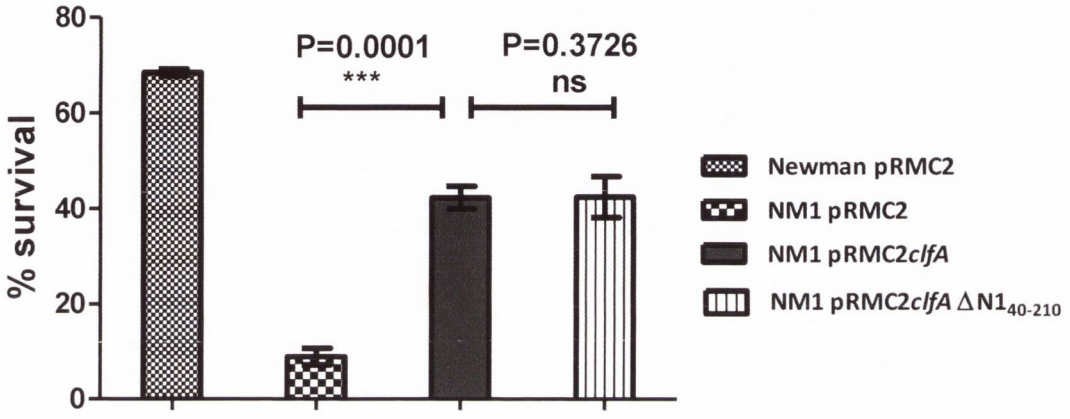


Figure 3.16 Survival of *S. aureus* in whole human blood.

Bacteria were incubated in fresh hirudin-treated human blood (50 μg/ml) for 3 h and viable counts were determined on TSA plates incorporating chloramphenicol. The percentage survival was calculated based on the number of input cfu. Error bars represent the standard deviation on the mean of three independent experiments performed on separate days with different blood donors.

Plasmid pALC2073 is the precursor of pRMC2 which allows constitutive expression in *S. aureus* due to weak TetR repression (Bateman *et al.*, 2001, Corrigan and Foster, 2009). Whole cell dot immunoblotting with polyclonal anti-ClfA IgG was performed on *S. aureus* NM1 cells carrying pRMC2*clfA* or pALC2073*clfA* under inducing or non-inducing conditions. Although no ClfA can be detected on the surface of cells carrying pRMC2*clfA* in the absence of ATc cells carrying pALC2073*clfA* express high levels of ClfA in both the presence and absence of inducing agent. No apparent difference in the level of ClfA expressed from pALC2073*clfA* was observed in the presence of ATc when compared to the uninduced control and in both cases the level of ClfA expressed from pALC2073*clfA* was higher than that achieved from induced pRMC2*clfA* (Fig 3.17).

To determine if expression of ClfAΔN1$_{40\text{-}220}$ is deleterious a deletion of residues 40-220 was made in plasmid pALC2073*clfA*. Plasmid pALC2073*clfA* was manipulated by inverse PCR to generate pALC2073*clfA*ΔN1$_{40\text{-}220}$. Equal amounts of the parental plasmid pALC2073*clfA* and pALC2073*clfA*ΔN1$_{40\text{-}220}$ DNA were purified from *E. coli* DC10B and used to transform *S. aureus* NM1. Transformation with pALC2073*clfA* yielded ~2 x 10^3 Cm resistant colonies (Fig. 3.18A). However, pALC2073*clfA*ΔN1$_{40\text{-}220}$ yielded fewer than 20 transformants (Fig. 3.18A). Both plasmids tested were able to transform *E. coli* with equal efficiently (~ 3 x 10^4 cfu per transformation, Fig. 3.18B) which demonstrates the integrity of the DNA. The failure to establish transformants with pALC2073*clfA*ΔN1$_{40\text{-}220}$ in *S. aureus* suggested that constitutive expression of a variant of ClfA lacking subdomain N1 inhibited growth and colony formation. PCR colony screening of pALC2073*clfA*ΔN1$_{40\text{-}220}$ transformants using primers directed against the multiple cloning site of plasmid pALC2073 revealed bands with a range of both higher and lower molecular weights when compared to pALC2073*clfA*ΔN1$_{40\text{-}220}$ DNA from *E. coli* (Fig 3.18C). This finding suggests that the plasmid had undergone rearrangements to reduce or prevent expression of truncated ClfA.

3.2.10 Construction and validation of an isogenic variant of strain NM1 which is sensitive to erythromycin (NM2).

To facilitate competitive growth experiments to determine if there is a deleterious effect on growth because of expression of ClfAΔN1$_{40\text{-}220}$ from pRMC2 an isogenic strain of NM1 which is erythromycin sensitive was constructed and designated NM2 (Table 2.1). The mutant was constructed by allelic replacement of the *clfB*::Em^R

cassette with a 1 kb fragment homologous to the up and downstream regions of the gene using the temperature sensitive integration vector pIMAY (a kind gift from Dr. Michelle Mulcahy). A schematic diagram of the allelic replacement by pIMAY is illustrated in Fig. 3.1. Strain NM2 was validated by Western immunoblotting of cell wall fractions to ensure that it lacked the cell wall proteins ClfA, ClfB and protein A (Fig. 3.19 A(i)(ii)(iii)). Anti-SdrE IgG was used as a control for the cell wall of *S. aureus* (Fig 3.19A(iv)). Antibiotic resistance markers were tested to ensure loss of the erythromycin resistance cassette and a growth curve was performed to ensure there was no difference in the fitness between NM1 and NM2 (Fig. 3.19B). To ensure *agr* activity delta toxin production was examined on sheep blood agar plates (Fig 3.19C).

3.2.11 Competitive growth of NM1 (pRMC2*clfA*) and NM2 (pRMC2*clfA*ΔN1$_{40\text{-}220}$)

In order to investigate if inducing expression of ClfAΔN1$_{40\text{-}220}$ in *S. aureus* harboring the tightly repressed pRMC2*clfA*ΔN1$_{40\text{-}220}$ would inhibit bacterial growth, competitive growth experiments were performed with bacteria harboring pRMC2*clfA* or pRMC2*clfA*ΔN1$_{40\text{-}220}$. To distinguish between cells harboring the different plasmids, pRMC2*clfA*ΔN1$_{40\text{-}220}$ was introduced into strain NM2, which is Em sensitive. Equal numbers of NM1 (pRMC2*clfA*) and NM2 (pRMC2*clfA*ΔN1$_{40\text{-}220}$) were mixed and co-cultured for two generations either in the presence or absence of the inducer ATc. Viable counts were obtained by plating on agar containing Cm. Replica plating onto agar incorporating Em allowed the number of Em resistant bacteria NM1 (pRMC2*clfA*) to be counted.

In the absence of inducer where ClfA expression was repressed (Fig. 3.8) equal numbers of NM1 (pRMC2*clfA*) and NM2 (pRMC2*clfA*ΔN1$_{40\text{-}220}$) were recovered (Fig. 3.20A). However when ClfA expression was induced and the bacteria were allowed to grow for approximately two generations the number of NM2 (pRMC2*clfA*ΔN1$_{40\text{-}220}$) recovered was 1.5 times lower than for NM1 (pRMC2*clfA*) (Fig. 3.20A). This indicates that expression of ClfA lacking subdomain N1 confers a growth disadvantage compared to expression of full-length ClfA. To investigate if expression of wild-type ClfA affected the fitness of *S. aureus*, control experiments were conducted with NM1 (pRMC2*clfA*) and NM2 carrying the empty vector pRMC2. The proportion of Em^S to Em^R bacteria was the same for cultures grown in ATc and the inducer-free control (Fig. 3.20B).

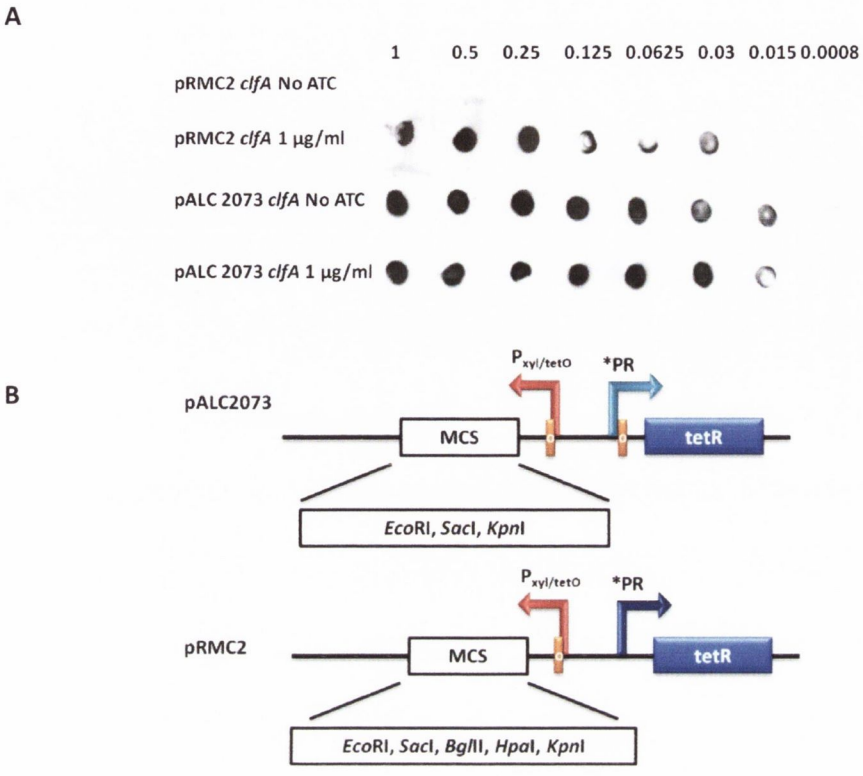


Figure 3.17 Comparing ClfA expression by pALC2073*clfA* and pRMC2*clfA*

(A) Whole cell dot immunoblot of NM1 (pRMC2*clfA*) or NM1 (pALC2073*clfA*). 5 µl each dilution of cells was spotted onto filter paper and probed with polyclonal anti-ClfA IgG and protein A peroxidase. Strain and level of inducing agent are listed at the side of the blot and OD_{600} of cells at the top of the blot. **(B)** Schematic of the $P_{xyl/tetO}$ and *PR promoter regions and multiple cloning sites (MCS) of pALC2073 and pRMC2. Expression of target genes is controlled by TetR expression from the *PR promoter. TetR binds to the *tetO* site within the $P_{xyl/tetO}$ promoter. The -10 box of the *PR promoter has been mutated in pRMC2 simultaneously disrupting its *tetO* site and relieving autorepression. In the presence of ATc TetR binding to *tetO* was disrupted at the $P_{xyl/tetO}$ promoter and high level expression achieved.

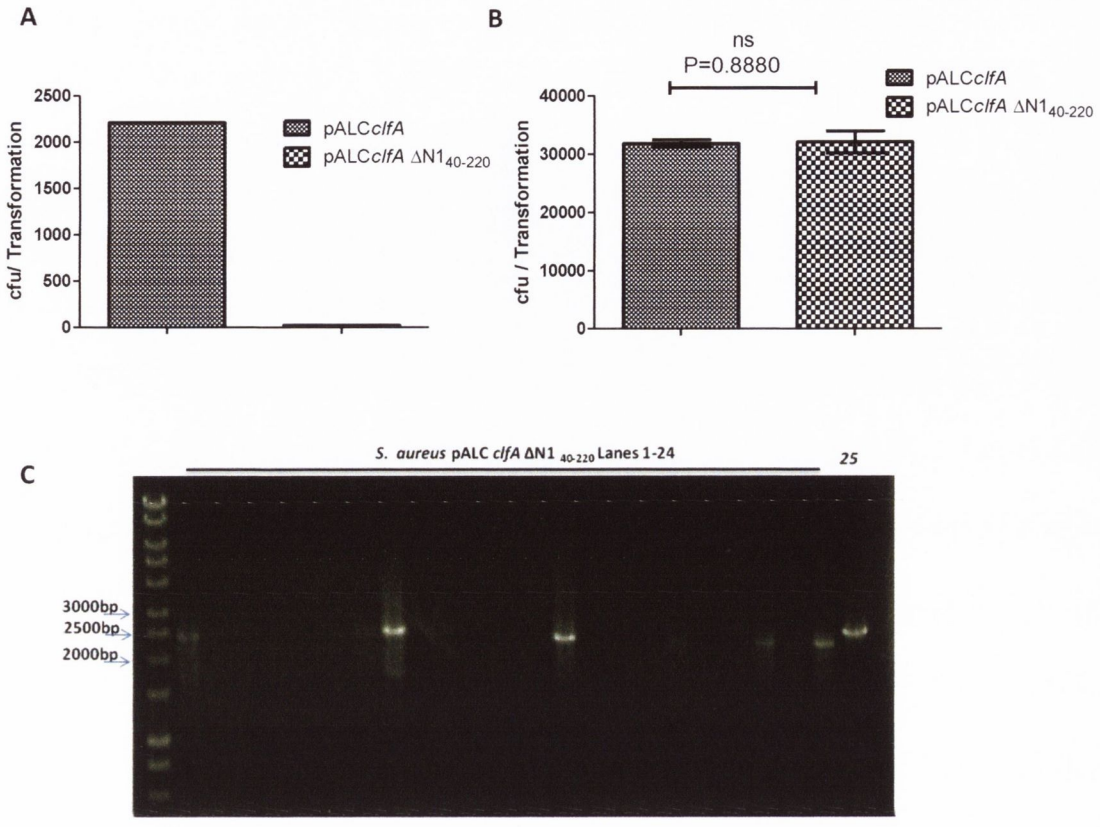


Figure 3.18 Transformation of *S. aureus* with constitutively expressing pALC2073*clfA*ΔN1$_{40\text{-}220}$

(A) Transformation efficiency of pALC2073*clfA* compared to pALC2073*clfA*ΔN1$_{40\text{-}220}$. NM1 cells were transformed with 5 µg of pALC2073*clfA* or pALC2073*clfA*ΔN1$_{40\text{-}220}$, plated on TSA containing Cm and the total cfu per transformation was calculated. Graph shown is representative of two independent comparative transformations. **(B)** Transformation efficiency of pALC2073*clfA* compared to pALC2073*clfA*ΔN1$_{40\text{-}220}$. *E. coli* DC10B cells were transformed with 100 ng plasmid DNA, plated on L agar containing ampicillin and the total cfu per transformation was calculated. Graph shown is representative of two independent comparative transformations. P values were calculated using Student's t-test **(C)** PCR colony screen of NM1 pALC2073*clfA*ΔN1$_{40\text{-}220}$ transformants performed using primers directed against the MCS of pALC2073. *E. coli* DC10B (pALC2073*clfA*ΔN1$_{40\text{-}220}$) was used as a positive control.

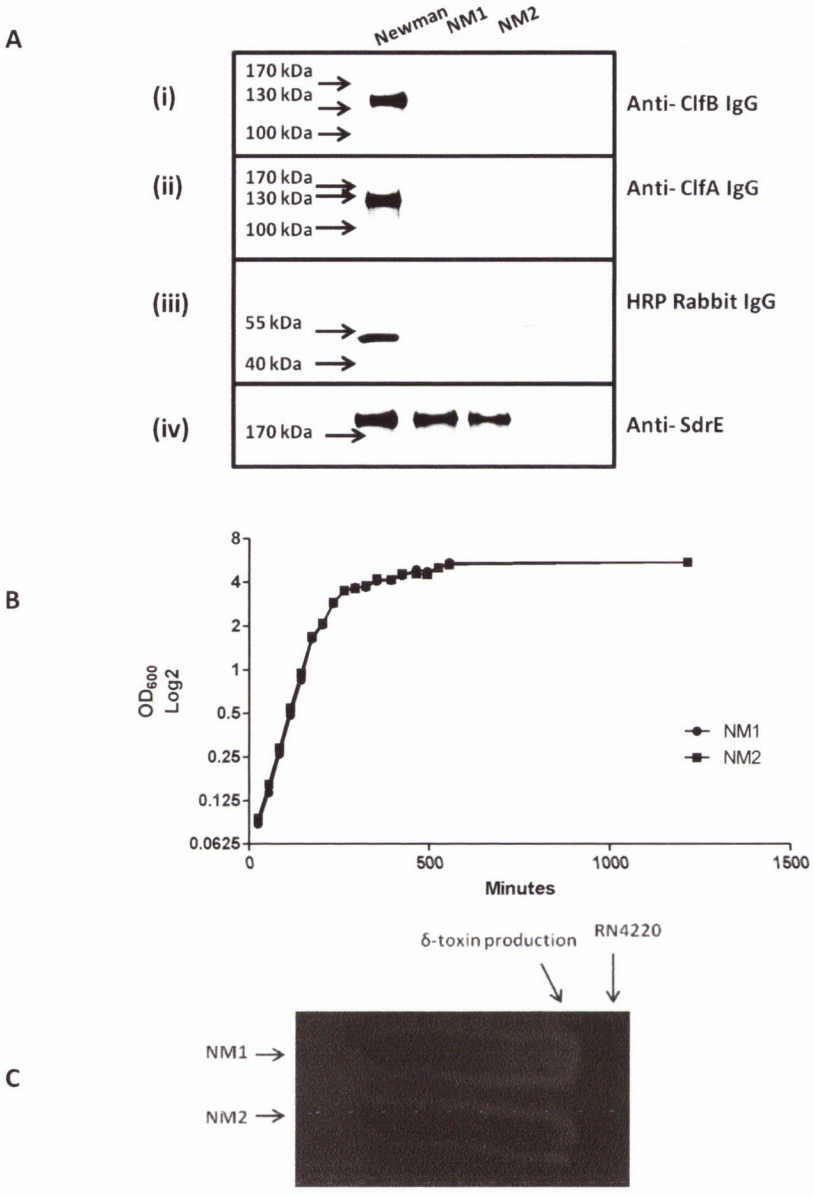
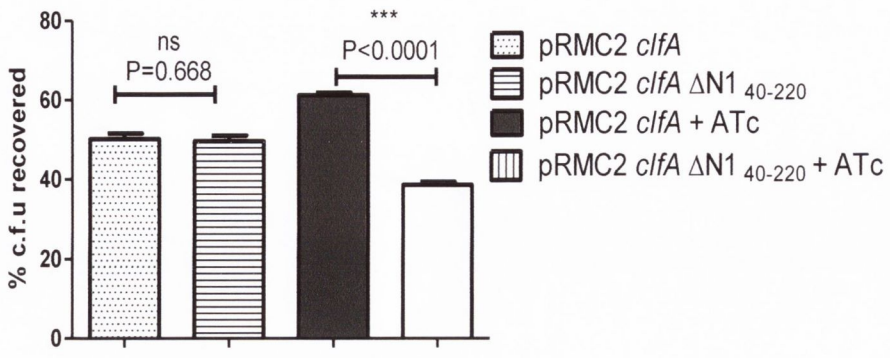


Figure 3.19 Construction and validation of strain NM2

(A) Western immunoblotting of cell wall fractions of strain Newman, NM1 and NM2 probed with (i) polyclonal anti-ClfB IgG, (ii) polyclonal anti-ClfA IgG, (iii) HRP-conjugated rabbit IgG or (iv) polyclonal anti-SdrE IgG. Bound antibodies were detected with HRP-conjugated anti rabbit IgG.**(B)** Growth curve comparing NM1 and the isogenic erythromycin sensitive mutant NM2. **(C)** *S. aureus* NM2 and its parental strain NM1 were cross-streaked against RN4220 on sheep blood agar plates in order to compare the haemolytic pattern. Enhanced haemolysis due to δ-toxin is indicated with black arrows.

A



B

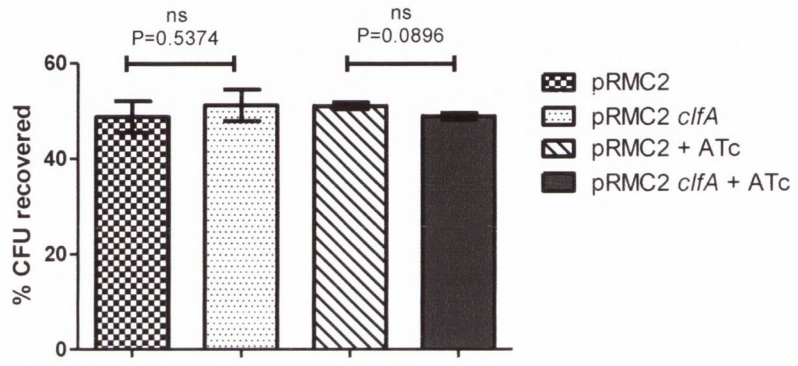
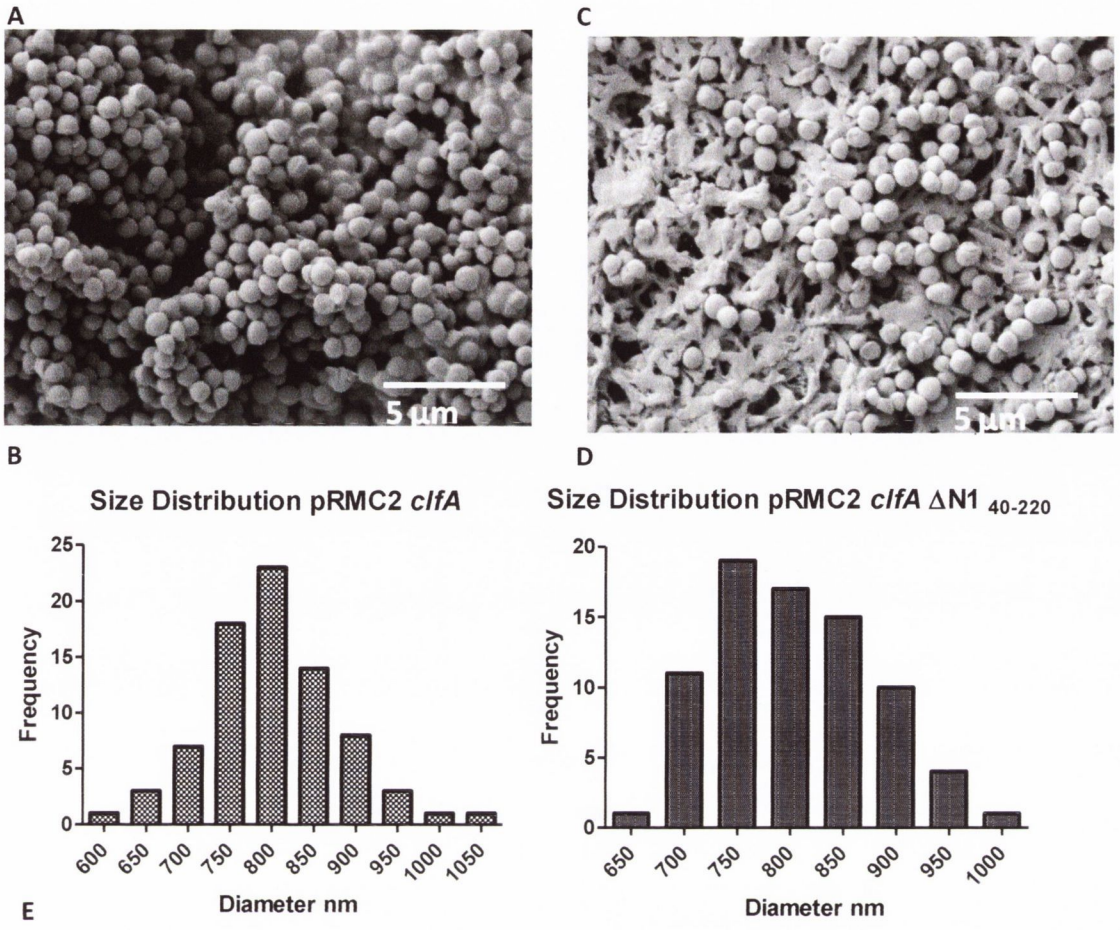


Figure 3.20 Competitive growth comparing NM1(pRMC2*clfA*) and NM2(pRMC2*clfA*ΔN1$_{40\text{-}220}$) or NM2(pRMC2).

(A) *S. aureus* NM1 (pRMC2*clfA*) and NM2 (pRMC2*clfA*ΔN1$_{40\text{-}220}$) cells were induced with ATc (1200 ng/ml) and plated on agar incorporating Cm. Colonies resistant to Cm were replica plated onto agar containing Em. **(B)** Competitive growth assay of NM1 (pRMC2*clfA*) and NM2 (pRMC2). *S. aureus* NM1 (pRMC2*clfA*) and NM2 (pRMC2) cells were induced with 1.2 µg/ml ATc and plated on TSA Cm. Colonies resistant to Cm were replica plated onto TSA containing Em. Bar charts represent the mean percentage cfu based on the total Cm resistant cfu. Error bars represent the standard deviation on the mean of three independent experiments. Statistical analysis was performed using Student's t-test.



E

NM1 pRMC2*clfA*	NM1 pRMC2*clfA*ΔN1$_{40-220}$
Mean Diameter 802 nm	Mean Diameter 799 nm
SD 75 nm	SD 81nm
SEM 8.5 nm	SEM 9 nm

Figure 3.21 Size distribution of *S. aureus* NM1 cells expressing ClfA or ClfAΔN1$_{40-220}$.

(A) SEM image of *S. aureus* cells expressing ClfA. **(B)** Histogram of size distribution of *S. aureus* cells expressing ClfA. **(C)** SEM image of *S. aureus* cells expressing ClfAΔN1$_{40-220}$. **(D)** Histogram of size distribution of *S. aureus* cells expressing ClfAΔN1$_{40-220}$. **(E)** Table of results obtained from size analysis of SEM images and histograms from (A-D).

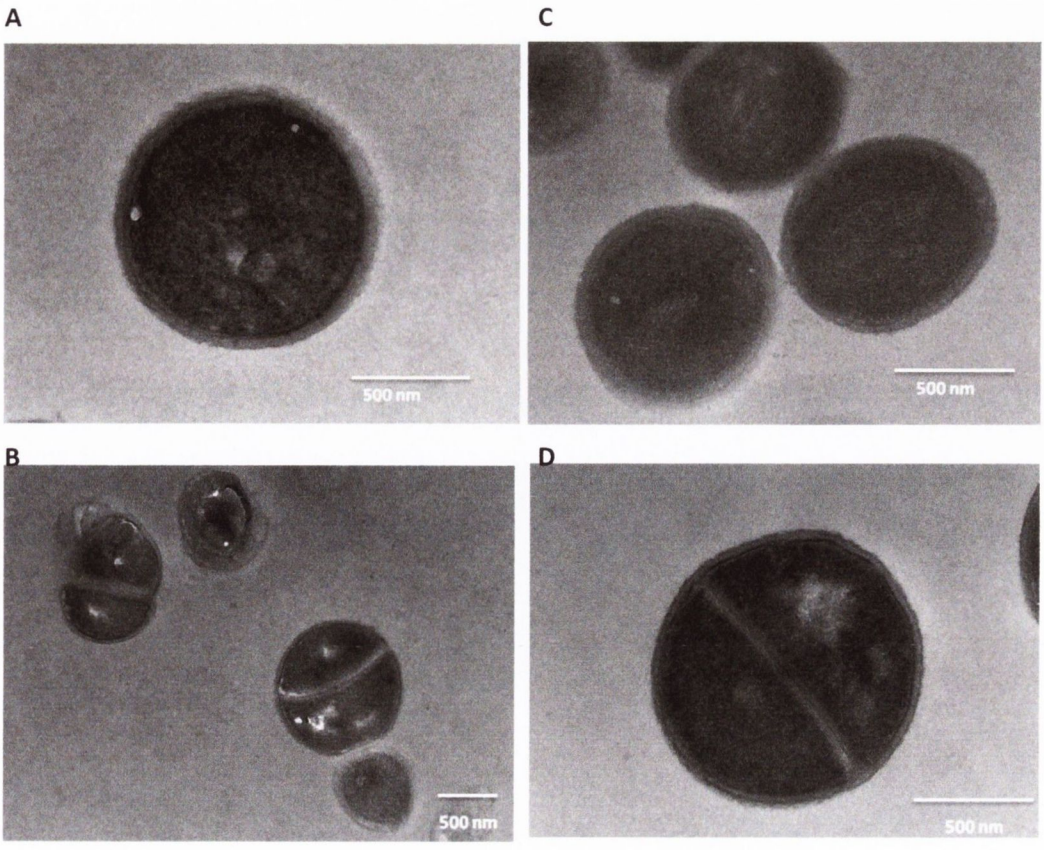


Figure 3.22 TEM analysis of *S. aureus* NM1 cells expressing ClfA or ClfAΔN1$_{40\text{-}220}$.

(A&B) Representative TEM images of NM1 expressing ClfA. **(C&D)** Representative TEM images of NM1 expressing ClfAΔN1$_{40\text{-}220}$.

3.2.12 Electron microscopy of *S. aureus* cells expressing ClfA or ClfAΔN1$_{40\text{-}220}$

To determine whether expression of ClfAΔN1$_{40\text{-}220}$ causes morphological defects in *S. aureus* scanning electron microscopy (SEM) was performed on *S. aureus cells* expressing ClfA or ClfAΔN1$_{40\text{-}220}$. NM1 (pRMC2*clfA*) or (pRMC2*clfA*ΔN1$_{40\text{-}220}$) were grown to an OD_{600} of 0.5 and induced with 1.2 µg/ml ATc for 2 hours. Cells were harvested, washed in PBS and fixed for 1 hr in 3 % glutaraldehyde. After fixation cells were dehydrated on an ethanol gradient. Critical point drying was performed to remove all liquid before mounting samples on SEM stubs and sputter coating in gold. Samples were imaged by SEM. Representative images for NM1 (pRMC2*clfA*) and NM1 (pRMC2*clfA*ΔN1$_{40\text{-}220}$) are shown in Fig 3.21 A & C respectively. Eighty cells from each group were chosen at random and their size distribution was analysed using imageJ software (Fig. 3.21 B&D). No statistical difference in cell diameter was observed between the control group expressing wildtype ClfA and the test group expressing ClfAΔN1$_{40\text{-}220}$. This data suggests that expression of ClfAΔN1$_{40\text{-}220}$ does not affect cell size.

Transmission electron microscopy (TEM) analysis was also performed on NM1 (pRMC2*clfA*) or (pRMC2*clfA*ΔN1$_{40\text{-}220}$) cells induced as above to allow closer investigation of the cell wall and division septum. Induced cells were washed and fixed with then dehydrated before embedding within the resin. Thin slices were stained and TEM images obtained. Figure 3.22 A & B show representative TEM images of NM1 (pRMC2*clfA*) or C & D of NM1 (pRMC2*clfA*ΔN1$_{40\text{-}220}$) cells respectively. In both samples a range of cell diameters was observed. This was expected as the processing of TEM samples requires thin slices to be taken. Depending on the location of the cocci within the sample different sectors of the cocci will be bisected resulting in the range of cell diameters observed. No difference in cell wall thickness or misplacement of division septa was observed for either cells expressing ClfA or ClfAΔN1$_{40\text{-}220}$. The electron microscopy results indicate that expression of ClfAΔN1$_{40\text{-}220}$ does not affect the division of staphylococci.

3.3 Discussion

Clumping factor A is the archetypal member of the MSCRAMM family of wall-anchored proteins of *S. aureus*. The defining features of these proteins are (i) the A region and (ii) the flexible unfolded region linking the A region to the cell wall spanning/anchoring domain. The A regions comprise two separately folded subdomains N2 and N3 composed of IgG-like folds. All MSCRAMMs which contain a long, flexible unfolded region linking the A region to the cell wall spanning/anchoring domain have an N-terminal subdomain N1 with no known function.

In this Chapter subdomain N1 was shown to be dispensable for ClfA mediated bacterial adherence to fibrinogen in agreement with previous studies demonstrating that recombinant N23 subdomain constructs bind to fibrinogen with the same affinity as recombinant N123 subdomains (Geoghegan *et al.*, 2010, Ganesh *et al.*, 2008). Here, this has been demonstrated using recombinant ClfA, ClfA expressed on the surface of *S. aureus* lacking the bulk of the N1 subdomain (ClfAΔN1$_{40\text{-}210}$) and using bacteria treated with aureolysin to shave the N1 subdomain from the surface of the cell. In all cases loss of the N1 subdomain did not affect fibrinogen binding showing conclusively that the N1 subdomain is not required for ClfA-mediated adherence to fibrinogen.

Intriguingly although the N1 subdomain is not required for recombinant His-tagged ClfA A regions to bind to immobilised fibrinogen it is necessary for immobilised recombinant His-tagged ClfA to bind to fibrinogen in the fluid phase. This was circumvented by the addition of a GST-tag. This observation suggests that the immobilised N23 form of the protein requires additional residues to allow binding. Since a GST tag was sufficient it is likely that specific residues of N1 are not required and it is tempting to speculate that due to the dynamic nature of the dock, lock, latch mechanism additional residues are required to facilitate the flexibility required for ligand binding.

Subdomain N1 plays no role in fibrinogen binding by ClfA located on the surface of *S. aureus*. To determine whether it is involved in evasion of opsonization each of the individual subdomains of ClfA were expressed as recombinant GST fusion proteins. The results demonstrate that the presence of the N1 subdomain does not affect recognition of recombinant ClfAN23$_{221\text{-}559}$ by antibodies in human or mouse sera. Furthermore the N1 subdomain itself was poorly immunogenic.

ClfA promotes survival of *S. aureus* in human blood (Smith *et al.*, 2011) by protecting the bacteria from phagocytosis by neutrophils and macrophages (Higgins *et al.*, 2006, Palmqvist *et al.*, 2004). This Chapter demonstrated that subdomain N1 is not required for ClfA to inhibit killing of *S. aureus* in human blood. ClfA binds to and activates the complement regulator factor I, resulting in cleavage of the complement opsonin C3b (Hair *et al.*, 2010). This result indicates indirectly that subdomain N1 is not involved in this interaction. The interaction between ClfA and factor I is not investigated in this Chapter and will be examined in greater detail in Chapter 5.

In this Chapter pRMC2 was used as an inducible expression system to help elucidate the function of the N1 subdomain. In agreement with previous studies, this Chapter has demonstrated that pRMC2 can express a target gene at a high level and is tightly repressed in the absence of inducer (Corrigan and Foster, 2009). Furthermore we have demonstrated the utility of pRMC2 when dealing with mutations in regions of unknown function. Constitutive expression of an N1 deletion mutant following transformation into *S. aureus* resulted in a very low yield of transformants which upon further examination appeared to have undergone rearrangements. This complication was avoided by the use of pRMC2 highlighting its value in the study of genes of unknown function by avoiding potential gene toxicity issues. Recently a new variant of pRMC2 has been developed with an additional *tetO* site within the $P_{xyl/tetO}$ promoter region. This vector, pRAB11, is reported to have improved repression in the absence of ATc due to the additional *tetO* site (Helle *et al.*, 2011). However under the conditions described in this thesis no leaky expression was detected from pRMC2 and it was deemed fit for purpose.

Neither ClfAΔN1$_{40\text{-}220}$ nor ClfAΔN1$_{40\text{-}228}$ were expressed on the cell surface following induction. Constitutive expression of an N1 deletion mutant of pALC2073*clfA* following transformation into *S. aureus* resulted in a very low yield of transformants, in the majority of which the plasmid appeared to have undergone rearrangements. Furthermore, induction of the truncated *clfA* mutant in *S. aureus* caused a significant loss of fitness compared to cells expressing wild-type ClfA which shows that attempting to express the protein lacking N1 is affecting cell growth and colony formation. These studies provide indirect evidence that although the ClfA ΔN1 variants are not surface-displayed attempts to express them have a deleterious effect on the cell. Intriguingly a plasmid constitutively expressing an FnBPA N1 deletion mutant cannot

be established in *S. aureus* (Geoghegan *et al.*, 2013). Since N1 subdomains are found in all MSCRAMMs it seems likely that they will have a conserved function. This will be explored in greater detail in Chapter 4.

In conclusion, this Chapter has demonstrated that pRMC2 is a powerful tool for inducible gene expression especially where associated with a deleterious growth phenotype. The N1 subdomain of ClfA is not required for fibrinogen binding by recombinant proteins or once the protein has been established on the surface of *S. aureus*. It was not possible to express a variant of ClfA lacking the entire N1 subdomain on the surface of the cell suggesting that it may play a role in surface elaboration of ClfA. However, the N1 subdomain of ClfA could be removed from the surface of *S. aureus* by aureolysin-treatment. Again loss of the N1 subdomain by aureolysin treatment did not affect adherence to fibrinogen. In this Chapter ten residues were identified at the C-terminus of subdomain N1 which were sufficient to mediate surface expression at a similar level to wild type ClfA with no loss ability to adhere to fibrinogen. The role of the N1 subdomain in surface display of ClfA and other MSCRAMMs will be examined in detail in Chapter 4.

Chapter 4

Investigating the role of subdomain N1 in the cellular localisation of ClfA and other MSCRAMMs

4.1 Introduction

The MSCRAMM family comprises proteins with a distinctive substructure. An N-terminal ligand-binding A region contains two tandemly arrayed subdomains N2 and N3 that are composed of IgG-like folds (Deivanayagam *et al.*, 2002) and the N-terminal N1 which, in the case of ClfB, is elongated and is not compact (Perkins *et al.*, 2001). The A domains are linked to the cell wall by an extended flexible region comprising repeats of the dipeptide Ser-Asp (ClfA, ClfB, SdrC, SdrD, SdrE, Bbp) or 10/11 fibronectin binding domains (FnBPA, FnBPB) (Hartford *et al.*, 1997, Schwarz-Linek *et al.*, 2003). Although ClfA is the main focus of this Chapter the MSCRAMMs ClfB and FnBPB will also be investigated.

ClfB has a very similar structural organisation to ClfA, sharing 93% amino acid identity in region R and 26% amino acid identity in the A region (Ni Eidhin *et al.*, 1998). ClfB binds to the α-chain of fibrinogen by the 'dock, lock, latch' mechanism (Walsh *et al.*, 2008, Ganesh *et al.*, 2011). In addition, ClfB also binds to the tail region of human cytokeratin 10 (Walsh *et al.*, 2004) and the Ω-loop rich loricrin, a major component of the human cornified envelope, making it an important factor in nasal colonisation (Mulcahy *et al.*, 2012).

The A regions of FnBPs are multifunctional. Both bind the γ-chain of fibrinogen by the 'dock, lock, latch' mechanism similarly to ClfA but in addition are capable of binding to elastin (Loughman *et al.*, 2008, Burke *et al.*, 2010). Adjacent to the A region are 10/11 tandemly arrayed fibronectin binding repeat regions. FnBPA contains 11 repeats while FnBPB contains only 10. These mediate Fn binding through a tandem β-zipper mechanism (Bingham *et al.*, 2008). FnBPs play an important role in immune evasion and pathogenesis by facilitating invasion of epithelial and endothelial cells via a fibronectin bridge between *S. aureus* and host cell integrins. FnBPs are also involved in platelet activation and aggregation (Fitzgerald *et al.*, 2006), coating of implanted medical devices (Arrecubieta *et al.*, 2008), and the intracellular accumulation phase of biofilm formation in certain MRSA strains (O'Neill *et al.*, 2008).

Although MSCRAMMs play a pivotal role during colonization and establishment of *S. aureus* infection very little is known about how these proteins are directed to the secretion apparatus (Sibbald *et al.*, 2006, Schneewind and Missiakas, 2012). Members of the MSCRAMM family possess an N-terminal signal sequence that

is predicted to mediate their translocation across the cytoplasmic membrane by the general secretory (Sec) pathway (Sibbald *et al.*, 2006, DeDent *et al.*, 2008). The Sec system in *S. aureus* consists of homologues of the canonical SecYEG translocation channel and the SecA ATPase (Sibbald *et al.*, 2006). However no homologue of the general secretory chaperone SecB or the signal recognition peptide (SRP) has ever been identified in Gram-positive organisms and there is a dearth of knowledge of protein specific chaperones.

S. aureus and other Gram-positive organisms contain an additional Sec system not found in Gram-negative organisms, denoted the accessory Sec system. The accessory Sec system is responsible for secretion of a subset of proteins which cannot be secreted via the canonical Sec system. It consists of an accessory ATPase (SecA2) and a second SecY protein (SecY2). SecA2 and SecY2 are thought form a novel translocation channel with the aid of accessory proteins, Asp1, Asp2 and Asp3. It has also been suggested that SecA2 may interact with the canonical SecYEG translocation channel to aid secretion of certain pre-proteins (Rigel and Braunstein, 2008).

In *Streptococcus gordonii* the accessory Sec system is required for export and surface-display of the serine rich glycoprotein GspB. GspB contains an accessory signal domain in addition to its signal sequence which is required to direct secretion via the accessory Sec system (Bensing and Sullam, 2010). GspB is an important virulence factor of *S. gordonii* which binds to and activates human platelets. In *S. gordonii*, the accessory Sec system is solely responsible for secretion of GspB. GspB is heavily glycosylated by four glycosyltransferases whose genes (*gly*, *nss*, *gtfA*, and *gtfB*) are located within the locus encoding the accessory Sec system. The glycosylation of GspB inhibits its secretion via the canonical Sec system.

In *S. aureus* SraP is the only protein known to be exported by the accessory Sec system. SraP is a member of the same family of serine rich glycoproteins as GspB although SraP is not thought to be as heavily glycosylated as GspB because *S. aureus* has only two glycosyl transferases *gtfA* and *gtfB*. Although ClfA has never been shown to be secreted via the accessory Sec system, it is glycosylated by the glycosyltransferases SdgA and SdgB encoded by *sdgA* and *sdgB* within the *sdr* locus (Hazenbos *et al.*, 2013).

A 'YSIRK/GS' motif present in the signal sequence of many MSCRAMMs directs secretion of the pre-proteins to sites of peptidoglycan biosynthesis at the region of septum formation (DeDent *et al.*, 2008). MSCRAMMs containing a YSIRK/GS motif include ClfA, ClfB, the Sdr proteins and the fibronectin binding proteins A and B. Proteins lacking the 'YSIRK/GS' motif, including SasA, SasD, SasF and SasK are localised to the cell pole. Reciprocal exchange of signal sequences containing the 'YSIRK/GS' motif and those that do not altered the localisation of the chimeric protein. However, the mechanisms involved in trafficking MSCRAMMs to these cellular compartments are yet to be elucidated (Schneewind and Missiakas, 2012, DeDent *et al.*, 2008). Surface display of *S. aureus* surface proteins containing the 'YSIRK/GS' motif is reduced in mutants lacking the *spdA, spdB or spdC* genes which encode three transmembrane proteins with abortive infectivity (ABI) domains. Unfortunately the molecular basis of this is not understood (Frankel *et al.*, 2010).

A sorting signal is located at the C-terminus of the protein. It comprises an LPXTG motif, a hydrophobic membrane-spanning domain and at the extreme C-terminus, a stretch of positively charged residues. The last two elements delay secretion across the membrane and facilitate recognition and cleavage of the LPXTG sequence by sortase A, a lipoprotein located on the outer face of the cytoplasmic membrane (Schneewind *et al.*, 1993). Sortase cleaves between threonine and glycine forming an acyl-enzyme intermediate which allows transfer of the MSCRAMM to the pentaglycine bridge of lipid II. Following transglycosylation the MSCRAMM becomes covalently anchored to peptidoglycan (Ton-That *et al.*, 2000).

In Chapter 3 the N1 subdomain of ClfA was shown to be dispensable for fibrinogen binding by recombinant proteins or that were expressed on the surface of *S. aureus*. It was not possible to express a complete ClfAΔN1 variant on the surface of the cell suggesting that the N1 subdomain may play a role in surface elaboration. Furthermore, attempted expression of a ClfA variant lacking subdomain N1 resulted in impaired growth and colony formation of *S. aureus.* Ten residues were identified at the C-terminus of subdomain N1 which were sufficient to mediate surface expression at a similar level to wild type ClfA with no loss ability to adhere to fibrinogen.

In this Chapter the subcellular localization of ClfAΔN1 variants is investigated. In Chapter 3 ten residues (211-220) were identified at the C-terminus of subdomain N1 which were sufficient to mediate surface expression of the remainder of the protein.

This Chapter investigates whether residues 211-220 are necessary as well as sufficient for surface elaboration of ClfA. In light of the growth defect observed in cells expressing ClfAΔN1$_{40\text{-}220}$, the subcellular localization of another MSCRAMM was investigated in *S. aureus* expressing ClfAΔN1$_{40\text{-}220}$. Furthermore this Chapter investigates whether the N1 subdomain is required for surface expression of another MSCRAMM FnBPB and determines whether the necessity for subdomain N1 in export of MSCRAMMs is due to their unique architecture.

4.2 Results

4.2.1 Subdomain N1 is required for ClfA to be localized to the *S. aureus* cell wall.

Whole cell immunoblotting indicated that ClfAΔN1$_{40\text{-}220}$ was not elaborated on the surface of *S. aureus* following induction with ATc (Chapter 3, Fig 3.9) but bacterial growth was significantly reduced (Chapter 3, Fig 3.20A) suggesting that subdomain N1 may be involved in surface display. Therefore, the subcellular location of ClfAΔN1$_{40\text{-}220}$ following induction was investigated. Fractions corresponding to the cytoplasm, the cytoplasmic membrane and proteins solubilized from the cell wall by lysostaphin treatment during protoplast formation (cell wall fraction) were analyzed by Western immunoblotting probing with the ClfA mAb.

In the wild type ClfA sample a band of 150 kDa was detected only in the cell wall fraction. This is consistent with normal sorting. In contrast, ClfAΔN1$_{40\text{-}220}$ was not detected in the cell wall fraction but was instead located in the membrane and cytoplasmic fractions (Fig. 4.1A). This protein migrated more slowly than expected and may represent translation products that had not been cleaved posttranslationally by signal peptidase and sortase A. In contrast ClfAΔN1$_{40\text{-}210}$ was only detected in the cell wall fraction in a similar fashion to full-length ClfA indicating that this truncate had been correctly secreted and sorted.

As a control for secretion and sorting activity in cells attempting to express the ΔN1 constructs, samples were also probed for the expression of SdrE, a member of the MSCRAMM family which is known to be sorted to the cell wall (O'Brien *et al.*, 2002). This protein was detected only in the cell wall fraction indicating that it had been exported and sorted correctly in all strains (Fig. 4.1B). Additional controls to ensure equal loading of membrane and cytoplasmic fractions were also performed. Fractions were probed with rabbit anti-*Micrococcus luteus* F_1/F_0ATPase serum which cross reacts with the staphylococcal protein as a control for the cell membrane (Fig 4.2 A). A cross reactive band of ~50 kDa was observed only in the membrane fractions consistent with the size of *S. aureus* ATPase (Downer *et al.*, 2002). Cytoplasm fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue to show equal loading of protein (Fig 4.2 B). To confirm that ClfAΔN1$_{40\text{-}220}$ was not released into the supernatant the concentrated supernatants of NM1 (pRMC2*clfA*) and NM1 (pRMC2*clfA*ΔN1$_{40\text{-}220}$) induced with 800 ng/ml and 1.2 μg/ml respectively was probed with ClfA mAb. No

ClfA variants were detected in the concentrated supernatant fractions (Fig 4.2 C). The cell wall fraction of stationary phase Newman *spa* was used as a positive control for ClfA expression. The concentrated supernatant of stationary phase Newman *spa* was used to determine wild type levels of secreted ClfA.

ClfAΔN1$_{40\text{-}220}$ was not detected in the cell wall fraction but was instead located in the membrane and cytoplasmic fractions (Fig. 4.1A). Similar results were obtained for ClfAΔN1$_{40\text{-}228}$ (Fig. 4.3A, B). The failure to localize to the cell wall explains why ClfAΔN1$_{40\text{-}220}$ and ClfAΔN1$_{40\text{-}228}$ were not detected on the surface of intact bacterial cells (Chapter 3, Fig 3.9). In contrast ClfAΔN1$_{40\text{-}210}$ was only detected in the cell wall fraction in a similar fashion to full-length ClfA indicating that this truncate had been correctly secreted and sorted.

To eliminate the possibility that removal of N1 subdomain interfered with the signal protease cleavage site an additional construct was generated with 2 additional residues at the N-terminus of the N1 subdomain, ClfAΔN1$_{42\text{-}220}$ (Fig 4.4A). Again, although cellular fractionation revealed the protein was well expressed in the cytoplasm and cytoplasmic membrane fractions no protein could be detected in the cell wall fraction (Fig 4.4B). These results indicate that the N1 subdomain is required for surface expression of ClfA.

4.2.2 The effect of ATc on the subcellular localization of ClfA.

ATc was chosen as an inducer because it displays little antibiotic activity (Bateman *et al.*, 2001). As a control for the effect of ATc on ClfA localisation the effect of increasing concentrations of ClfA surface expression was examined. Stationary phase Newman cells were subcultured and grown to mid-exponential phase then re-adjusted to an OD_{600} of 0.05 grown to an OD_{600} of 0.5 and then treated for 2 hours with ATc. Cellular fractions of Newman grown in the presence of ATc were probed with polyclonal anti-ClfA IgG. In the uninduced control a band of ~150 kDa was observed in the cell wall fraction only. No difference in the level of ClfA or subcellular localization was observed at even the highest concentration of ATc used (Fig 4.5A). Cytoplasm fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. No difference in overall protein composition was observed (Fig 4.5B). These results demonstrate that the presence of ATc did not affect proper secretion and sorting of ClfA.

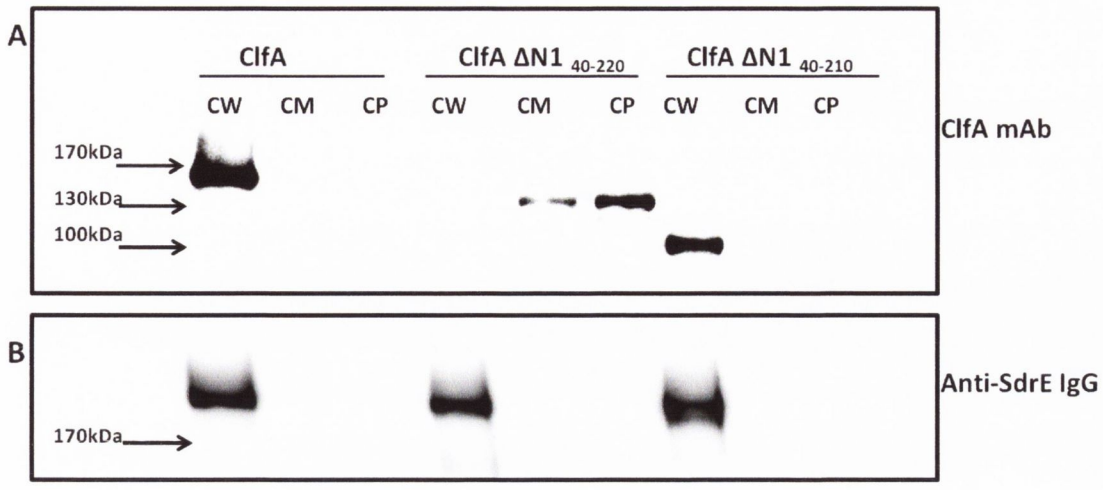


Figure 4.1 Subcellular localization of ClfA, ClfAΔN1$_{40-220}$ and ClfAΔN1$_{40-210}$.

Cellular fractions from *S. aureus* expressing ClfA, ClfAΔN1$_{40-220}$ or ClfAΔN1$_{40-210}$ were probed with **(A)** ClfA mAb and detected with HRP-conjugated rabbit anti-mouse IgG or **(B)** anti-SdrE IgG and detected with HRP-conjugated goat anti-rabbit IgG in a Western immunoblot. Fractions are labelled cell wall (CW), cell membrane (CM) and cytoplasm (CP).

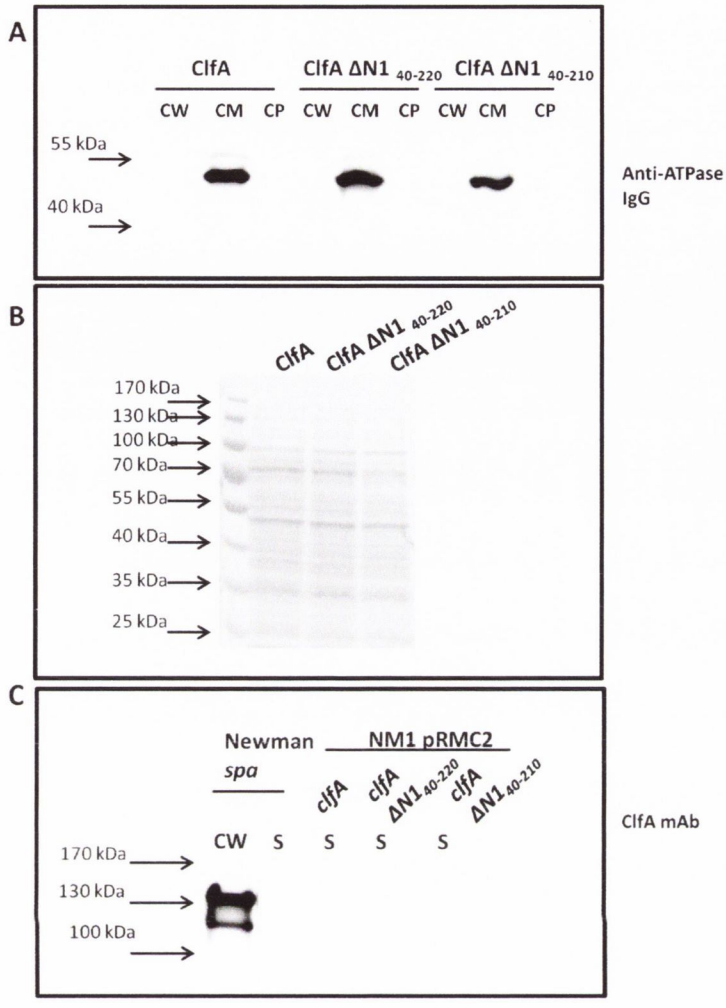


Figure 4.2 Membrane, cytoplasm and supernatant controls for ClfAΔN1 variants

(A) Subcellular localization of the membrane F_1/F_0 ATPase in *S. aureus* expressing ClfA, ClfAΔN1$_{40\text{-}220}$ and ClfAΔN1$_{40\text{-}210}$. Western immunoblot of cellular fractions from *S. aureus* expressing ClfA induced with 800 ng/ml ATc, ClfAΔN1$_{40\text{-}220}$ or ClfAΔN1$_{40\text{-}210}$ induced with 1.2 µg/ml Atc were probed with rabbit anti-ATPase serum and detected with HRP-conjugated goat anti-rabbit IgG. Fractions are labelled cell wall (CW), cell membrane (CM) and cytoplasm (CP). **(B)** Cytoplasm fractions as in (A) separated by SDS-PAGE and stained with Coomassie Brilliant Blue. **(C)** Concentrated supernatant fractions from NM1 (pRMC2*clfA*), (pRMC2*clfA*ΔN1$_{40\text{-}220}$) and (pRMC2*clfA*ΔN1$_{40\text{-}210}$) were probed with ClfA mAb and detected with HRP-conjugated rabbit anti-mouse IgG. The CW fraction of stationary phase Newman *spa* was used as a positive control for ClfA. The concentrated supernatant of stationary phase Newman *spa* was used to determine wild type levels of secreted ClfA. Cell wall (CW) or supernatant (S) fractions are labelled.

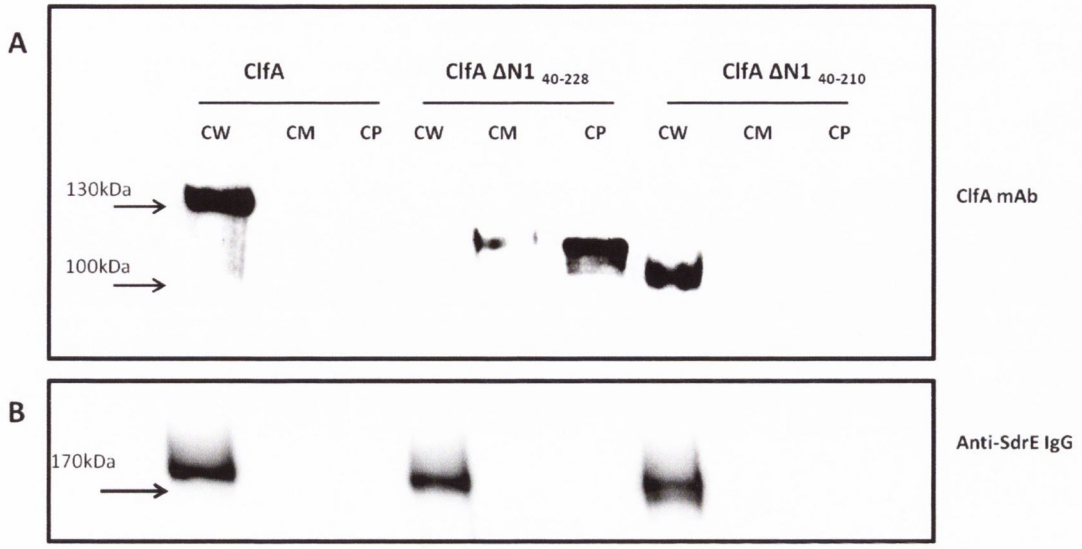


Figure 4.3 Subcellular localization of ClfA, ClfAΔN1$_{40-228}$ and ClfAΔN1$_{40-210}$.

Cellular fractions from *S. aureus* expressing ClfA, ClfAΔN1$_{40-228}$ or ClfAΔN1$_{40-210}$ were probed with **(A)** ClfA mAb and detected with HRP-conjugated rabbit anti-mouse IgG or **(B)** anti-SdrE IgG and detected with HRP-conjugated goat anti-rabbit IgG in a Western immunoblot. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions are labelled.

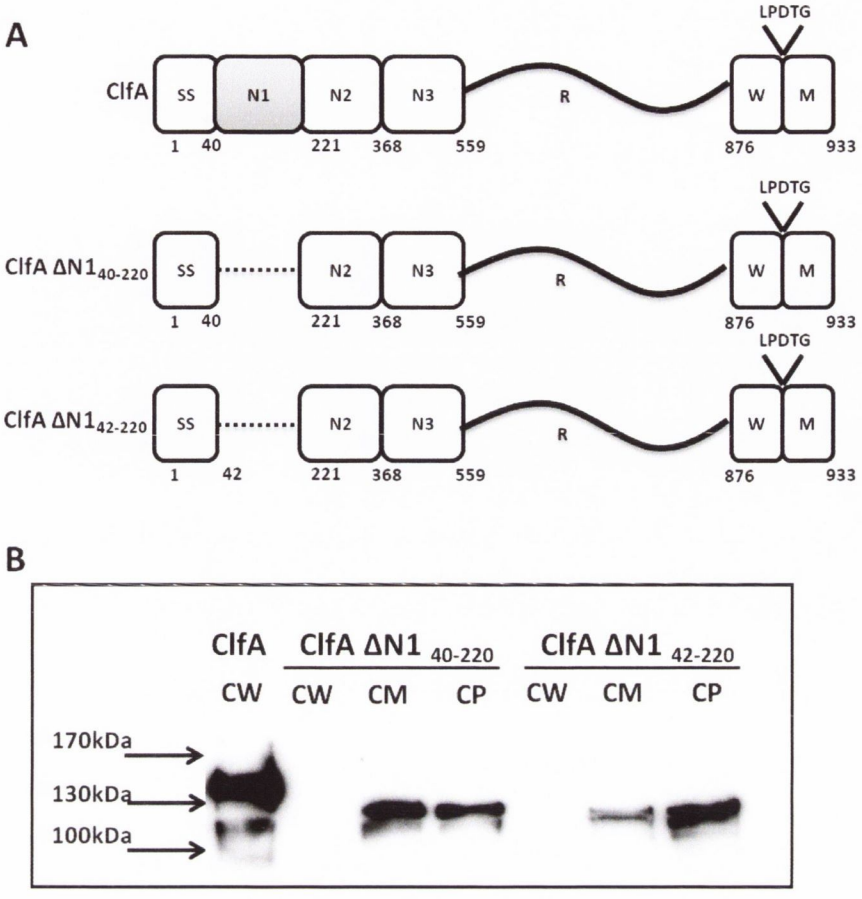


Figure 4.4 Subcellular localisation of ClfAΔN1$_{42-220}$.

(A) Schematic representation of ClfA, ClfAΔN1$_{40-220}$ and ClfAΔN1$_{42-220}$. **(B)** Western immunoblot of cellular fractions of NM1 expressing ClfA, ClfAΔN1$_{40-220}$ and ClfAΔN1$_{42-220}$. The blot was probed with ClfA mAb and HRP-conjugated rabbit anti-mouse IgG. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions are labelled.

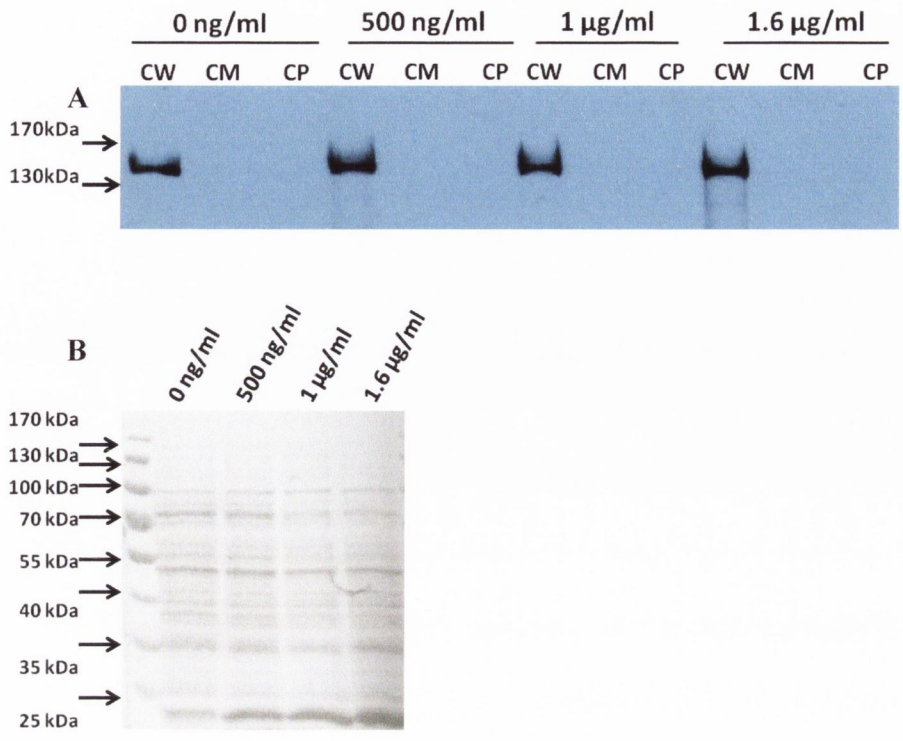


Figure 4.5 Subcellular localization of ClfA expressed from Newman (pRMC2) grown in ATc.

(A) Western immunoblot of cellular fractions from *S. aureus* Newman (pRMC2) incubated with increasing concentrations of ATc. The concentration of ATc is indicated at the top of the blot. The blot was probed with polyclonal anti-ClfA IgG and detected with protein A-peroxidase. Fractions are labelled cell wall (CW), cell membrane (CM) and cytoplasm (CP). **(B)** Cytoplasm fractions as in (A) separated by SDS-PAGE and stained with Coomassie Brilliant Blue.

4.2.3 The effect of ClfAΔN1$_{40-220}$ on the localization of the MSCRAMM SdrE.

Deletion of subdomain N1 of ClfA results in mislocalisation of ClfAΔN1$_{40-220}$. To determine if mislocalisation of ClfA variants affects correct localisation of another MSCRAMM, cellular fractions of cells expressing ClfAΔN1$_{40-220}$ were probed for SdrE. SdrE is a cell wall-anchored surface protein of *S. aureus* which is expressed in both the exponential and stationary phases of growth. *S. aureus* cells carrying pRMC2*clfA* or pRMC2*clfA*ΔN1$_{40-220}$ were grown to an OD_{600} of 0.5. Before induction with ATc cells were treated with trypsin for 1 hour at 37°C to remove all cell wall-anchored proteins. This ensured removal of any cell wall-bound SdrE that was secreted prior to the induction of ClfAΔN1$_{40-220}$ expression and allowed the surface expression of SdrE to be examined in the presence of ClfAΔN1$_{40-220}$. Cells were washed and resuspended in the original volume of BHI and induced with 1.2 µg/ml ATc for two hours (Fig 4.6A). Cell wall fractions of cells treated with trypsin or cells treated with trypsin then induced with ATc were probed with ClfA mAb. No cross reactive bands were detected in trypsin-treated cell wall fractions, indicating that the protease had removed all cell wall-anchored protein (Fig 4.6B). NM1 (pRMC2*clfA*) cells that were induced with ATc after trypsin treatment displayed two bands in the cell wall, one at ~150 kDa and a second at ~100 kDa. The 150 kDa band corresponds to the size of full length ClfA, while the 100 kDa band is likely to be a break down product due to protease that was not removed by washing prior to induction. Trypsin-treated fractions were also probed with anti-SdrE IgG (Fig 4.6C). No difference in the level of cell wall SdrE was detected in cells expressing ClfA or ClfAΔN1$_{40-220}$. As with ClfA, a double band was observed for the SdrE protein. The higher band of greater than 170 kDa corresponds to the full length SdrE protein, while the lower band is likely due to breakdown due to contaminating protease. To determine if cells expressing ClfAΔN1$_{40-220}$ contained SdrE in other subcellular fractions, whole cell fractionation was performed on the same cells as panel B and C. SdrE was only detected in the cell wall fraction of cells expressing ClfA or ClfAΔN1$_{40-220}$ showing that expression of ClfAΔN1$_{40-220}$ did not affect correct secretion and sorting of SdrE (Fig 4.6D).

4.2.4 Identification of residues in subdomain N1 required for ClfA to be localized to the cell wall.

The N1 truncate ClfAΔN1$_{40-210}$ was sorted efficiently to the cell wall of *S. aureus* while ClfAΔN1$_{40-220}$ and ClfAΔN1$_{40-228}$ were found in the cytoplasm and

membrane (Fig 4.1A and Fig 4.3A) suggesting that residues 211 - 220 may be required for efficient translocation and cell wall localization. The Phyre2 program predicts that the N1 subdomain is largely disordered, except for a short region at the C-terminus beginning at residue 209 (Fig 4.7). This structure may be important for functional surface expression of ClfA. In order to investigate the importance of residues 211-220 further, plasmid pRMC2*clfA* was manipulated to create a derivative lacking DNA encoding amino-acids 211-220 of ClfA (plasmid pRMC2*clfA*$\Delta_{211\text{-}220}$). A second variant where residues 211-220 were substituted with 10 alanines was also constructed (Fig 4.8A).Whole cell dot immunoblotting using ClfA mAb with cells expressing ClfA$\Delta_{211\text{-}220}$ (Fig 4.8B, upper panel) or ClfAΔN1$_{40\text{-}220+10Ala}$ (Fig 4.8B, lower panel) revealed that they were not surface expressed. Fractionation revealed ClfA$\Delta_{211\text{-}220}$ was not detected in the cell wall but was present in the cell membrane and cytoplasm (Fig 4.8C). This shows that residues 211-220 are necessary for cell wall localization. While ClfAΔN1$_{40\text{-}210}$ was only detected in the cell wall fraction (Fig 4.8C), a variant where residues 211-220 were substituted with ten alanines (ClfAΔN1$_{40\text{-}220+10Ala}$) was not (Fig 4.8C). SdrE was detected only in the cell wall fraction indicating that it had been exported and sorted correctly in all strains (Fig 4.8D). These results demonstrate that residues 211-220 are necessary and sufficient for surface expression of the remainder of the ClfA protein. To reflect the two different definitions of subdomain N1 (40-220 and 40-228), an additional variant of ClfA was constructed lacking residues 211-228 (ClfA$\Delta_{211\text{-}228}$; Fig 4.9A). ClfA$\Delta_{211\text{-}228}$ was detected in the cytoplasm and membrane fractions similarly to ClfA$\Delta_{211\text{-}220}$ (Fig 4.9B).

In ClfAΔN1$_{40\text{-}220+10Ala}$ residues 211-220 could not be functionally substituted with ten alanine residues. Residue 211 is a proline. Proline residues are commonly associated with bends in secondary structure or at the beginning of an α-helix. To determine whether proline was important for the function of residues 211-220, plasmid pRMC2*clfA* was manipulated to delete residues 40-210 and to simultaneously substitute proline$_{211}$ with an alanine (Fig 4.10A). This was achieved by incorporating a single alanine codon into the forward primer designed for inverse PCR. Figure 4.10B shows an agarose gel electrophoresis image of the inverse PCR product. To confirm loss of DNA encoding the N1 subdomain a restriction digest with EcoRI and BglII was performed on plasmid pRMC2*clfA*ΔN1$_{40\text{-}210P211*A}$ (Fig 4.10C). The cell wall fraction of NM1 expressing ClfAΔN1$_{40\text{-}210}$P$_{211}$*A$_{211}$ induced with 1 μg/ml ATc was probed with ClfA

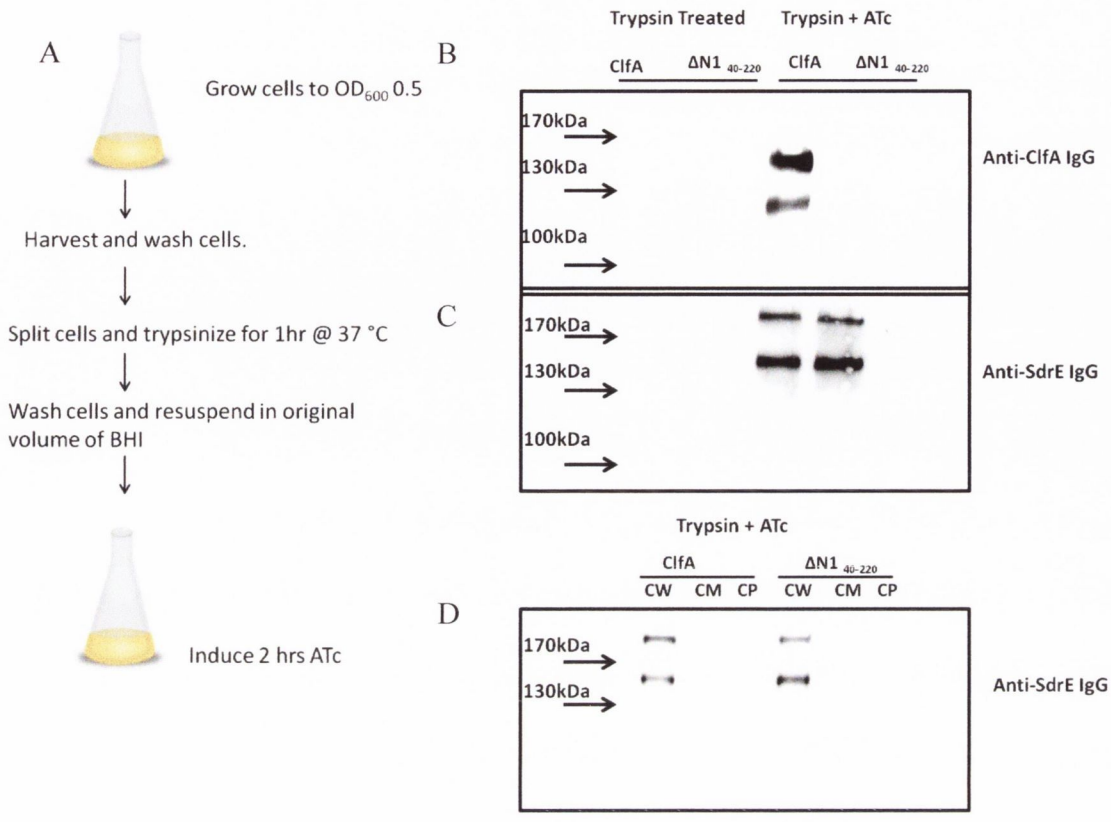


Figure 4.6 Subcellular localisation of SdrE in trypsin-treated cells expressing ClfAΔN1$_{40-220}$

(A) Schematic representation of the experiment to remove all surface proteins by treatment with trypsin before induction with ATc. **(B)** Western immunoblot of cell wall fractions of NM1 expressing ClfA or ClfAΔN1$_{40-220}$ treated with trypsin or following trypsin treatment and induction with 1.2 μg/ml ATc. The blot was probed with ClfA mAb and detected with HRP-conjugated rabbit anti-mouse IgG. **(C)** Cell wall fractions as in B. The blot probed with anti-SdrE IgG and detected with protein A-peroxidase. **(D)** Cellular fractions of NM1 expressing ClfA or ClfAΔN1$_{40-220}$ following trypsin treatment and induction with 1.2 μg/ml ATc. The blot was probed with rabbit anti-SdrE IgG and protein A-peroxidase. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions are labelled.

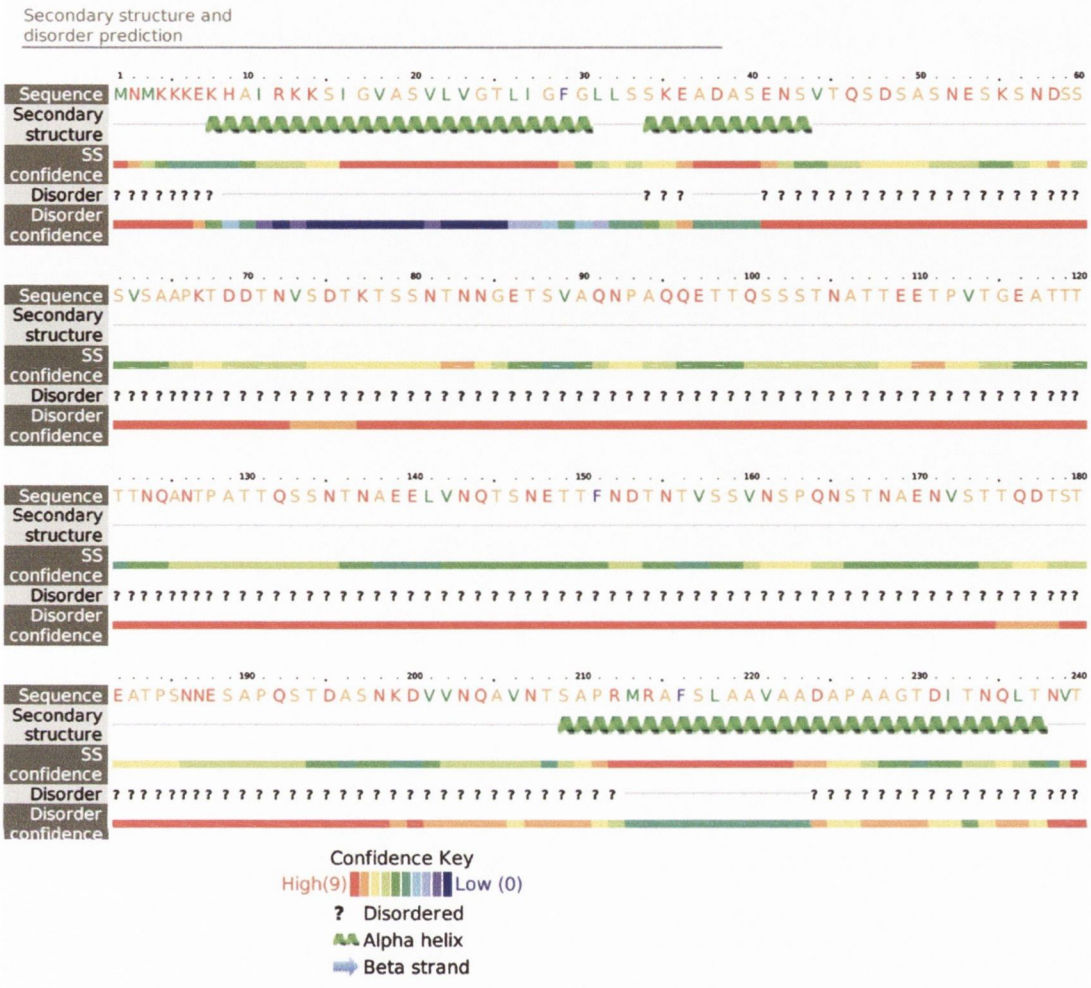


Figure 4.7 Phyre 2 prediction of secondary structure of the N1 subdomain of ClfA.

Regions within the signal sequence and the C-terminal region of the N1 subdomain of ClfA are predicted to have α-helical secondary structures are indicated by the green helices.

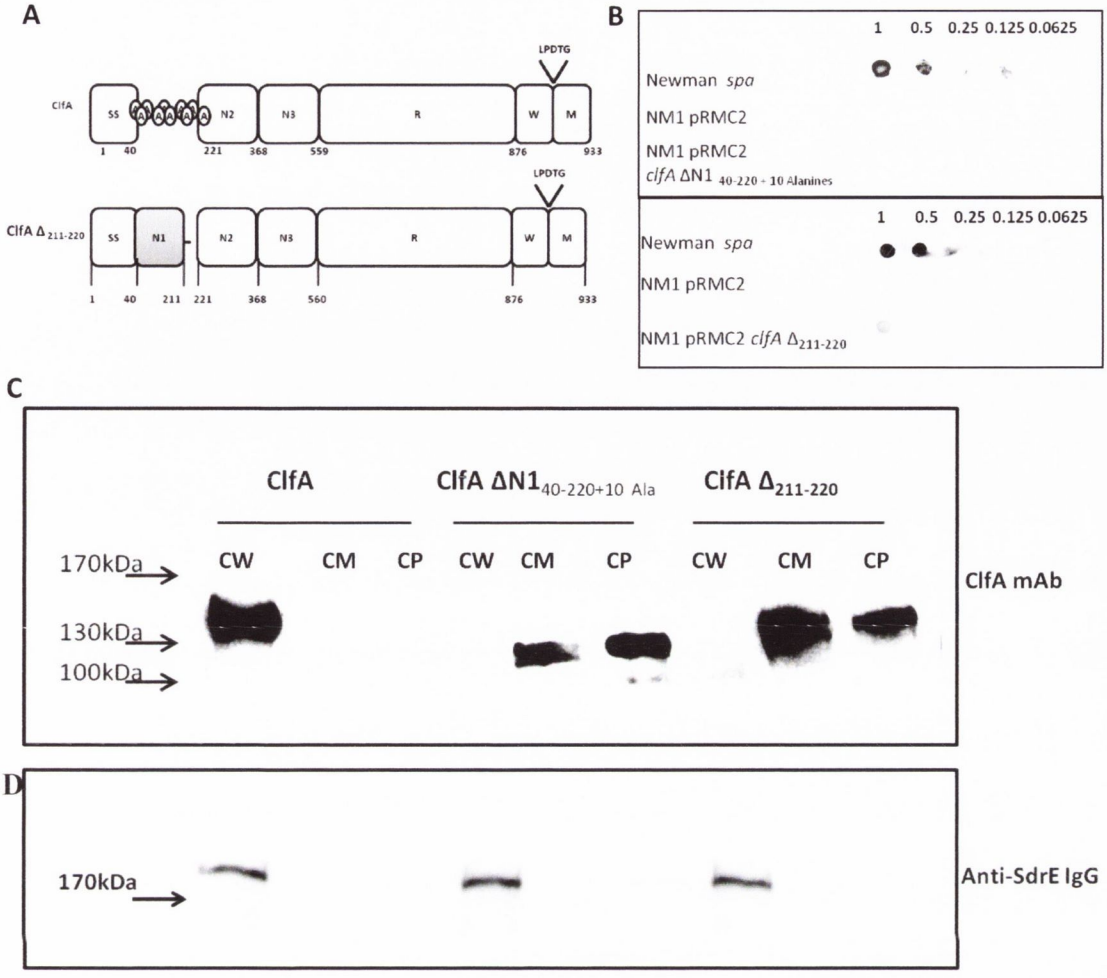


Figure 4.8 Subcellular localisation of ClfAΔN1$_{40\text{-}220+10Alas}$ and ClfAΔ$_{211\text{-}220}$.

(A) Schematic representation of ClfAΔN1$_{40\text{-}220+10Alas}$ and ClfAΔ$_{211\text{-}220}$. **(B)** Whole cell dot immunoblot of NM1 (pRMC2*clfA*ΔN1$_{40\text{-}220+10Alas}$) [upper panel], or NM1 (pRMC2*clfA* Δ$_{211\text{-}220}$) [lower panel]. Newman *spa* was used as a positive control for ClfA surface expression from a single chromosomal copy of the gene. NM1 (pRMC2) was used as a negative control. The OD_{600} of cells is listed across the top of the blot, and strain is listed at the side. A suspension of cells (5 µl) was spotted onto the membrane. Blots were probed with ClfA mAb and detected with HRP-conjugated rabbit anti-mouse IgG. **(C)** Subcellular location of ClfA, ClfAΔN1$_{40\text{-}220+10Ala}$ and ClfAΔ$_{211\text{-}220}$. Cellular fractions from *S. aureus* expressing ClfA induced with 800 ng /ml ATc or ClfAΔN1$_{40\text{-}220+10\ Ala}$ and ClfAΔ$_{211\text{-}220}$ induced with ATc (1200 ng/ml) probed with ClfA mAb and detected with HRP-conjugated rabbit anti-mouse IgG. **(D)** Fractions as in (C) probed with anti-SdrE IgG detected with protein A-peroxidase in a Western immunoblot. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) are labelled.

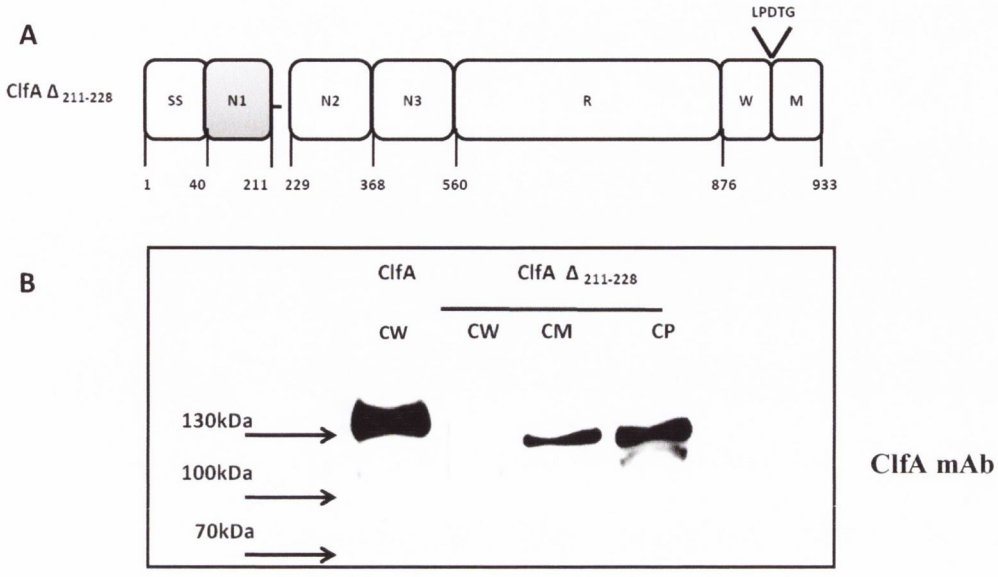


Figure 4.9 Subcellular localisation of ClfAΔ$_{211-228}$

(A) Schematic representation of ClfAΔ$_{211-228}$. **(B)** Cellular fractions from *S. aureus* expressing ClfAΔ$_{211-228}$ induced with 1200 ng/ml ATc. The cell wall fraction of NM1 expressing ClfA induced with 800 ng/ml was included as a positive control for ClfA expression. The blot was probed with ClfA mAb and detected with HRP-conjugated rabbit anti-mouse IgG. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions are labelled.

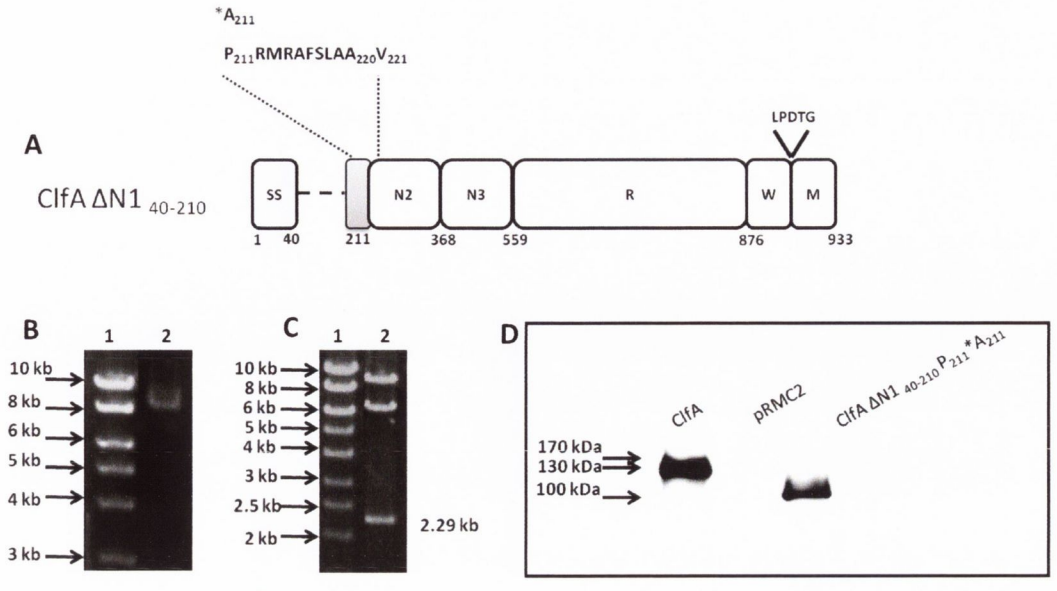


Figure 4.10 Construction and localisation of ClfAΔN1$_{40\text{-}210}$P$_{211}$*A$_{211}$

(A) Schematic representation of ClfAΔN1$_{40\text{-}210}$P$_{211}$*A$_{211}$. **(B)** Agarose gel electrophoresis (L1) DNA size marker (L2) inverse PCR product for pRMC2 *clfA*ΔN1$_{40\text{-}210}$P$_{211}$*A$_{211}$. **(C)** Agarose gel electrophoresis (L1) DNA size marker, (L2) restriction digest of pRMC2 *clfA*ΔN1$_{40\text{-}210}$P$_{211}$*A$_{211}$ with EcoRI and BglII. The band at 2.29 kb corresponds to the size of ClfAΔN1$_{40\text{-}210}$P$_{211}$*A$_{211}$. The band at 6.4 kb corresponds to the size of cut pRMC2. The band at 8.7 kb corresponds to pRMC2 *clfA*ΔN1$_{40\text{-}210}$P$_{211}$*A$_{211}$ which has not been cut to completion by EcoRI and BglII. **(D)** Cell wall fractions from NM1 expressing ClfA or ClfAΔN1$_{40\text{-}210}$P$_{211}$*A$_{211}$ induced with 1 μg/ml ATc. NM1 (pRMC2) was used as a negative control. Cell wall fractions were probed with ClfA mAb and detected with HRP-conjugated rabbit anti-mouse IgG in a Western immunoblot.

mAb. A cross reactive band was detected at ~100 kDa indicating that P_{211} is not necessary for surface expression of ClfA.

4.2.5 Subdomain N1 is not required for cell wall localization of a ClfA variant lacking residues comprising the serine-aspartate repeat region.

The A regions of MSCRAMMs which contain an N1 subdomain with no known structure or function are linked to the cell wall by an extended flexible region. In the case of ClfA this comprises Ser-Asp dipeptide repeats. To determine if the N1 subdomain is required for correct localisation of MSCRAMMs containing long flexible unstructured repeat regions additional variants were constructed. Plasmids pRMC2*clfA* and pRMC2*clfA*$\Delta N1_{40\text{-}220}$ were manipulated in order to create variants lacking DNA encoding the Ser-Asp dipeptide repeat region (residues 559-875) generating plasmids pRMC2*clfA*$\Delta SD_{559\text{-}875}$ and plasmid pRMC2*clfA*$\Delta N1_{40\text{-}220}\Delta SD_{559\text{-}875}$, respectively (Fig 4.11A). ClfA$\Delta SD_{559\text{-}875}$ was only detected in the cell wall (Fig 4.11B). Similarly, ClfA$\Delta N1_{40\text{-}220}\Delta SD_{59\text{-}875}$ was present only in the cell wall fraction (Fig. 4.11B). This demonstrates that the N1 subdomain is not required for export or cell wall localization of ClfA lacking the flexible Ser-Asp repeat region and is only required for expression of the repeat-containing protein.

To determine whether the N1 subdomain was required for surface export of the Ser-Asp repeat region alone or export of the Ser-Asp repeat region in conjunction with the IgG-like folds of the N23 region plasmid pRMC2*clfA* was manipulated to remove DNA encoding the A region. DNA encoding a FLAG-epitope was introduced into both constructs N-terminal to the Ser-Asp repeat region to facilitate detection of the proteins in the absence of the A region. Two additional constructs were generated by inverse PCR to create ClfA$\Delta N123_{40\text{-}559FLAG}$ lacking the entire A region and ClfA$\Delta N23_{40\text{-}220FLAG}$ lacking the N23 region (Fig 4.12A). Cellular fractions of NM1 expressing ClfA$\Delta N123_{40\text{-}559FLAG}$ and ClfA$\Delta N23_{40\text{-}220FLAG}$ were probed with monoclonal anti-FLAG IgG and detected with HRP-conjugated rabbit anti-mouse IgG. Cellular fractions of NM1 expressing ClfA or carrying pRMC2 (empty vector) were included as additional controls for non-FLAG tagged proteins. A crossreactive band was detected at ~50 kDa in all membrane fractions. This is likely to be Sbi, a membrane-localized IgG binding protein reacting with the secondary antibody. ClfA$\Delta N123_{40\text{-}559FLAG}$ does not appear to be expressed in any fraction suggesting the construct is unstable or not reacting with the

antibody. However, a band of ~130 kDa was detected in the membrane of cells expressing ClfAΔN23$_{40\text{-}220FLAG}$, suggesting that the N1 subdomain is not sufficient for surface display of the Ser-Asp repeats in the absence of the N23 region. Unexpectedly a cross-reactive band of ~55 kDa was detected in the cytoplasm of the pRMC2*clfA* non-FLAG-tagged control (Fig 4.12B). This suggests that when ClfA is expressed from pRMC2 a small breakdown product of ClfA can cross react with the anti-FLAG antibodies. This phenomenon was observed with two distinct monoclonal FLAG antibodies. This complicated interpretation of the data and further investigation in this area.

4.2.6 Investigation of the mechanism by which subdomain N1 mediates surface expression of ClfA

It is unclear why a region distinct from the signal sequence should be necessary to mediate correct cell wall localisation of a pre-protein. It is possible that subdomain N1 acts as a molecular chaperone and binds to the N23 region of ClfA. To investigate the mechanism by which subdomain N1 mediates surface expression of ClfA, recombinant GST-tagged ClfAN1$_{LONG40\text{-}228}$ was examined for its ability to bind to immobilised His-tagged ClfAN23$_{220\text{-}559}$. No binding was detected (Fig 4.13A). The Sec pathway mediates secretion of unfolded pre-proteins. If the N1 subdomain acts a chaperone for the remainder of ClfA by binding the N23 region it would bind directly to this region when it is unfolded. Recombinant His-tagged ClfAN23$_{220\text{-}559}$ migrated under reducing and denaturing conditions by SDS-PAGE was probed in a ligand affinity blot with GST-tagged ClfAN1$_{LONG40\text{-}228}$ and detected with anti-GST IgG. Cross-reactive bands corresponding to the size of His-tagged ClfAN123$_{40\text{-}559}$ and ClfAN23$_{220\text{-}559}$ were detected when probed with GST-tagged ClfAN1$_{LONG40\text{-}228}$. However, this was attributed to non-specific binding of the GST-tag as similar bands were also observed in the GST control (Fig 4.13B).

Residues 211-220 are necessary for surface expression of ClfA. To investigate the possible role of this region a His-tagged peptide comprising residues 211-220 was synthesised commercially (HisClfA$_{211\text{-}220}$). The ability of HisClfA$_{211\text{-}220}$ to bind to immobilised GST-tagged ClfAN123$_{40\text{-}559}$ and N23$_{220\text{-}559}$ was examined. No interaction was detected indicating that residues 211-220 do not bind to the N23 region (Fig

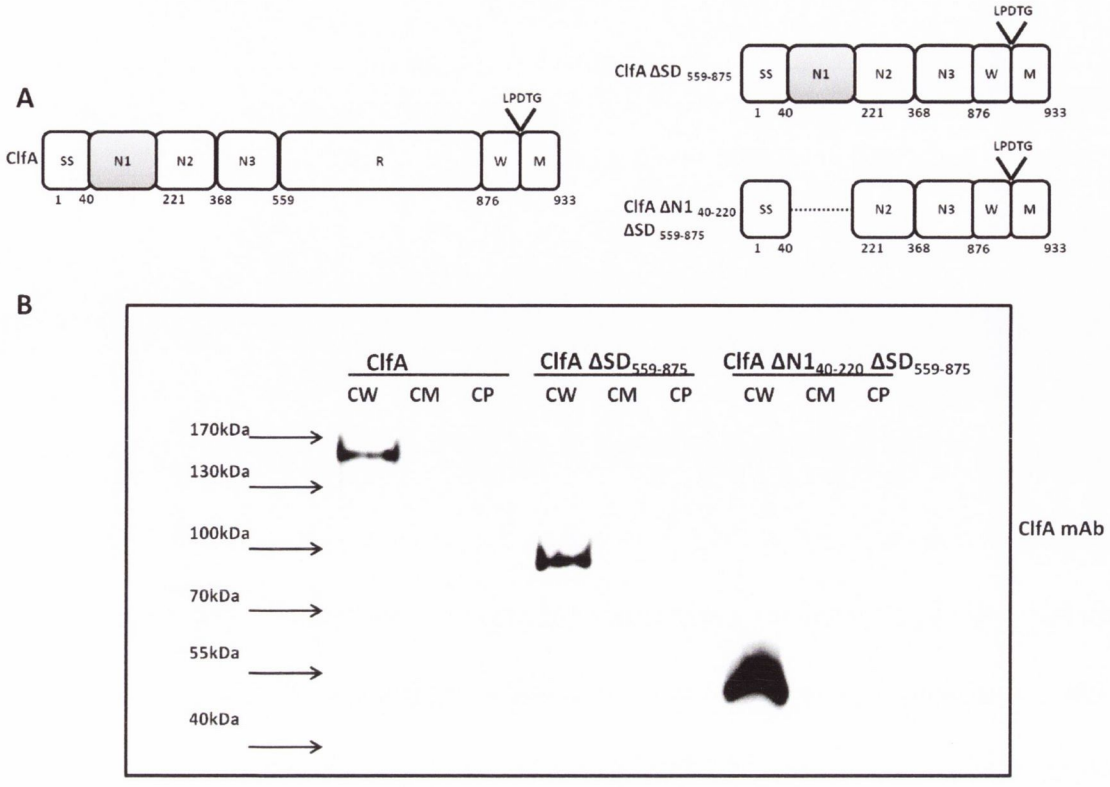


Figure 4.11 Subcellular localisation of ClfAΔSD$_{559-875}$ and ClfAΔN1$_{40-220}$ΔSD$_{559-875}$

(A) Schematic representation of ClfA, ClfAΔSD$_{559-875}$ and ClfAΔN1$_{40-220}$ΔSD$_{559-875}$. **(B)** Subcellular location of ClfA, ClfAΔSD$_{559-875}$ and ClfAΔN1$_{40-220}$ΔSD$_{559-875}$. Cellular fractions from *S. aureus* were probed with ClfA mAb and HRP-conjugated rabbit anti-mouse IgG in a Western immunoblot. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions are labelled.

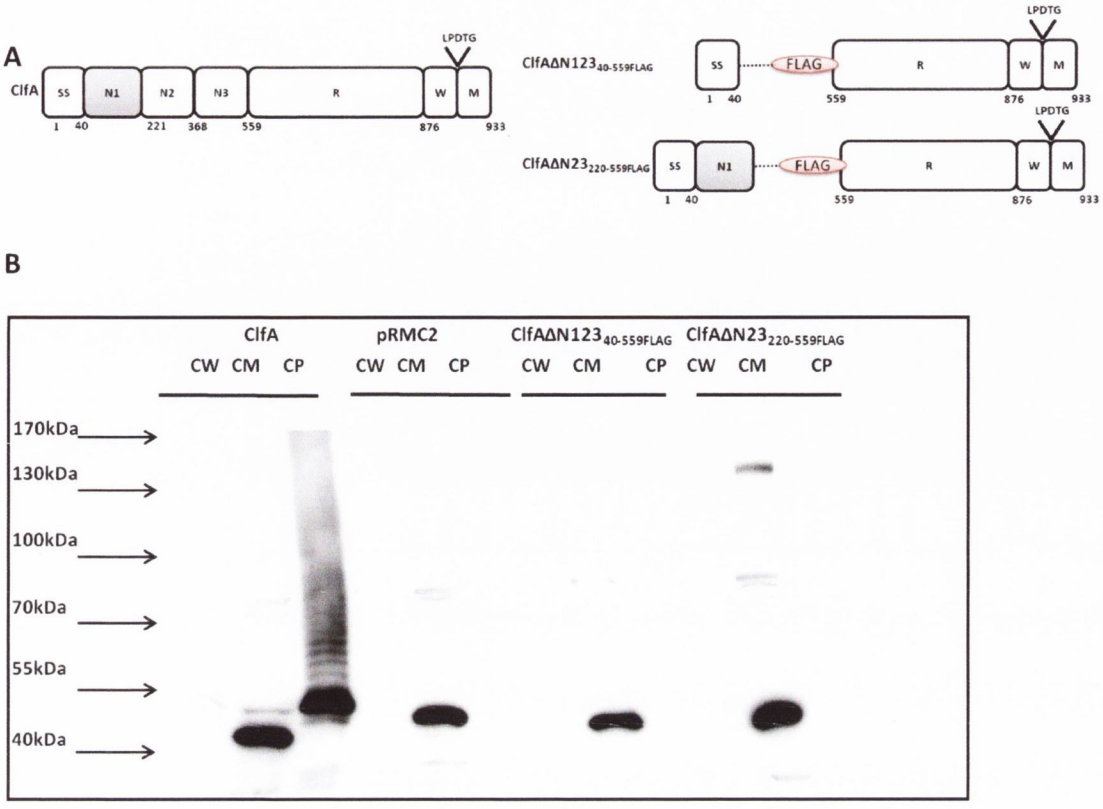


Figure 4.12 Subcellular localisation of ClfAΔN123$_{40\text{-}559FLAG}$ and ClfAΔN23$_{40\text{-}220FLAG}$

(A) Schematic representation of ClfA, ClfAΔN123$_{40\text{-}559FLAG}$ and ClfAΔN23$_{40\text{-}220FLAG}$. **(B)** Subcellular location of ClfA, ClfAΔN123 $_{40\text{-}559FLAG}$ and ClfAΔN23$_{40\text{-}220FLAG}$ after induction with 1 µg/ml ATc. Cellular fractions were probed with murine monoclonal anti-FLAG IgG and HRP-conjugated rabbit anti-mouse IgG. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions are labelled.

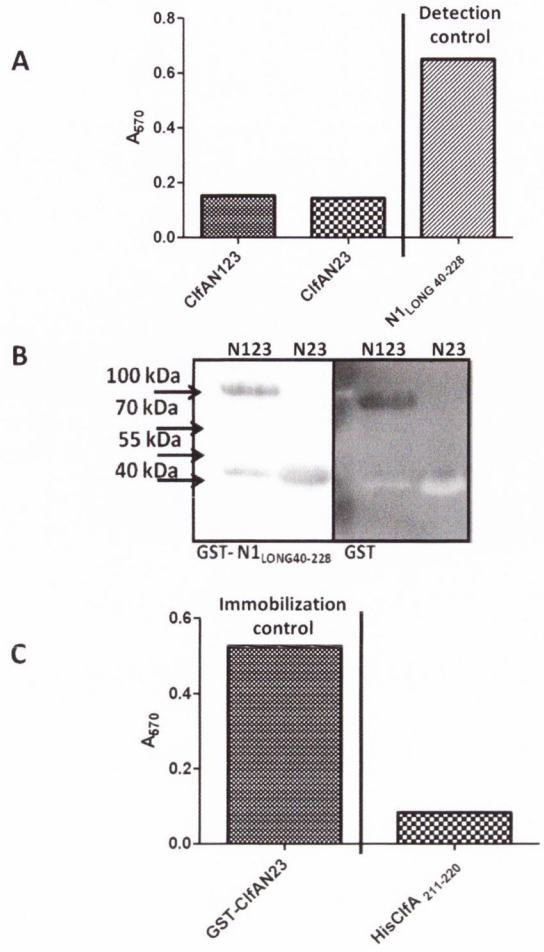


Figure 4.13 Recombinant GST-tagged ClfA $N1_{LONG40-228}$ binding to His-tagged $ClfAN123_{40-559}$ and $ClfAN23_{221-559}$

(A) Recombinant His-tagged $ClfAN123_{40-559}$ and $ClfAN23_{221-559}$ were immobilised on a microtitre plate and incubated with GST-tagged ClfA $N1_{LONG40-228}$. Immobilised ClfA $N1_{LONG40-228}$ was used as a positive control for GST-tagged ClfA $N1_{LONG40-228}$ recognition. Bound GST was recognised by HRP-conjugated anti-GST IgG. **(B)** Recombinant His-tagged $ClfAN123_{40-559}$ and $ClfAN23_{221-559}$ were electrophoresed by SDS-PAGE, transferred to a membrane and probed with GST-tagged ClfA $N1_{LONG40-228}$ or GST only. Bound GST was recognised by HRP-conjugated anti-GST IgG. **(C)** Immobilised GST-tagged $ClfAN23_{221-559}$ was incubated with the synthetic peptide $HisClfA_{211-220}$. $HisClfA_{211-220}$ was recognised using murine polyclonal anti-His IgG and HRP-conjugated anti-mouse IgG. Immobilised GST-tagged $ClfAN23_{220-559}$ was used as a positive control for GST-tagged $ClfAN23_{220-559}$ immobilisation and was detected using HRP-conjugated anti-GST IgG. ELISA plate wells were incubated with a chromogenic substrate and the absorbance at A_{450} was determined. Data points represent the mean of triplicate wells and the graph is representative of three independent experiments.

4.13C). However, this does not preclude the possibility that residues 211-228 can bind in this region.

4.2.7 The effect of protein glycosylation on surface expression of ClfA and a ClfAΔN1$_{40-220}$.

ClfA has been shown to be glycosylated via glycosyltransferases encoded by the *sdgA* and *sdgB* genes (Hazenbos *et al.*, 2013). These modifications occur in the Ser-Asp repeat region of ClfA. In *S. gordonnii*, GspB a serine rich glycoprotein has been shown to be exported via the accessory Sec pathway. Furthermore, glycosylation of GspB is refractory to secretion via the canonical Sec pathway (Bensing and Sullam, 2010). To determine whether glycosylation affects the cellular localisation of ClfA or ClfAΔN1$_{40-220}$ a strain of *S. aureus* SH1000 containing a Ka^R determinant which disrupts the *sdgA* and *sdgB* genes (SH1000 *sdgAB*) was employed (Table 2.1). The Ka^R determinant was moved into a strain of Newman lacking *clfA* due to a frame shift mutation and *clfB* due to disruption with an erythromycin resistance cassette (Newman *clfA5clfB*) by generalized transduction forming NM3 (Table 2.1). NM3 grew normally when compared to Newman *clfA5clfB* (Fig 4.14A) and expression of δ-toxin indicated that the *agr* locus was intact (Fig 4.14B). This suggests that secondary mutations affecting growth and Agr had not been acquired during transduction. Strain NM3 was transformed with plasmids pRMC2*clfA* or pRMC2*clfA*ΔN1$_{40-220}$. Cellular fractions of NM3 expressing ClfA showed no significant change in protein localization with the majority of the protein detected in the cell wall fraction. Cellular fractions of NM3 expressing ClfAΔN1$_{40-220}$ revealed cross reactive protein present in the membrane and cytoplasm fraction similar to expression of ClfAΔN1$_{40-220}$ in strain NM1 (Fig 4.14C). These results indicate that lack of glycosylation does not affect the surface elaboration of ClfAΔN1$_{40-220}$.

4.2.8 Construction and validation of pRMC2*clfB* and pRMC2*clfB* ΔN1$_{48-200}$

Region A of ClfB shares ~26% amino-acid identity with the corresponding region of ClfA and is organised into N1, N2 and N3 subdomains (Ni Eidhin *et al.*, 1998) (Fig 4.15A). To determine whether the N1 subdomain plays a similar role in secretion and surface expression of other MSCRAMMs the full *clfB* gene including its ribosome binding site was amplified from strain Newman and cloned into pRMC2 between the EcoRI and BglII sites. The construct was validated by DNA sequencing but failed to

express high levels of ClfB. This was demonstrated by whole cell dot immunoblotting of NM1 (pRMC2*clfB*) cells induced with 1.2 µg/ml ATc after 1hr, 2hr, or 3hr (Fig 4.15B). Cell wall fractions were obtained from cells in panel B, and probed with anti-ClfB IgG. A immunoreactive band of ~150 kDa was detected which corresponds to the size of ClfB in the cell wall fraction of Newman *spa* from the exponential phase of growth. However, very poor levels of ClfB were obtained (Fig 4.15C). Cells from panel B and C were tested for their ability to adhere to immobilised human cytokeratin 10. Cytokeratin 10 is a ligand that is only recognised by ClfB in contrast to fibrinogen which is bound by several MSCRAMMs and can be used to monitor ClfB expression. NM1(pRMC2*clfB*) induced with 1.2 µg/ml ATc could adhere to human cytokeratin 10 but not to the same level as Newman ClfB^{+} (Fig 4.15D). Plasmid pRMC2*clfB* was manipulated to remove DNA encoding residues 48-200 comprising the N1 subdomain (Fig 4.15E). Sequence analysis confirmed complete deletion of subdomain N1. However, no ClfB was detected in any cellular fractions of NM1 (pRMC2*clfB*ΔN1$_{48\text{-}200}$) induced with 1.2 µg/ml (Fig 4.15F). Due to poor levels of expression of ClfB and the ClfBΔN1$_{48\text{-}200}$ variant this MSCRAMM was not investigated further.

4.2.9 Construction and validation of pRMC2*fnbB*

Region A of FnBPB shares ~ 25% amino acid identity with the corresponding region of ClfA and is organised into N1, N2 and N3 subdomains (Deivanayagam *et al.*, 2002). FnBPB does not have a Ser-Asp repeat region and instead harbours ten fibronectin binding repeats at the C-terminus of the protein (Fig 4.16A). In order to determine if subdomain N1 was required for localisation of other *S. aureus* MSCRAMMs, the subcellular localisation of FnBPB and FnBPBΔN1 variants was studied. The *fnbB* gene from strain 8325 was cloned into pRMC2 between the EcoRI and BglII sites to generate pRMC2*fnbB*. Plasmid DNA was isolated from putative positive clones and the insertion of *fnbB* was confirmed by cleavage with EcoRI and BglII, revealing bands of 6.4 kb corresponding to pRMC2 backbone and 2.7 kb corresponding to the *fnbB* gene (Fig 3.16B). Plasmid pRMC2*fnbB* was sequenced to confirm the integrity of the *fnbB* gene. Plasmids were transformed into a strain of the MRSA isolate BH1CC lacking both FnBPA and FnBPB (BH1CC Δ*fnbAB*, Table 2.1) for analysis of expression. Strain BH1CC was selected because it is protease deficient and allows expression of FnBPs into stationary phase (Geoghegan *et al.*, 2013a).

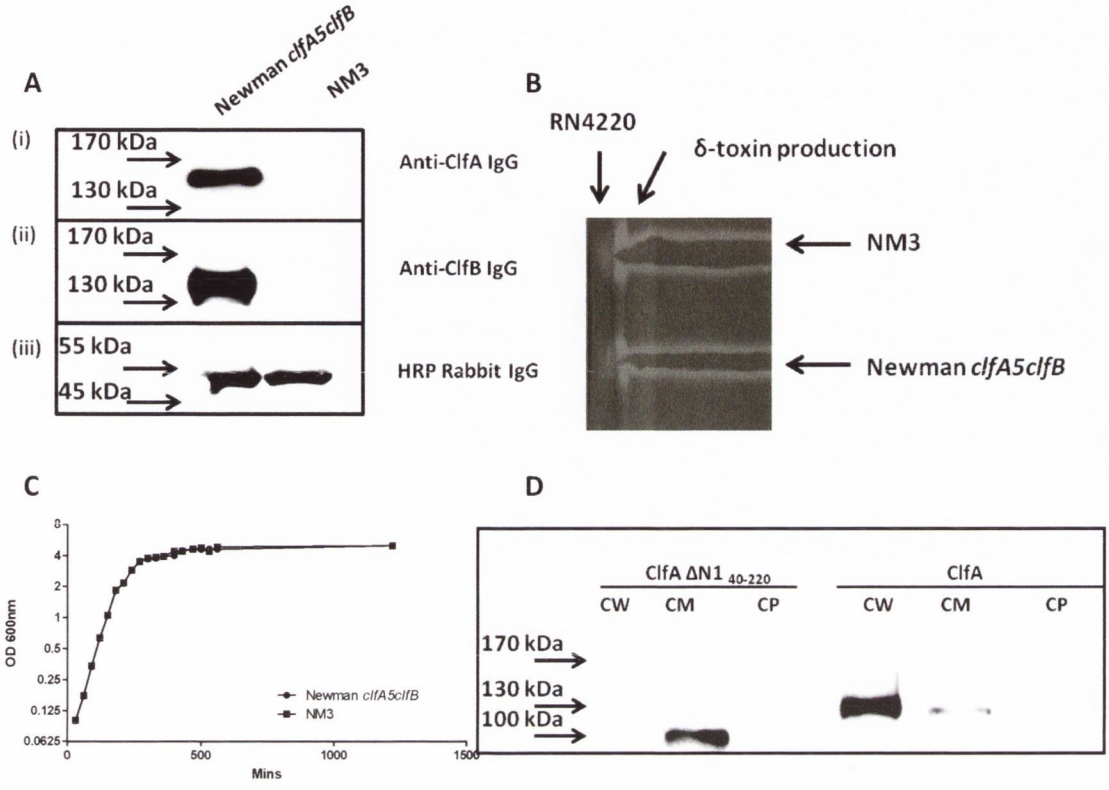


Figure 4.14 Construction and validation of strain NM3

(A) Western immunoblotting of cell wall fractions of strain Newman *clfA5clfB* and NM3 probed with polyclonal anti-ClfA IgG, polyclonal anti-ClfB IgG or HRP-conjugated rabbit IgG. Bound antibodies were detected with HRP-conjugated anti-rabbit IgG and exposure to a chemiluminescent substrate. **(B)** *S. aureus* NM3 and its parental strain Newman *clfA5clfB* were cross-streaked against RN4220 on sheep blood agar plates in order to compare the haemolytic pattern. Enhanced haemolysis due to δ-toxin is indicated with black arrows. **(C)** Growth curve comparing NM3 and its parental strain Newman *clfA5clfB*. **(D)** Cellular fractions from NM3 expressing ClfAΔN1$_{40\text{-}220}$ or ClfA. The blots were probed with ClfA mAb and detected with HRP-conjugated rabbit anti-mouse IgG. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions are labelled.

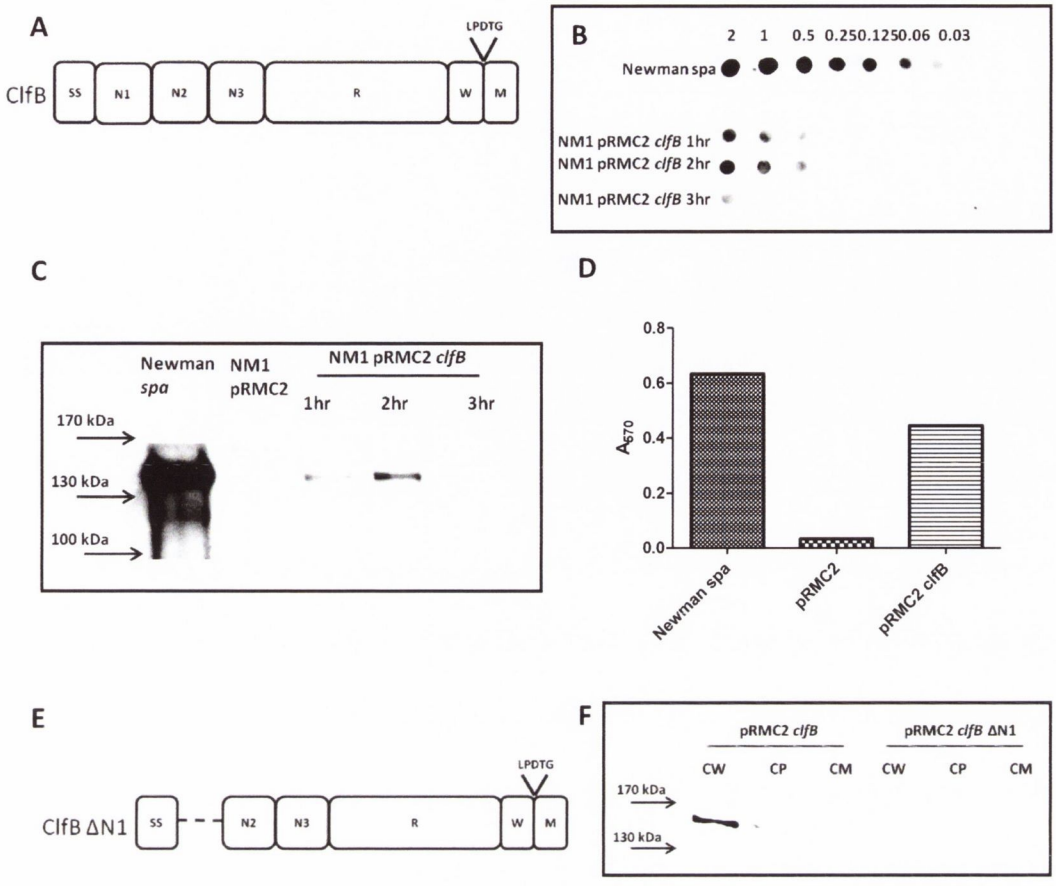


Figure 4.15 Construction and validation of pRMC2*clfB*

(A) Schematic representation of ClfB **(B)** Whole cell dot immunoblot of NM1 expressing ClfB induced with 1.2 µg/ml ATc after 1hr, 2hr or 3hr. Newman *spa* cells from the exponential phase of growth were used as a positive control for ClfB. NM1 (pRMC2) was used as a negative control. **(C)** Western immunoblot of cell wall fractions from (B) separated by SDS-PAGE. Blots in B and C were probed with polyclonal rabbit anti-ClfB IgG and protein A-peroxidase. **(D)** Bacterial adherence to immobilised human cytokeratin 10 by NM1 (pRMC2*clfB*) induced with 1.2 µg/ml ATc. Newman *spa* and NM1 (pRMC2) were used as positive and negative controls. Adherent cells were detected by staining with crystal violet and the A_{570} measured. **(E)** Schematic representation of ClfB $\Delta N1_{48\text{-}200}$. **(F)** Cellular fractions of NM1 (pRMC2*clfB*) and NM1 (pRMC2*clfB*$\Delta N1_{48\text{-}200}$) induced with 1.2 µg/ml ATc. The blot was probed with polyclonal anti-ClfB IgG and protein A-peroxidase. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions are labelled.

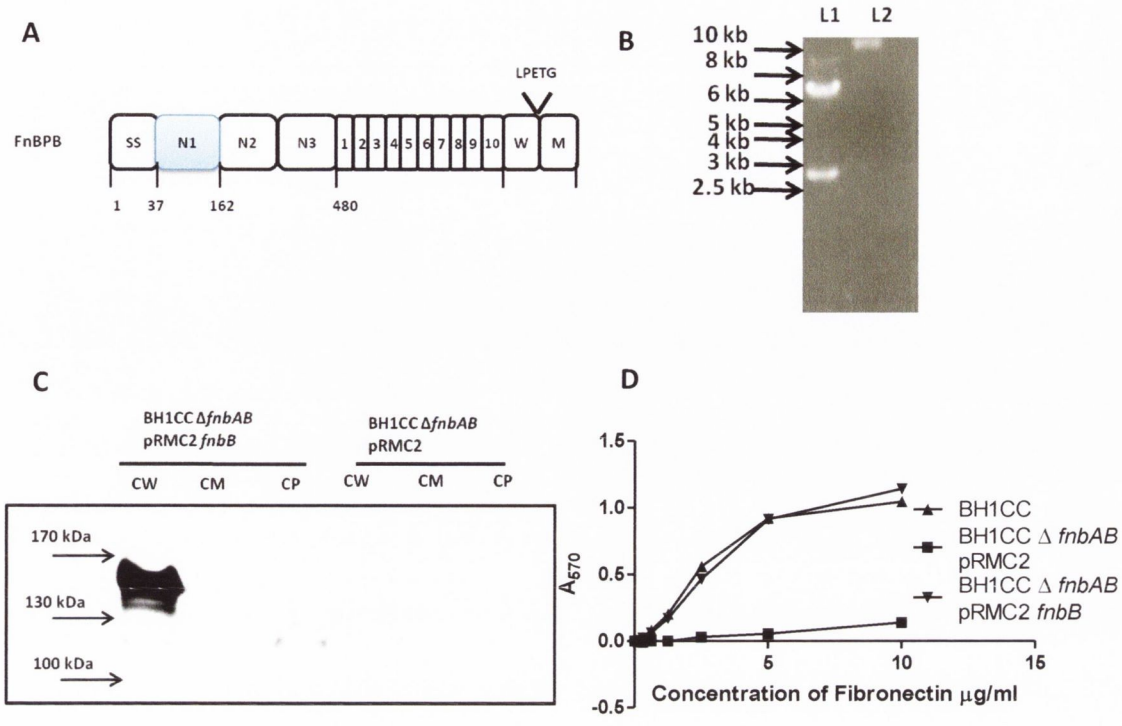


Figure 4.16 Construction and validation of pRMC2*fnbpB*

(A) Schematic representation of FnBPB. **(B)** (L1) agarose gel electrophoresis of restriction digest of pRMC2*fnbB* cleaved with EcoRI and BglII (L2) uncut pRMC2 *fnbB*. **(C)** Ligand affinity blot analysis of cellular fractions from BH1CC Δ*fnbAB* (pRMC2*fnbB*) and BH1CC Δ*fnbAB* (pRMC2) induced with 1 μg/ml ATc, probed with biotinylated fibronectin and detected with HRP-conjugated streptavadin. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions are labelled. **(D)** Bacterial adherence to immobilised fibronectin by cells expressing FnBPB from pRMC2 induced with 1 μg/ml ATc. Exponential phase cells of BH1CC were used as a positive control. BH1CC Δ*fnbAB* (pRMC2) was used as a negative control. Adherent cells were detected by staining with crystal violet and the A_{570} measured. Data points represent the mean of triplicate wells. The graph is representative of three experiments performed on different days.

Cellular fractions of BH1CC Δ*fnbAB* (pRMC2 *fnbB*) induced with 1 µg/ml ATc were probed with biotinylated fibronectin and bound ligand was detected with HRP-conjugated streptavadin. Biotinylated fibronectin was used as antibodies raised against the A region were not available. BH1CC Δ*fnbAB* (pRMC2) was used as a negative control. A reactive band of ~160 kDa corresponding to the size of full length FnBPB was detected in the cell wall fraction of BH1CC Δ*fnbAB* (pRMC2*fnbB*) (Fig 4.16C). No reactive bands were detected in the BH1CC Δ*fnbAB* (pRMC2) control. BH1CC Δ*fnbAB* (pRMC2*fnbB*) induced with 1 µg/ml ATc was tested for adherence to immobilized fibronectin and compared to exponential phase BH1CC. BH1CC Δ*fnbAB* (pRMC2*fnbB*) could adhere to fibronectin in a dose responsive and saturable manner at levels similar to BH1CC. BH1CC Δ*fnbAB* (pRMC2) did not adhere to fibronectin (Fig 4.16D).

4.2.10 Construction and validation of pRMC2*fnbB*ΔN1$_{38\text{-}162}$

In order to determine if the N1 subdomain is necessary for correct localisation of FnBPB plasmid pRMC2*fnbB* was manipulated by inverse PCR to remove the entire N1 subdomain spanning residues 38-162 to yield pRMC2*fnbB*ΔN1$_{38\text{-}162}$ (FIG 4.17A). Loss of DNA encoding the N1 subdomain was validated by cutting with EcoRI and BglII. A band of 6.4 kb corresponds to the size of pRMC2 backbone and the band at 2.3 kb corresponds to the size of the *fnbB* gene lacking DNA encoding the N1 subdomain (FIG 4.17B). Plasmids were transformed into BH1CCΔ*fnbAB*. Cell wall, membrane and cytoplasmic fractions were probed for FnBPB expression in a ligand affinity blot using biotinylated fibronectin. FnBPBΔN1$_{38\text{-}162}$ was not detected in the cell wall fraction. Reactive bands with a molecular weight of ~120 kDa were observed in the cell membrane and cytoplasm fractions (Fig 4.17C). This indicates that the N1 subdomain of FnBPB is required for cell wall localization of the protein.

4.2.11 Construction and subcellular localization of FnBPBΔN1 variants

In an attempt to restore surface expression of FnBPB lacking the N1 subdomain, in a similar strategy to ClfA, pRMC2*fnbB* was manipulated to yield pRMC2*fnbB*ΔN1$_{38\text{-}138}$ encoding a variant of FnBPB lacking most of the N1 domain but containing residues at the C-terminus of the N1 subdomain (Fig 4.18A). Cellular fractions of BH1CCΔ*fnbAB* (pRMC2*fnbB*ΔN1$_{38\text{-}138}$) were probed with biotinylated fibronectin and detected with HRP-conjugated streptavidin. Interestingly, FnBPBΔN1$_{38\text{-}138}$ was not detected in the cell wall, however reactive bands of similar molecular weight to

FnBPBΔN1$_{38\text{-}162}$ were detected in the membrane and cytoplasm fractions (Fig 4.18B). This suggests that this region is not sufficient to mediate surface expression of FnBPB.

To determine whether residues 211-220 of ClfA could mediate cell wall-localization of an FnBPB variant lacking N1 these residues were inserted between the signal sequence and the beginning of the N2 domain. 5′phosphorylated primers for inverse PCR were designed with additional flanking sequences containing five codons on each primer. This generated a ten residue linker region consisting of the "PRMRAFSLAA" motif of ClfA between the signal sequence and the first residue of the N2 subdomain in place of the N1 subdomain (FnBPBΔN1$_{38\text{-}162+ClfA211\text{-}220}$, Fig 4.19A). Cellular fractions of BH1CCΔ*fnbAB* (pRMC2*fnbB*ΔN1$_{38\text{-}162+ClfA211\text{-}220}$) were probed with biotinylated fibronectin and detected with HRP-conjugated streptavidin. This construct was not detected in the cell wall suggesting that these residues are unable to mediate surface expression of FnBPB (Fig 4.19B).

Plasmid pRMC2*fnbB* was manipulated by inverse PCR to remove DNA encoding most of the N1 subdomain leaving additional residues at the C-terminus of the N1 domain (pRMC2*fnBPB*ΔN1$_{38\text{-}134}$, Fig 4.19A). Cellular fractions of BH1CCΔ*fnbAB* (pRMC2*fnbB*ΔN1$_{38\text{-}134}$) were probed with biotinylated fibronectin and detected with HRP-conjugated streptavidin. As with the FnBPBΔN1$_{38\text{-}138}$ variant, FnBPBΔN1$_{38\text{-}134}$ was not detected in the cell wall. However, reactive bands of similar molecular weight to FnBPBΔN1$_{38\text{-}138}$ were detected in the membrane and cytoplasm fractions suggesting that these residues are unable to mediate surface expression of FnBPB (Fig 4.19C).

Analysis of the N1 subdomain of FnBPB by Phyre 2 predicted that residues 132-148 have an α-helical secondary structure similar to ClfA (Fig 4.20A). However, unlike ClfA two distinct additional regions of the N1 subdomain of FnBPB at residues 65-73 and 115-118 are also predicted to be α-helical. Since residues 135-162 are not sufficient to mediate surface expression of FnBPB it is possible that one or more of these regions of the N1 subdomain are also necessary.

Intriguingly, in a different study with a related protein to FnBPB a plasmid constitutively expressing an FnBPA N1 deletion mutant could not be established in *S. aureus* until a short region at the C-terminus of the N1 subdomain was restored (Geoghegan *et al.*, 2013b). Phyre 2 analysis predicted that the N1 subdomain of FnBPA has an α-helical secondary structure in this region (Fig 4.20B).

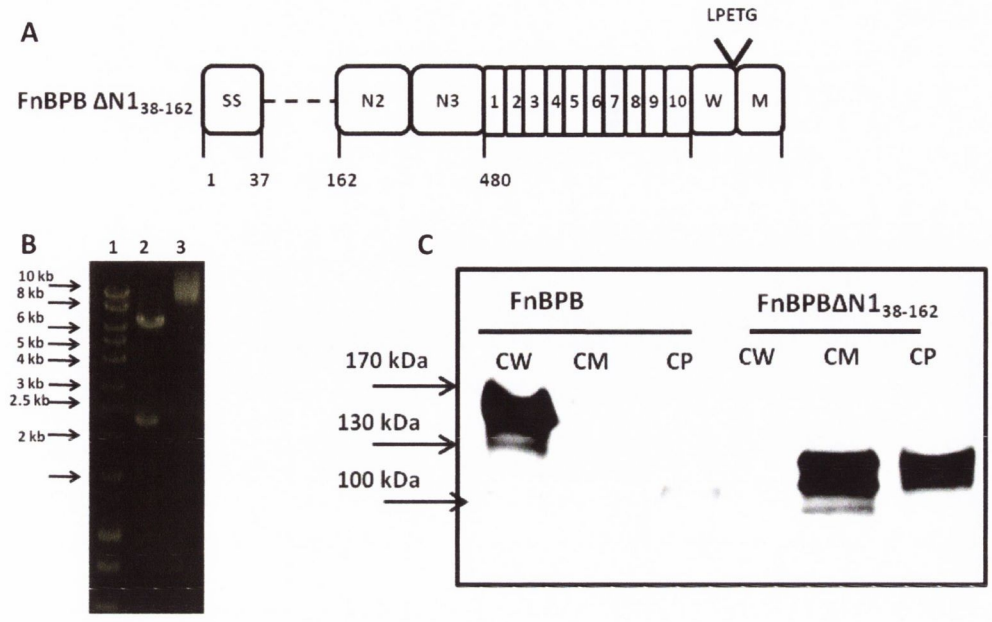


Figure 4.17 Construction, validation and localisation of FnBPBΔN1$_{38\text{-}162}$

(A) Schematic representation of FnBPBΔN1$_{38\text{-}162}$. **(B)** Agarose gel electrophoresis (1) DNA size marker, (2) pRMC2*fnbB*ΔN1$_{38\text{-}162}$ cleaved with EcoRI and BglII, and (3) uncut pRMC2 *fnbB*ΔN1$_{38\text{-}162}$. **(C)** Western ligand affinity blot of cellular fractions from BH1CC Δ*fnbAB* (pRMC2*fnbB*) and BH1CC Δ*fnbAB* (pRMC2*fnbB*ΔN1$_{38\text{-}162}$) induced with 1 μg/ml ATc, probed with biotinylated fibronectin and detected with HRP-conjugated streptavadin. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions are labelled.

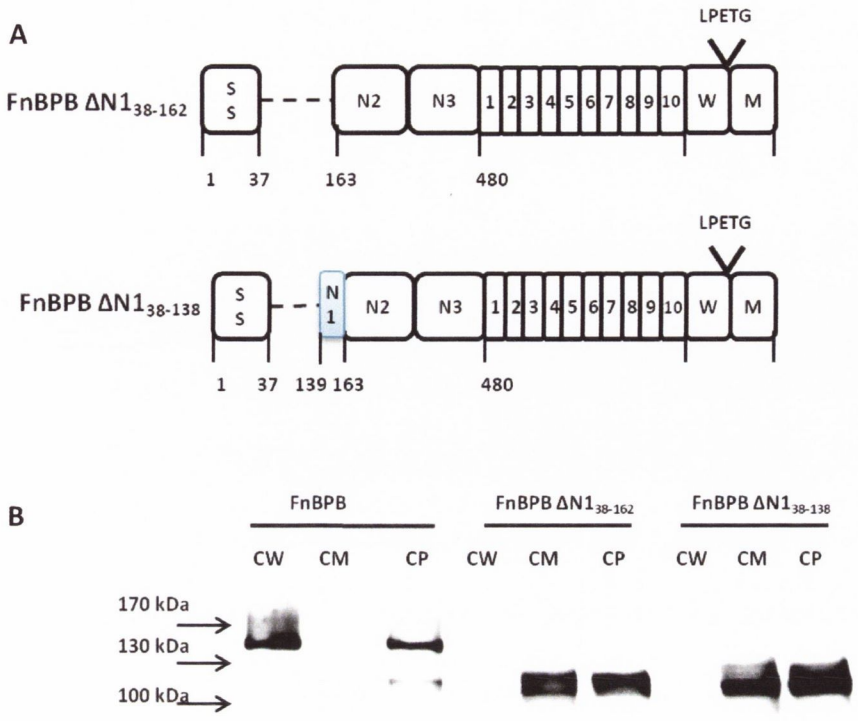


Figure 4.18 Subcellular localisation of FnBPBΔN1$_{38-162}$ and FnBPBΔN1$_{38-138}$

(A) Schematic representations of FnBPBΔN1$_{38-162}$ and FnBPBΔN1$_{38-138}$. **(B)** Cellular fractions from *S. aureus* expressing FnBPB, FnBPBΔN1$_{38-162}$ or FnBPBΔN1$_{38-138}$ induced with 1 μg/ml ATc probed with biotinylated fibronectin and detected with HRP-conjugated streptavadin in a ligand affinity blot. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions are labelled.

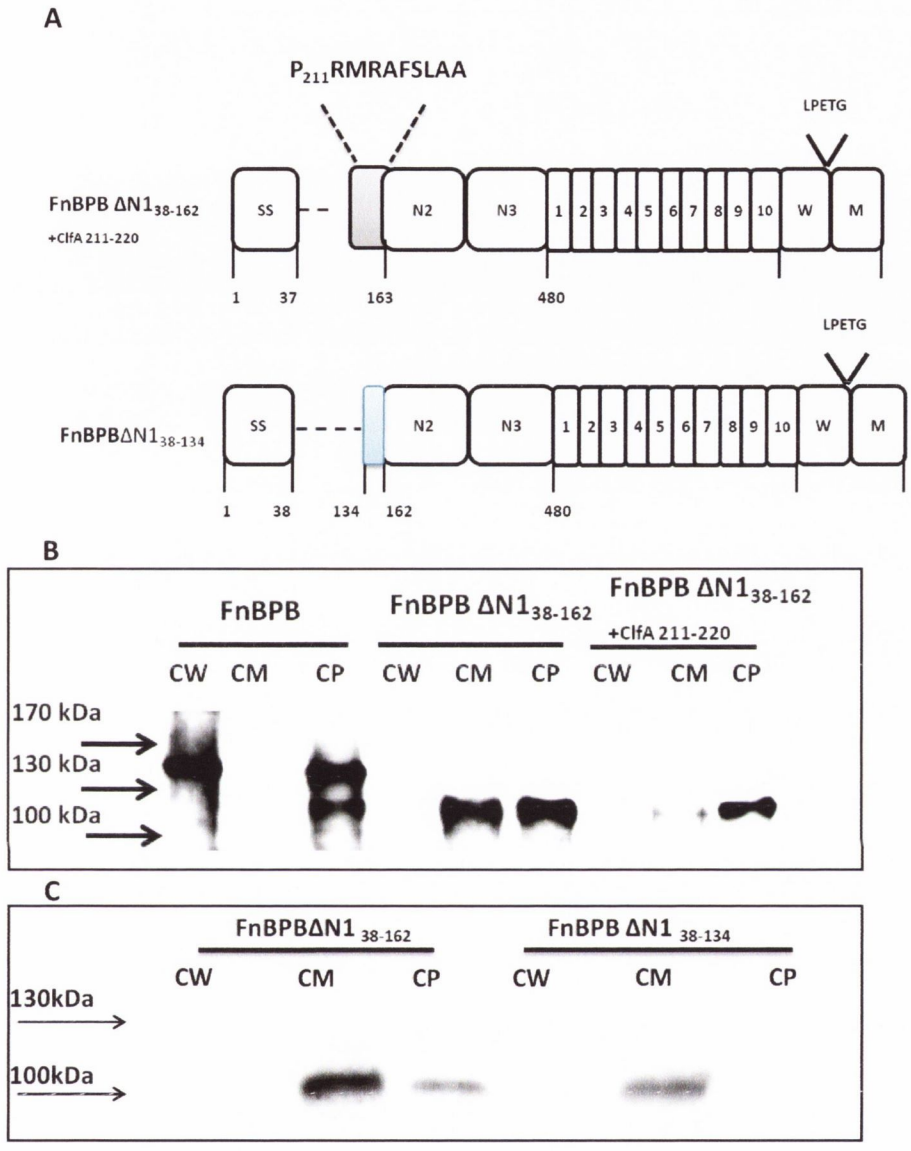


Figure 4.19 Subcellular localisation of FnBPBΔN1$_{38-162+ClfA211-220}$ and FnBPBΔN1$_{38-134}$

(A) Schematic representations of FnBPBΔN1$_{38-162+ClfA211-220}$ and FnBPBΔN1$_{38-134}$. Western ligand affinity blot of cellular fractions from **(B)** *S. aureus* expressing FnBPB, FnBPB ΔN1$_{38-162}$ or FnBPB ΔN1$_{38-162+ClfA211-220}$ induced with 1 μg/ml ATc. **(C)** *S. aureus* expressing FnBPBΔN1$_{38-162}$ or FnBPBΔN1$_{38-134}$ induced with 1 μg/ml ATc. Blots in B and C were probed with biotinylated fibronectin and detected with HRP-conjugated streptavadin. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions are labelled.

Figure 4.20 Phyre 2 prediction of secondary structure in the N1 subdomains of FnBPB and FnBPA

(A) Phyre 2 prediction of secondary structure of N1 subdomain of FnBPB. **(B)** Phyre 2 prediction of secondary of the N1 subdomain of FnBPA Regions within the signal sequence and the C-terminal region of the N1 subdomain that are predicted to have α-helical secondary structures are indicated by the green helices.

4.2.12 Subdomain N1 is not required for cell wall-localization of FnBPB lacking the long unstructured repeat region.

The N1 subdomain of ClfA was not necessary for correct localisation of the pre-protein in the absence of the long unstructured Ser-Asp repeat region. To determine if the N1 subdomain is necessary for export of FnBPB in the absence of the fibronectin binding repeat regions, pRMC2*fnbB*ΔN1$_{38\text{-}162}$ was manipulated to yield pRMC2*fnbB*ΔN1$_{38\text{-}162}$ΔFnBR$_{481\text{-}811FLAG}$. A FLAG epitope tag was introduced by primer extension during PCR to facilitate detection of the protein in the fibronectin binding repeat region (FnBR) (Fig 4.22A). FnBPBΔN1$_{38\text{-}162}$ΔFnBR$_{481\text{-}811FLAG}$ has a predicted molecular weight of ~50 kDa. To avoid interference by protein A in Western immunoblotting, the *spa*::Ka^R cassette from Newman *spa* was transduced into strain BH1CC Δ*fnbAB* to generate strain BH1CC Δ*fnbAB spa*::Ka^R (Table 2.1). Dot immunoblotting with HRP-conjugated rabbit-IgG was performed to confirm the loss of protein A (Fig 4.21A). A PCR colony screen using primers directed against the *fnbAB* region was performed to confirm the BH1CC Δ*fnbAB* background (800 bp), BH1CC was used as a positive control for the *fnbAB* region (7 kb) (Fig 4.21B). A growth curve was performed to ensure no growth defect was associated with acquisition of the *spa*::Ka^R cassette (Fig 4.21C). Strain BH1CC Δ*fnbAB spa*::Ka^R was transformed with pRMC2 *fnbB*, pRMC2*fnbB*ΔN1$_{38\text{-}162}$, pRMC2*fnbB*ΔN1$_{38\text{-}162}$ΔFnBR$_{481\text{-}811FLAG}$ for expression analysis.

Cellular fractions of BH1CC Δ*fnbAB spa*::Ka^R (pRMC2*fnbB*) and (pRMC2*fnbB*ΔN1$_{38\text{-}162}$) were probed with biotinylated fibronectin and HRP-conjugated streptavidin to confirm the subcellular localisation of FnBPB and FnBPBΔN1$_{38\text{-}162}$. As was observed in BH1CC Δ*fnbAB*, FnBPB was localised to the cell wall fraction. However, FnBPBΔN1$_{38\text{-}162}$ was confined to the cytoplasm and cytoplasmic membrane (Fig 4.22B). When probed with monoclonal anti-FLAG IgG and HRP-conjugated rabbit anti-mouse IgG cellular fractions of BH1CC Δ*fnbAB spa*::Ka^R (pRMC2*fnbB*ΔN1$_{38\text{-}162}$ΔFnBR$_{481\text{-}811FLAG}$) revealed a reactive band with a molecular weight of ~60 kDa in the cell wall fraction only (Fig 4.22C). This demonstrates that the N1 subdomain is not required for export or cell wall localization of FnBPB lacking the flexible fibronectin binding repeat region.

In Fig 4.12B cross reaction was observed between the anti-FLAG IgG and ClfA expressed from pRMC2. To determine whether the reactive band identified in the cell

wall fraction of BH1CC Δ*fnbAB spa*::Ka^R(pRMC2*fnbB*Δ$N1_{38\text{-}162}$Δ$FnBR_{481\text{-}811FLAG}$) was due to an artefact of the experiment pRMC2*fnbB*, pRMC2 and pRMC2*fnbB*Δ$N1_{38\text{-}162}$Δ$FnBR_{481\text{-}811FLAG}$ were transformed into strain NM1 lacking ClfA. Cellular fractions of NM1 (pRMC2*fnbB*), (pRMC2) and (pRMC2*fnbB*Δ$N1_{38\text{-}162}$Δ$FnBR_{481\text{-}811FLAG}$) were probed with monoclonal anti-FLAG IgG and no cross reaction was detected for either NM1 (pRMC2*fnbB*) or NM1 (pRMC2).

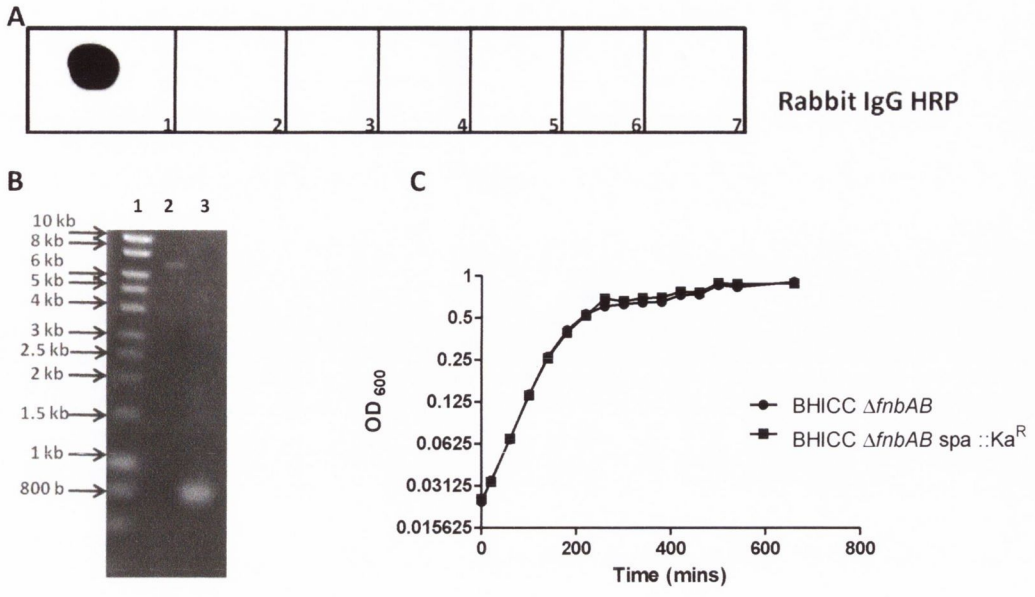


Figure 4.21 Construction and validation of BH1CC Δ*fnbAB spa*::Ka^R

(A) Whole cell dot immunoblot of BH1CC Δ*fnbAB spa*::Ka^R (panels 3-7). BH1CC Δ*fnbAB* was included as a positive control for protein A expression (panel 1). NM1 was included as a negative control (panel 2). A suspension of cells of OD 1 (5 µl) of each strain was spotted on a membrane. Blots were probed with HRP-conjugated rabbit IgG. **(B)** Agarose gel electrophoresis of PCR colony screen for the *fnbA* and *fnbB* genes using primers to amplify the *fnbAB* region. (1)DNA size marker 1, (2) BH1CC, (3) BH1CC Δ*fnbAB spa*::Ka^R. **(C)** Growth curve comparing BH1CCΔ*fnbAB* and BH1CC Δ*fnbAB spa*::Ka^R.

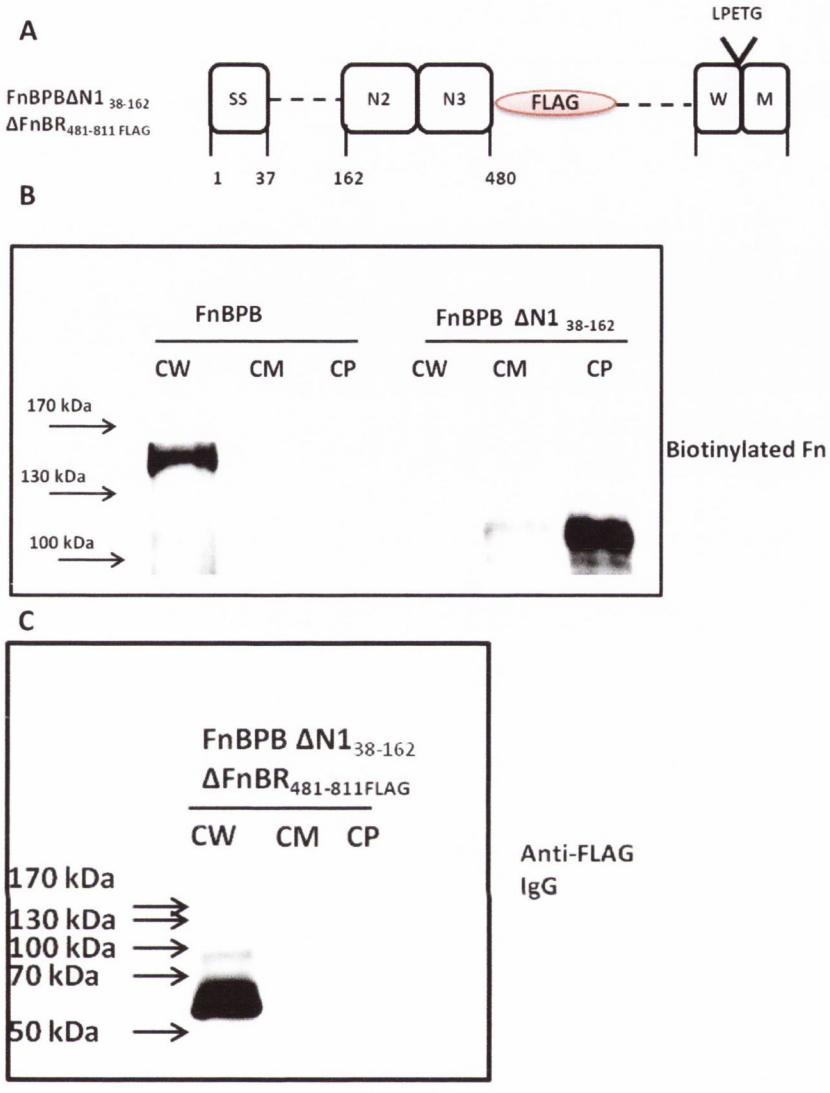


Figure 4.22 Subcellular localisation of FnBPBΔN1$_{38\text{-}162}$ΔFnBR$_{481\text{-}811FLAG}$

(A) Schematic representation of FnBPBΔN1$_{38\text{-}162}$ΔFnBR$_{481\text{-}811FLAG}$. **(B)** Western ligand affinity blot of cellular fractions from BH1CC Δ*fnb*AB *spa*::Ka^R expressing FnBPB or FnBPB ΔN1$_{38\text{-}162}$ induced with 1 µg/ml ATc were probed with biotinylated fibronectin and detected with HRP-conjugated streptavadin. **(C)** Western immunoblot of cellular fractions from BH1CC Δ*fnb*AB *spa*::Ka^R expressing FnBPBΔN1$_{38\text{-}162}$ΔFnBR$_{481\text{-}811}$ probed with monoclonal anti-FLAG IgG and detected with HRP-conjugated rabbit anti-mouse IgG. Cell wall (CW), cell membrane (CM) and cytoplasm (CP) fractions were labelled.

4.3 Discussion

In this Chapter a role for subdomain N1 in export and surface expression of MSCRAMMs was verified. Residues comprising the N1 subdomain of ClfA were shown to be required for the protein to be translocated across the membrane so that it can be anchored by sortase to cell wall peptidoglycan. Deletion mutants lacking N1 (residues 40-220, 42-220 and 40-228) were not exported and instead accumulated in the cytoplasm and the membrane fractions. This could only be detected in bacteria where ClfA expression was tightly regulated because constitutive expression of an N1 deletion mutant following transformation into *S. aureus* resulted in a very low yield of transformants where the plasmid had undergone rearrangements (Chapter 3, Fig 3.18).

Dissection of the N1 subdomain of ClfA revealed that a stretch of 10 residues close to the boundary between N1 and N2 is necessary for surface expression, and that only this region plus the 8 residues that join it to the beginning of N2 as defined by the X-ray crystal structure are necessary. Residues 211-220 could not be substituted by 10 alanines suggesting that they have a specific function. Secondary structure analysis of this region with Phyre 2 revealed that residues 209-228 are predicted to have α-helical structure. It is possible that this structure is required for the function of this region. Surface expression of ClfAΔN1$_{40\text{-}210}$ was lower than wild type ClfA (Fig 3.9), suggesting that perhaps these residues are not optimal for surface expression. The presence of additional residues upstream from 210 may improve surface expression of ClfA ΔN1 variants. However, as ClfAΔN1$_{40\text{-}210}$ was not detected in the cytoplasm or membrane fractions it is also possible that mRNA stability has been affected consequently lowering expression levels.

To determine whether growth of *S. aureus* in ATc affected the cellular localisation of wild type ClfA strain Newman (pRMC2) was subcultured and grown with ATc at the same concentrations used to induce expression from pRMC2. No affect on the level of expression of ClfA or its localisation was observed even at the highest concentration of ATc tested (1.2 μg/ml). This demonstrates that mislocalisation of ClfA variants is not caused by the presence of ATc.

Since expression of ClfAΔN1$_{40\text{-}220}$ affects growth and the protein is mislocalised to the cytoplasm and the membrane, the subcellular localisation of SdrE was examined in cells expressing ClfAΔN1$_{40\text{-}220}$ to investigate if secretion and anchoring of another

MSCRAMM was affected. Cells were trypsinized prior to induction to remove SdrE expressed before the addition of ATc. This allowed the surface expression of SdrE to be examined while ClfAΔN1$_{40\text{-}220}$ was being expressed. Expression of ClfAΔN1$_{40\text{-}220}$ did not affect expression of SdrE indicating that secretion and sorting of the protein was not affected by expression of ClfAΔN1$_{40\text{-}220}$.

A ClfA truncate lacking the Ser-Asp repeat region was exported and anchored to the cell wall in the absence of N1 suggesting that the N1 subdomain is required to aid transport of the long flexible unstructured Ser-Asp repeat region. Intriguingly the N1 subdomain was not sufficient to mediate surface expression of the Ser-Asp repeat region in the absence of the N23 region. This suggests that the N1 subdomain may interact with the N23 region whilst mediating surface expression. However, this result may be an artefact of the experiment due to cross-reaction with the anti-FLAG IgG.

To investigate the mechanism through which subdomain N1 mediates surface expression of ClfA, recombinant GST-tagged ClfA N1 was tested for its ability to bind to immobilised His-tagged ClfAN23. No binding was detected. However, if the N1 subdomain acts a chaperone for the remainder of ClfA by binding the N23 region it might bind to the unfolded N23 region. Recombinant His-tagged ClfA N23 denatured prior to SDS-PAGE and was probed with GST-tagged N1. Although binding was detected this was attributed to non-specific binding of the GST-tag which complicated further investigation.

Since residues 211-220 are necessary for surface expression of ClfA the ability of a His-tagged synthetic peptide comprising residues 211-220 to bind to GST-tagged ClfAN23 was examined. No interaction was detected. However, this does not rule out the possibility that residues 211-228 can bind in this region.

Subdomain N1 is not required for export of ClfA lacking the Ser-Asp repeat region. It is possible that subdomain N1 interacts directly with the Ser-Asp repeat region. This was not investigated in this Chapter but it presents an intriguing avenue for future work.

In *S. gordonii* the surface glycoprotein GspB has been shown to require an accessory signal domain in addition to its signal sequence to direct secretion via the accessory Sec system (Bensing and Sullam, 2010). To determine whether the N1 subdomain is required for surface expression of ClfA because of glycosylation of

residues in the Ser-Asp repeat region, the localisation of ClfAΔN1$_{40\text{-}220}$ was examined in a mutant of *S. aureus* lacking *sdgA* and *sdgB* (NM3). Unglycosylated ClfA was detected predominantly in the cell wall whereas ClfAΔN1$_{40\text{-}220}$ was localised to the cytoplasm and membrane fractions. This demonstrates that subdomain N1 is not required specifically for surface elaboration of glycosylated ClfA. Furthermore, ClfA has never been shown to be secreted via the accessory Sec system and is glycosylated by glycosyltransferases encoded on the *sdr* locus suggesting that this is a distinct mechanism.

This Chapter has demonstrated that the N1 subdomain of ClfA is necessary for surface display of ClfA. We hypothesised that if this is a feature of the MSCRAMM family and not just a property specific to ClfA then the N1 subdomains of other MSCRAMMs will also be required for surface expression. Accordingly deletion of the N1 subdomain of FnBPB prevented surface expression of that protein. Further support comes from an observation that a plasmid constitutively expressing an FnBPA N1 deletion mutant could not be established in *S. aureus* (Geoghegan *et al.*, 2013b). The level of amino acid sequence similarity between the N1 subdomains of the MSCRAMMs is very low so that it is not possible to determine if a similar "motif" is present in this region. Attempts to restore surface expression of FnBPB lacking the N1 subdomain were unsuccessful demonstrating that residues 134-162 are not sufficient for surface expression. Secondary structure analysis of FnBPB by Phyre 2 demonstrated additional regions within subdomain N1 distinct from the C-terminus with predicted alpha helical secondary structure. These regions may be necessary for N1 to mediate surface expression of FnBPB.

A ClfA truncate lacking the Ser-Asp repeat region was exported and cell wall anchored in the absence of N1 suggesting that the N1 subdomain is required to aid transport of the long flexible repetitive Ser-Asp repeat region. Interestingly, a FnBPB truncate lacking both the flexible FnBR region and the N1 subdomain was also cell wall anchored. These data suggest that the N1 subdomain is necessary for exporting MSCRAMMs with a complex structure containing IgG-like folds followed by a long unstructured repeat region.

In conclusion this Chapter shows an important function for the N1 subdomain in surface elaboration of the MSCRAMMs ClfA and FnBPB. The mechanism by which N1 supports export of the MSCRAMMs is not yet understood. This Chapter suggests

that the N1 subdomain of the MSCRAMM family of surface proteins contains an additional export domain required for appropriate surface display of the protein. This study is the first of its kind to indicate that additional domains, distinct from the signal sequence, are necessary in *S. aureus*. It is possible that the N1 subdomain acts as a cytoplasmic chaperone by directly binding to a region of the protein. Perhaps its role is to prevent the N2N3 subdomains folding prematurely into globular IgG-like folds or in aiding the formation of a hairpin loop to facilitate export by Sec. The Sec translocon facilitates the export of unfolded pre-proteins and would not tolerate the large folded IgG-like domains. However, CNA contains subdomains which form IgG-like folds but does not contain a region adjacent to the signal sequence of unknown structure and function. It is possible that in the absence of a long flexible repeat region it does not require a dedicated export domain.

Alternatively the N1 subdomain may engage an additional cytoplasmic factor which guides the long pre-protein from the ribosome to the Sec translocation apparatus. Recently ClfA has been shown to bind ClpC, a molecular chaperone. Although the biological significance of this interaction has not been investigated it is possible that subdomain N1 engages ClpC as a chaperone to ensure safe passage of the pre-protein to the Sec apparatus. Alternatively, the N1 subdomain may engage SecA or other components of the Sec machinery directly to facilitate export.

Chapter 5

Investigating the interaction between staphylococcal surface proteins and complement regulatory factors.

5.1 Introduction

Clumping Factor A (ClfA) is an important MSCRAMM of *S. aureus*. Its ability to bind human fibrinogen is well characterized (McDevitt *et al.*, 1995). ClfA binds fibrinogen by the 'dock, lock, latch' mechanism (Geoghegan *et al.*, 2010, Ganesh *et al.*, 2008). In the proposed mechanism for binding, the C-terminal residues of the γ-chain of fibrinogen dock in the trench formed between the independently folded N2 and N3 subdomains. When the ligand binds, a flexible extension of the C-terminus of the N3 subdomain is reoriented to encase the ligand peptide, locking it in place and then forms a β-strand which is complementary to a β-sheet of the N2 domain, creating the latch.

A second fibrinogen binding site was discovered in the ClfA A region. ClfA has a higher affinity for whole fibrinogen than for the γ-chain peptide. The γ-chain peptide mimics the C-terminal fibrinogen residues that bind to the trench in 'dock, lock, latch'. This observation, coupled with the fact that binding of recombinant ClfA A region to fibrinogen could only be inhibited by 60-70% by the fibrinogen γ-chain peptide suggested the presence of a second fibrinogen binding site in the A domain of ClfA. Conversely fibronectin binding protein A (which also binds to the γ-chain of fibrinogen) could be inhibited to 100% by the fibrinogen γ-chain peptide (Wann *et al.*, 2000). The second binding site was mapped to the top of the N3 subdomain and forms part of the epitope of the function blocking monoclonal ClfA antibody 12-9. This antibody inhibited recombinant ClfAN23 binding to fibrinogen, but not to a His-tagged γ-chain peptide. Single alanine substitutions in residues located in this region of the N3 subdomain reduced fibrinogen binding significantly. This led to the proposal of a model where ClfA initially interacts with the extreme C-terminus of the γ-chain of fibrinogen by a variation of the dock, lock, latch mechanism. The second binding site then interacts with a second site within the globular D-domain on fibrinogen (Ganesh VK, (manuscript in preparation)).

Recently a second ligand for ClfA has been discovered. ClfA binds and activates human complement factor I (FI) (Hair *et al.*, 2008), a serine protease which regulates activation of all three complement cascades. Factor I is an 88 kDa multidomain glycoprotein which is modified posttranslationally by proteolytic cleavage to generate a heavy chain (50 kDa) and a light chain (38 kDa) which remain linked by a disulphide bond. The serine protease activity of factor I is located in the light chain. Little is known

about the specific functions of the individual domains in the heavy chain. These have been named based upon their predicted structural similarity to other proteins (Tsiftsoglou *et al.*, 2005). The crystal structure of factor I has not yet been elucidated so the overall structure of the protein is also unknown. To modulate complement activation factor I normally requires a cofactor from the Regulators of Complement Activation (RCA) family.

Factor H (FH) is a member of the RCA family. This family of proteins is characterised by the presence of short consensus repeats known as complement control protein (CCP) repeats. Factor H works in two distinct ways to regulate complement activation. Firstly it can accelerate the dissociation of factor Bb from the C3bBb complex, inactivating the alternative pathway C3 convertase. Secondly, factor H recognises C3b that has been inappropriately deposited on non-activating host cell surfaces. Factor H acts as a cofactor for factor I which is then responsible for cleavage to the opsonin C3b (see Fig 1.13), generating iC3b to release C3f. This alters both the conformation and binding affinity of C3b (Janssen *et al.*, 2006). While iC3b is still recognized by receptors on the surface of neutrophils it is no longer capable of binding factor B to form the alternative pathway C3 convertase (See Fig 1.12). As the alternative pathway acts as a positive amplification loop for all of the complement pathways, the generation of a dead-end substrate stymies further opsonization of bacteria and subsequent phagocytosis (Walport, 2001). It is unclear exactly how factor H activates factor I but it is thought that factor H first binds C3b, then factor I binds the C3b-factor H complex (Wu *et al.*, 2009).

C4 binding protein (C4BP) is another fluid phase member of the RCA family. Similarly to factor H, C4BP can accelerate decay of C3 convertases. However, its binding specificity is for C4b making it active against the C3 convertase of the classical and lectin pathways, C4b2a. In addition C4BP can act as a cofactor for factor I to facilitate degradation of the opsonin C4b. Factor I cleaves C4b at two sites in the α' chain to yield C4c and C4d. As yet no biological functions or ligands have been identified for C4c or C4d.

The interaction between ClfA and factor I occurs in the A region of ClfA but the exact location and mechanism of activation of factor I is as yet unclear (Hair *et al.*, 2010b). *S. aureus* expressing a ClfA variant containing two substitutions which

abrogates fibrinogen binding, ClfA P336A Y338S, is less virulent then the wild type *in vivo* (Higgins *et al.*, 2006). *S. aureus* ClfA P336A Y338S was shown to bind factor I more tightly than the wild type ClfA but was unable to activate its serine protease activity (Hair *et al.*, 2010b). This result suggests that fibrinogen and factor I may occupy at least partially overlapping binding sites.

To date, no other surface proteins of *S. aureus* have been shown to bind complement factor I. It has been suggested that ClfA and factor I interact directly although this has not been demonstrated experimentally. Only two viral proteins have ever been shown to manipulate factor I directly. Vaccinia virus and Kaposi's sarcoma-associated Herpes virus express proteins with structural features thought to mimic the CCP repeats of the RCA family capable of directly binding factor I (Mark *et al.*, 2007, Mullick *et al.*, 2005).

In this Chapter the interaction between factor I and ClfA is investigated. The ability of each of the individual subdomains of region A of ClfA in factor I binding is examined. Variants of recombinant ClfA with reduced fibrinogen binding were studied for their ability to bind factor I. Experiments were performed to determine whether the interaction between ClfA and factor I is direct, and attempts to identify potential co-factors for the ClfA-factor I interaction were made. Futhermore, this Chapter demonstrates that the functional redundancy often observed in staphylcocci extends to factor I binding, and that numerous staphylococcal surface proteins are capable of binding other complement regulatory proteins.

5.2 Results

5.2.1 Clumping factor A binds human complement factor I

The A region of ClfA has been shown to bind to and activate complement factor I (Hair *et al.*, 2010a, Hair *et al.*, 2008). To confirm that ClfA could bind factor I in serum ligand affinity blotting was performed. Recombinant GST-tagged ClfAN123$_{40\text{-}559}$ was analysed by SDS-PAGE and stained with Coomassie Brilliant Blue. A band of ~100 kDa was observed. This is consistent with the correct molecular weight for full length undegraded GST-tagged ClfAN123$_{40\text{-}559}$ (Fig 5.1A(i)). GST-tagged ClfAN123$_{40\text{-}559}$ analysed by SDS-PAGE was transferred to a PVDF membrane which was incubated with 10% normal human serum and probed for factor I binding with monoclonal anti-factor I IgG. A band corresponding to the size of GST-tagged ClfAN123$_{40\text{-}559}$ was detected demonstrating that ClfAN123$_{40\text{-}559}$ can bind factor I in serum (Fig 5.1A(ii)).

In Chapter 3 the N1 subdomain was shown to be dispensible for fibrinogen binding. To determine if the N1 subdomain was required for the interaction between ClfA and factor I purified GST-tagged ClfAN123$_{40\text{-}559}$ and ClfAN23$_{221\text{-}559}$ were examined. Proteins were immobilized on a microtitre plate and examined by ELISA for their ability to capture complement factor I from normal human serum. Monoclonal anti-Factor I IgG was used to detect factor I binding in a dose-responsive and saturable manner (Fig 5.1B). Both ClfAN123$_{40\text{-}559}$ and ClfAN23$_{221\text{-}559}$ proteins were capable of binding to factor I with a similar high affinites (half maximum binding of 42 nm and 32 nm respectively). Interestingly ClfAN23$_{221\text{-}559}$ reached saturation before ClfAN123$_{40\text{-}559}$. This difference of binding at saturation may be due to a minor difference in the kinetics of the interaction. However, the N1 subdomain does not appear to be necessary for ClfA binding to factor I. Similar to observations made in Chapter 3 for fibrinogen binding, immobilised His-tagged ClfAN23$_{221\text{-}559}$ was not able to promote factor I binding.

5.2.2 Investigation of the regions within ClfA responsible for factor I binding

To determine which subdomains within the A region of ClfA were responsible for mediating the interaction with factor I, each of the individual subdomains were examined for their ability to capture factor I. DNA encoding each of the individual subdomains of ClfA was cloned into pGEX-4T-2 as described in Chapter 3. To reflect the two different definitions of subdomain N1 two distinct GST-tagged recombinant

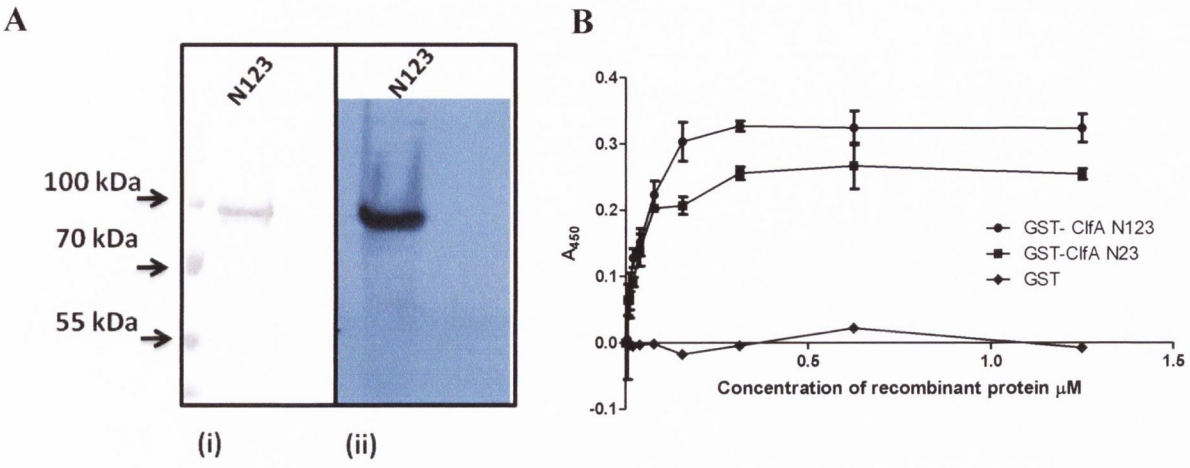


Figure 5.1 Clumping factor A binds human complement factor I

(A) Recombinant GST-tagged ClfA $N123_{40\text{-}559}$ was analysed by SDS-PAGE and (i) stained with Coomassie Brilliant Blue or (ii) transferred to a PVDF membrane, incubated with 10 % normal human serum and probed with monoclonal anti-factor I IgG. Bound anti-factor I IgG was detected with HRP-conjugated rabbit anti-mouse IgG and incubation with a chemiluminescent substrate. **(B)** Investigating the ability of immobilised GST-tagged recombinant ClfA to capture factor I from 10% normal human serum. Wells of a microtitre plate were incubated overnight with 0-1.5 μM recombinant GST-tagged ClfA $N123_{40\text{-}559}$ or ClfA $N23_{221\text{-}559}$. GST was included as a negative control. Immobilised protein was incubated with 10% normal human serum. Bound factor I was detected with monoclonal anti-factor I IgG and HRP-conjugated rabbit anti-mouse IgG. Wells were incubated with a chromogenic substrate and the A_{450} determined. Data points represent the mean of triplicate wells. The graph is representative of three independent experiments.

protein constructs were generated. $N1_{SHORT}$ comprises residues 40-220 and reflects the N1 subdomain as defined by its aureolysin cleavage site. $N1_{LONG}$ comprises residues 40-228 and reflects the N1 subdomain as defined by the crystal structure of the minimum ligand binding domain of $ClfAN23_{229-545}$.

1 μM of each recombinant GST-tagged ClfA subdomain was incubated overnight to coat the surface of a microtitre plate. Immobilised protein was incubated with doubling dilutions of serum. Bound factor I was detected with monoclonal anti-factor I IgG and HRP-conjugated rabbit anti-mouse IgG. None of the individual subdomains could promote binding of factor I (Fig 5.2). This indicates that the N1 subdomain is not required for the interaction between ClfA and factor I and neither individual subdomain can interact with factor I alone.

Fibrinogen binding by the A region of ClfA requires both the N2 and N3 regions to be intact. To determine whether residues which are necessary for fibrinogen binding are important for factor I, binding amino acid substitutions which were originally isolated to map the fibrinogen binding sites in ClfA were utilised (Ganesh VK, (manuscript in preparation)). DNA encoding the $ClfAN23_{221-559}$ variants ClfA Y512A, ClfA I408A, ClfA D481A and ClfA P336S Y338A D321A (ClfAPYD) was cloned into pGEX 4T-2. Figure 5.3A shows a ribbon diagram of the crystal structure of the N23 region highlighting residues important in fibrinogen binding. Proteins were purified to homogeniety and analysed by SDS-PAGE. All GST-tagged $ClfAN23_{221-559}$ variants migrated as a single band at ~60 kDa similar to the wild type GST-tagged $ClfAN23_{221-559}$ (Fig 5.3B). Doubling dilutions of GST-tagged rClfA variants were immobilized on the surface of a microtitre plate. To confirm that all constructs coated the plate with equal efficiency bound protein was detected using polyclonal anti-GST IgG and HRP-conjugated rabbit anti-goat IgG. All constructs coated the plate in a dose-responsive and saturable manner. No difference in the level of immobilized protein was detected (Fig 5.3C).

The recombinant ClfA variants were examined for their ability to capture factor I from serum compared to wild type GST-tagged $ClfAN23_{221-559}$. Wells of microtitre plates were incubated overnight with 0-500 nM of GST-tagged $ClfAN23_{221-559}$ and its variants to coat wells. Immobilised proteins were incubated with 10% normal human serum. Bound factor I was detected with monoclonal anti-factor I IgG. All variants tested showed minor reductions in factor I capture but no significant difference in K_D at

half maximal binding was observed (Fig 5.4 A-D). These results indicate that the ability of ClfA to bind fibrinogen is not necessary for factor I binding. Furthermore they suggest that factor I binding involves distinct residues not involved in the 'dock, lock, latch' mechanism employed by ClfA binding to fibrinogen. However, this does not preclude the possibility that residues that are involved in fibrinogen binding can affect the dissociation rate of bound factor I as this cannot be accurately measured by ELISA.

5.2.3 Investigating the interaction between ClfA and purified factor I.

In order to determine whether ClfA and factor I interact directly, purified factor I was examined for ClfA binding ability. Wells of a microtitre plate were incubated overnight with 1 μM of GST-tagged ClfAN123$_{40\text{-}559}$. Immobilised protein was incubated with 40 μg/ml factor I. 10% normal human serum was used as a positive control for factor I binding binding (Fig 5.5A). Although immobilised ClfAN123$_{40\text{-}559}$ can capture factor I from serum, it cannot bind to purified factor I. Factor I was captured from serum by an immobilised monoclonal antibody and ClfA binding was examined. Wells of a microtitre plate were incubated overnight with monoclonal anti-factor I IgG. Coated wells were incubated with normal human serum to capture factor I on the surface of the plate. Bound factor I was then incubated with GST-tagged ClfAN123$_{40\text{-}559}$. Bound ClfA was detected with HRP-conjugated anti-GST IgG. As a control for factor I capture on the surface of the microtitre plate bound protein was detected with polyclonal goat anti- factor I IgG and HRP-conjugated rabbit anti-goat IgG (Fig 5.5 B). ClfAN123$_{40\text{-}559}$ could not bind to factor I captured from human serum. This result, combined with observations that ClfA cannot bind commercially purified factor I suggests that ClfA does not interact with factor I alone and requires one or more additional cofactors that present in serum.

5.2.4 Investigating potential cofactors for the ClfA factor I interaction

ClfA cannot bind to purified factor I but can capture factor I from human serum. This suggests that additional cofactors present in human serum are required. ClfA is known to bind to fibrinogen and in its natural function regulating complement activation, factor I interacts with factor H, C4BP and C3b. To determine if these proteins are involved as cofactors for factor I and ClfA, factor I was pre-incubated with either fibrinogen, factor H, C3b, C4BP, C3 or fibrinogen and factor H together. The concentrations used represent those that occur in blood. Wells of a microtitre plate were

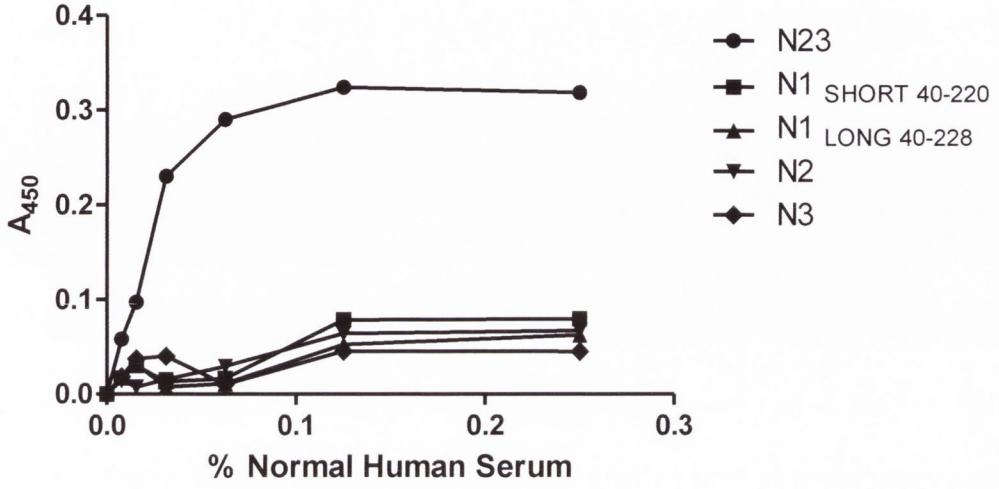


Figure 5.2 Factor I binding by individual subdomains of ClfA.

Recombinant GST-tagged ClfA subdomains were tested for their ability to capture factor I from normal human serum. Wells of a microtitre plate were incubated overnight with 1 μM of each of the recombinant GST-tagged ClfA subdomains. Immobilised protein was incubated with dilutions of normal human serum ranging from 0-2.5 %. GST-tagged ClfA $N23_{221-559}$ was included as a positive control for factor I binding. Bound factor I was detected with monoclonal anti-factor I IgG and HRP-conjugated rabbit anti-mouse IgG. Wells were incubated with a chromogenic substrate and the A_{450} determined. Data points represent the mean of triplicate wells. The graph is representative of three independent experiments.

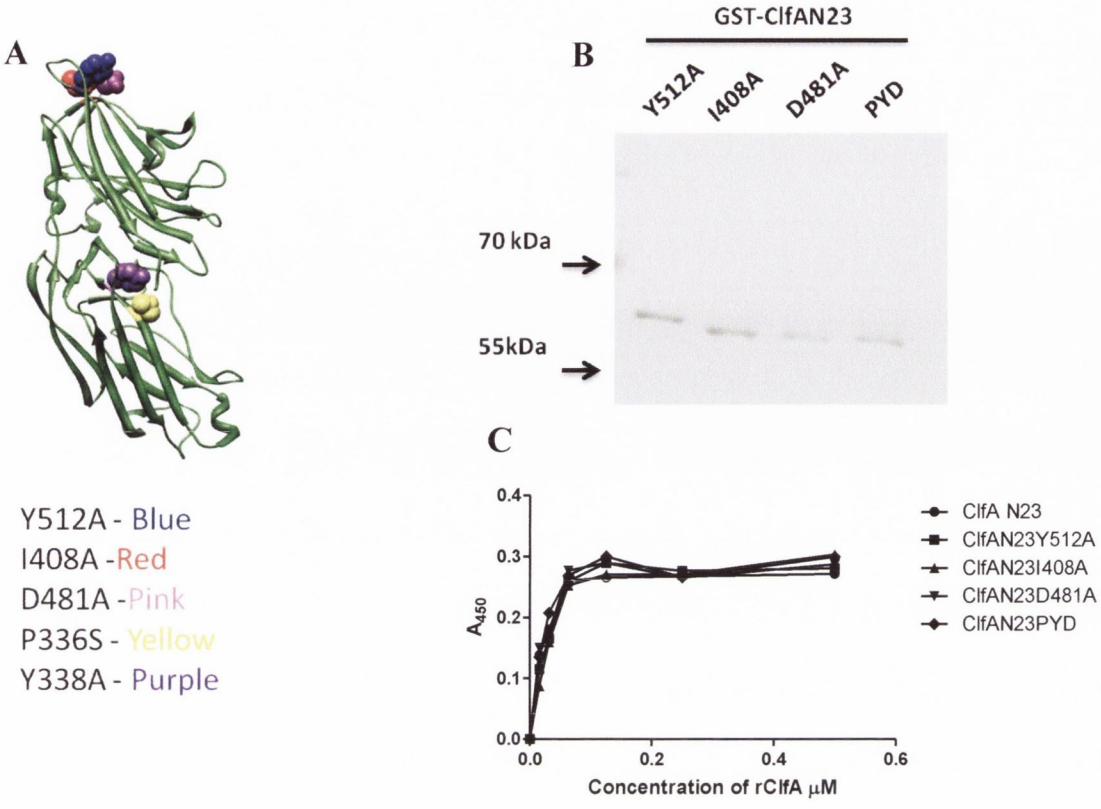


Figure 5.3 Expression of ClfA variants deficient in fibrinogen binding

(A) Ribbon model of the crystal structure of ClfA $N23_{229\text{-}545}$. Residues important for fibrinogen binding in the trench (P336, Y338) and in the second fibrinogen binding site at the top of the N3 subdomain (Y512, I408, D481) are highlighted and amino acid coordinates indicated. **(B)** Purified ClfA variants were analysed by SDS-PAGE and stained with Coomassie Brilliant Blue. L1: ClfAN23Y512A, L2: ClfAN23 I408A, L3: ClfAN23 D481, L4: ClfAN23 P336S, Y338A, D321A. Molecular weight markers are indicated with black arrows. (C) GST-tagged ClfA variants were tested to ensure equal immobilisation on the surface of a microtitre plate. Wells were incubated overnight with 0-500 nM of rClfA. Bound protein was detected with goat anti-GST IgG and HRP-conjugated rabbit anti-goat IgG. Wells were incubated with a chromogenic substrate and the A_{450} determined. Data points represent the mean of triplicate wells and the graph is representative of three independent experiments.

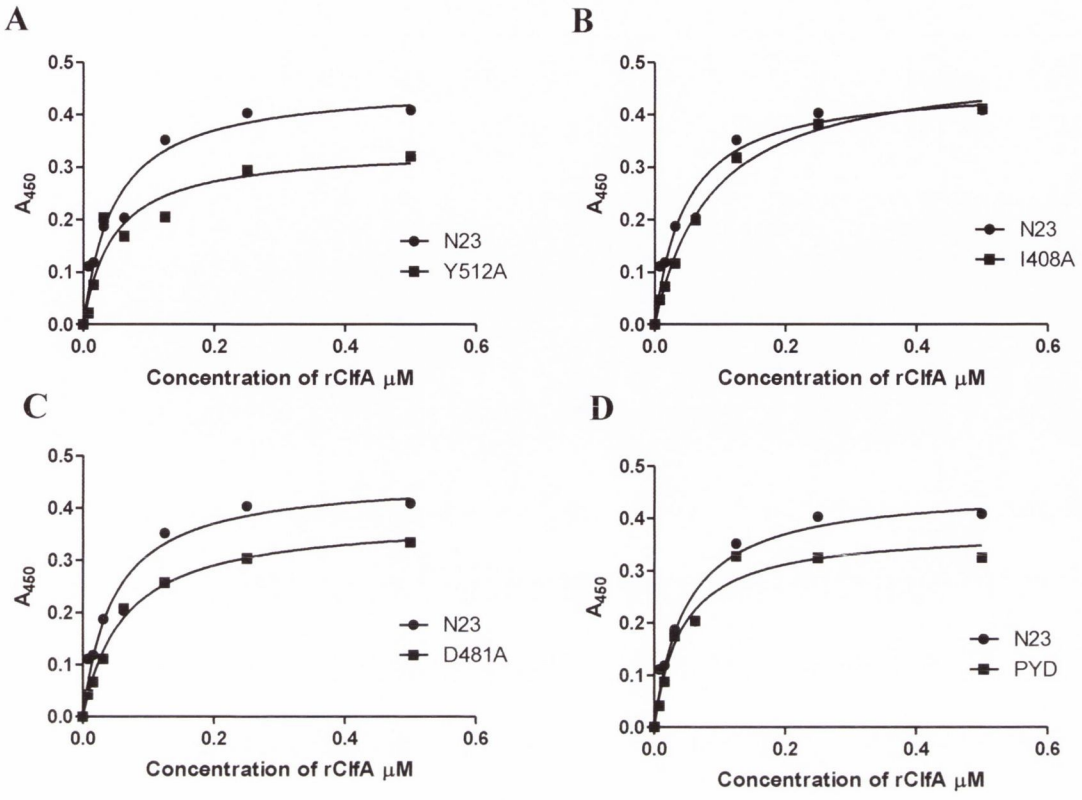


Figure 5.4 Capture of factor I by ClfA variants deficient in fibrinogen binding

Wells were incubated over night with 0-500 nM of recombindant GST-tagged ClfA N23 and **(A)** ClfA Y512A **(B)** ClfA I408A **(C)** D481A and **(D)** ClfA PYD. Wells were incubated with 10% normal human serum. Bound factor I was detected with monoclonal anti-factor I IgG and HRP-conjugated rabbit anti-mouse IgG. Wells were incubated with a chromogenic substrate and the A_{450} determined. Data points represent the mean of triplicate wells. Graphs are representative of three independent experiments

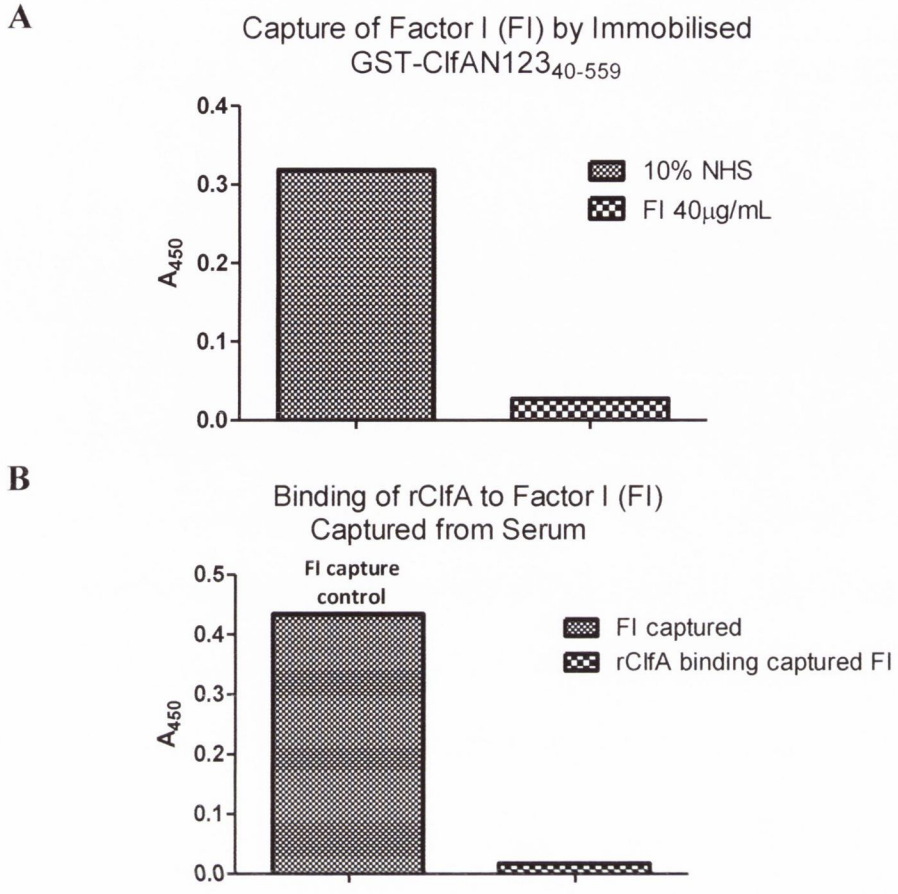


Figure 5.5 Investigating the interaction between recombinant ClfA N123$_{40-559}$ and purified factor I.

(A) Wells were incubated with 1 µM recombinant GST-tagged ClfA N123$_{40-559}$ overnight. Immobilised protein was incubated with either 10% normal human serum or 40 µg/ml purified factor I. Bound factor I was detected with murine monoclonal anti-factor I IgG and HRP-conjugated rabbit anti-mouse IgG. **(B)** Wells were incubated overnight with monoclonal anti-factor I IgG. Wells coated with IgG were incubated with normal human serum to capture factor I followed by 8 µM recombinant GST-tagged ClfA N123$_{40-559}$. Bound ClfA was detected with HRP-conjugated anti-GST IgG. As a control for factor I capture by immobilised anti-factor I IgG bound protein was detected with polyclonal goat anti-factor I IgG and HRP-conjugated rabbit anti-goat IgG. Wells were incubated with a chromogenic substrate and the A_{450} determined. Data points represent the mean of triplicate wells. Graphs are representative of three independent experiments.

incubated overnight with GST-tagged ClfA $N123_{40-559}$ and then incubated with normal human serum, factor I alone or factor I pre-incubated with a potential cofactor (Fig 5.6). Although immobilized ClfA was able to capture factor I from normal human serum, it was not able to interact with factor I which had been pre-incubated with any of the other serum proteins tested. This result suggests that fibrinogen, factor H, C4BP, C3b, C3 or fibrinogen and factor H are insufficient to act as cofactors for factor I binding by ClfA. This does not preclude the possibility that ClfA may interact with these proteins and that more than one cofactor is necessary.

To determine whether ClfA interacts with serum components other than factor I, microtitre plates were incubated overnight with GST-tagged ClfA$N123_{40-559}$. Coated wells were incubated with 10% normal human serum. Bound factor H, fibrinogen or C3 fragments were detected with monoclonal anti-factor H IgG, polyclonal anti-fibrinogen IgG or polyclonal anti-C3 IgG respectively (Fig 5.7A,B,C). ClfA bound all three plasma proteins in a dose responsive and saturable manner.

5.2.5 Studying the interaction of staphylococcal surface proteins with complement factor I

Staphylococcal surface proteins display a high degree of functional redundancy with multiple surface protiens capable of binding to the same ligands. It is possible that staphylococcal surface proteins other than ClfA may also bind factor I. To investigae this, the surrogate host *Lactococcus lactis* was used for heterologous expression of surface proteins from *S. aureus*, *S. lugdunensis* and *S. epidermidis*. The MSCRAMMs ClfA, ClfB, Fbl, SdrC, SdrD, SdrE and SdrG along with the surface protein IsdH were expressed from the constitutive expression vector pKS80. To confirm surface expression of each protein whole cell dot immunoblotting was performed with specific antibodies (Table 2.4). *L. lactis* carrying pKS80 empty vector was used as a negative control. All proteins were detected on the surface of *L. lactis*. Fbl appeared to be expressed at a low level. However, in the absence of specific anti-Fbl IgG, polyclonal anti-ClfA IgG was used to detect Fbl which is only partially cross-reactive (Fig 5.8A). Bacterial adherence to immobilised fibrinogen was tested with cells expressing ClfA, ClfB, Fbl and SdrG. Each was capable of promoting a high level of adherence to immobilised fibrinogen when compared to the negative control (Fig 5.8B). This demonstrates that the fibrinogen binding MSCRAMMs are functionally expressed by *L. lactis*.

To determine if surface proteins could bind factor I whole cell dot ligand affinity blotting was performed on *L. lactis* expressing ClfA, ClfB, Fbl, IsdH, SdrC, SdrD, SdrE or SdrG. Lactococci expressing staphylococcal proteins were spotted onto a membrane and incubated with 10% normal human serum. Bound factor I was detected with monoclonal anti-factor I IgG and HRP-conjugated rabbbit anti-mouse IgG. *L.lactis* expresing ClfA was capable of capturing factor I. This is consistent with observations that recombinant ClfA can bind factor I. Interestingly ClfB, IsdH and SdrD were all capable of binding factor I (Fig 5.9). IsdH has previously been shown to have a role in C3b degradation on the surface of *S. aureus* but factor I binding was not tested (Visai *et al.*, 2009). Interestingly, ClfB interacted strongly with Factor I. Intruigingly although Fbl shares 60% homology with ClfA in the N23 region, it was not able to capture factor I from serum.

To investigate the interactions between factor I and staphylococcal fibrinogen binding MSCRAMMs further equimolar amounts of recombinant His-tagged ClfAN123$_{40\text{-}559}$, Fbl N123$_{40\text{-}553}$ and ClfB N23$_{201\text{-}542}$ proteins were incubated overnight on a microtitre plate and examined by ELISA for their ability to capture human complement factor I from normal human serum. Monoclonal anti-factor I IgG was used to detect factor I binding. Interestingly ClfB bound ~1.5-fold more factor I than ClfA (Fig 5.10). Fbl did not bind factor I which is consistent with the results obtained from whole cell dot ligand affinity blotting.

5.2.6 Investigating the interaction of staphylococcal surface proteins and members of the regulators of complement activation family, factor H and C4BP

Complement factor H is a member of the Regulators of Complement Activation (RCA) family of proteins. In order to inactivate complement that has become inappropriately fixed on host cell surfaces factor H normally recruits factor I to mediate cleavage of C3b. To determine whether staphylococcal surface proteins can bind factor H whole cell dot ligand affinity blotting was performed. *L. lactis* expressing ClfA, ClfB, Fbl, IsdH, SdrC, SdrD, SdrE or SdrG were spotted onto a membrane and incubated with 10% normal human serum. Bound factor H was detected with monoclonal anti-factor H IgG and HRP-conjugated rabbbit anti-mouse IgG (Fig 5.11). Although recombinant ClfA was capable of capturing factor H, *L. lactis* expressing ClfA was not. Of the

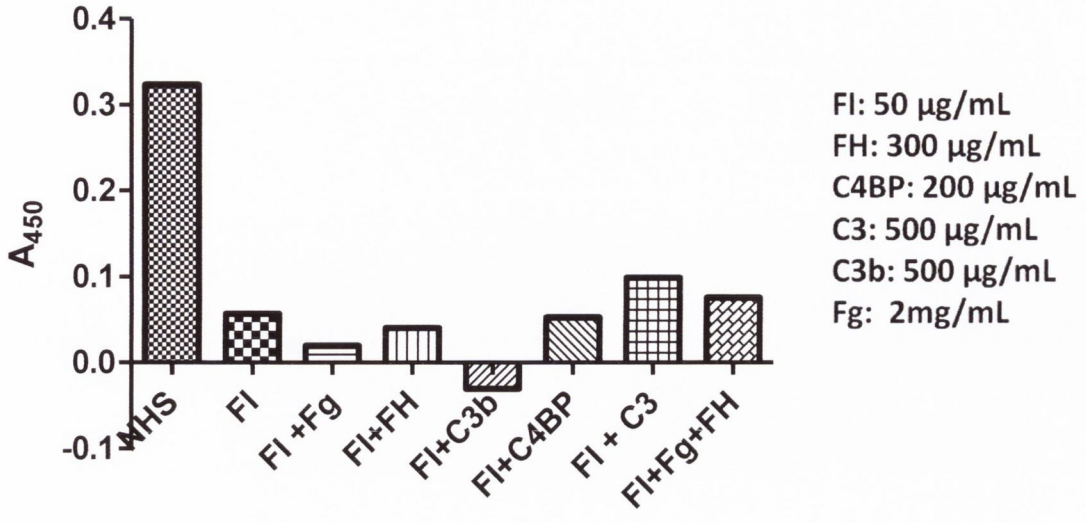


Figure 5.6 Investigating possible cofactors for ClfA and Factor I binding.

Wells of a microtitre plate were incubated overnight with 1 µM of recombinant GST-tagged ClfA $N123_{40\text{-}559}$. Immobilised protein was incubated with 10% normal human serum (NHS), purified factor I or purified factor I co-incubated with either fibrinogen, factor H (FH), C3b, C4 binding protein (C4BP), C3 or factor H and fibrinogen. All proteins were used at physiological blood concentrations listed at the side. Bound factor I was detected with monoclonal anti-factor I IgG and HRP-conjugated rabbit anti-mouse IgG. Wells were incubated with a chromogenic substrate and the A_{450} determined. Data points are the mean of triplicate wells. Graph is representative of three independent experiments.

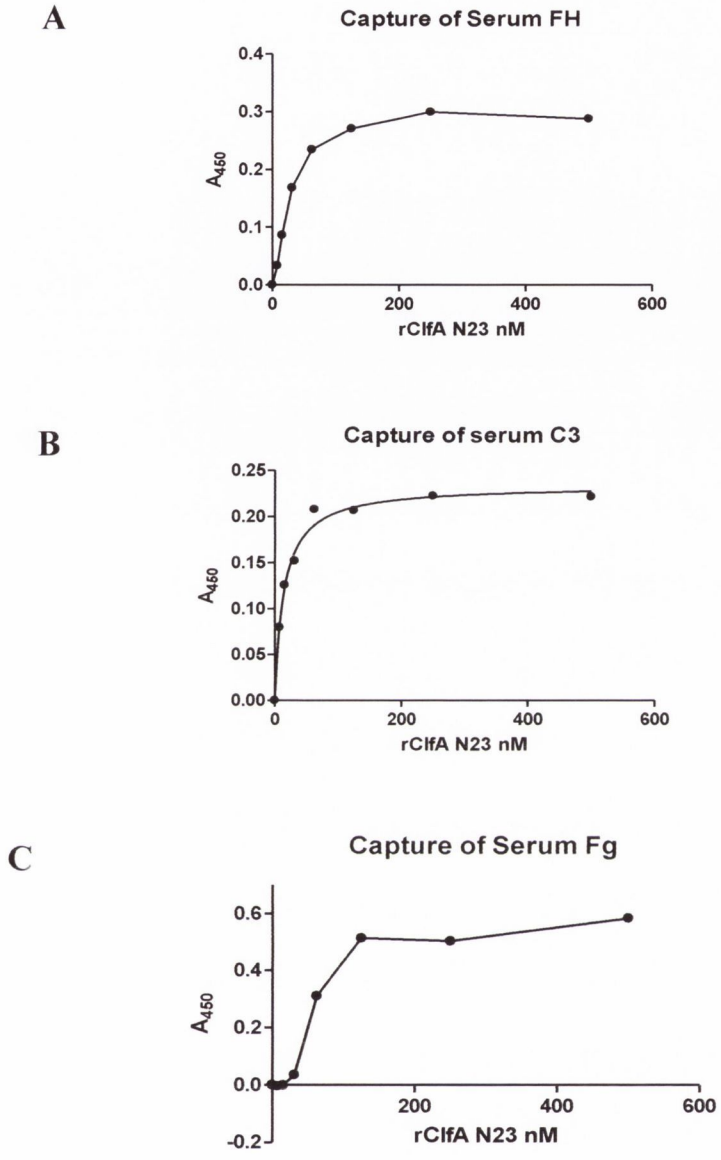


Figure 5.7 Capture of serum components from human serum by immobilized ClfA N23$_{221-559}$.

Wells of a microtitre plate were incubated overnight with 0-500 nM of recombinant GST-tagged ClfAN23$_{221-559}$. Immobilised protein was incubated with 10% normal human serum. **(A)** Bound factor H was detected using monoclonal anti-factor H IgG and HRP-conjugated rabbit anti-mouse IgG. **(B)** Bound C3 was detected by polyclonal anti C3 serum and HRP-conjugated rabbit anti-goat IgG. **(C)** Bound fibrinogen was detected using HRP-conjugated rabbit anti-fibrinogen IgG. Wells were incubated with a chromogenic substrate and the A_{450} determined. Data points represent the mean of triplicate wells. Graphs are representative of three independent experiments.

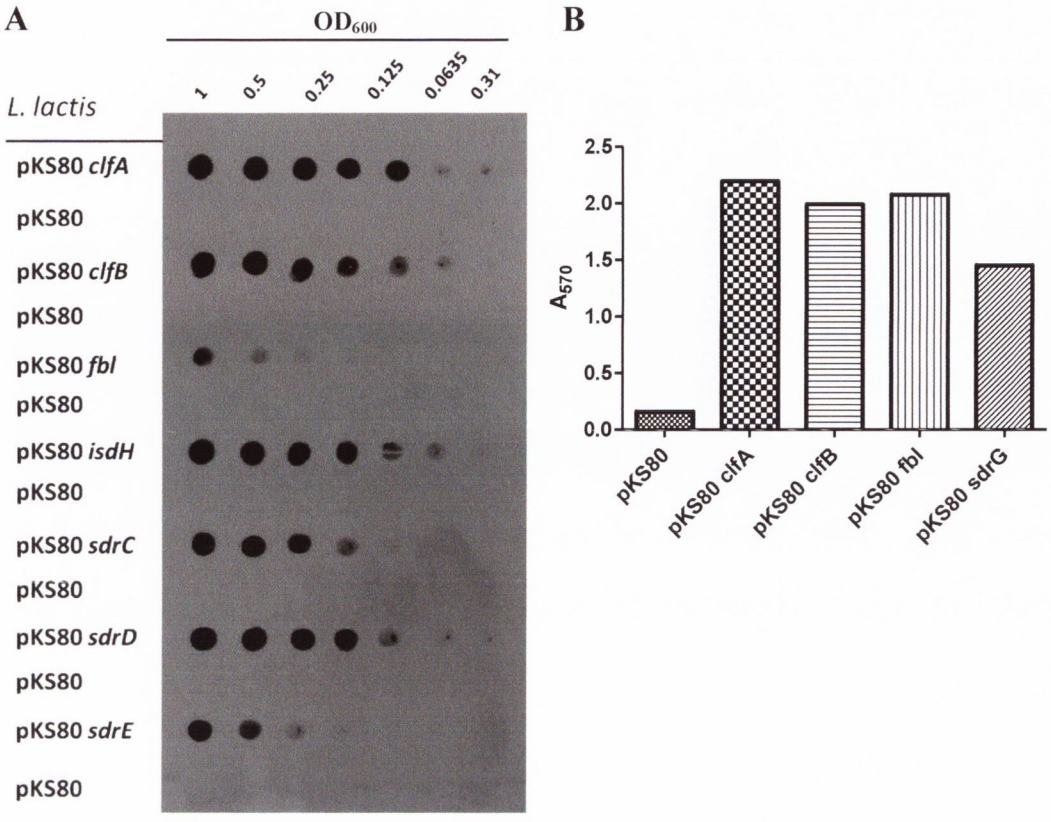


Figure 5.8 Heterologous expression of staphylococcal surface proteins by *Lactococcus lactis*.

(A) Whole cell dot immunoblot of *L. lactis* carrying pKS80 *clfA*, pKS80 *clfB*, pKS80 *fbl*, pKS80 *isdH*, pKS80 *sdrC*, pKS80 *sdrD*, or pKS80 *sdrE*. The OD_{600} of the cells is indicated across the top of the blot. A suspension of cells (5 µl) at each concentration was spotted onto the membrane and probed with specific antibodies. *L. lactis* carrying pKS80 empty vector was used as a negative control. **(B)** Bacterial adhesion to immobilized fibrinogen by *L. lactis* expressing ClfA, ClfB, Fbl or SdrG. Wells of a microtitre plate were incubated overnight with 10 µg/ml human fibrinogen. Coated wells were incubated with a suspension of bacterial cells OD_{600} 1. Adherence to fibrinogen was measured by staining with crystal violet and measuring the A_{570}. Data points represent the mean of triplicate wells. The graph is representative of three independent experiments.

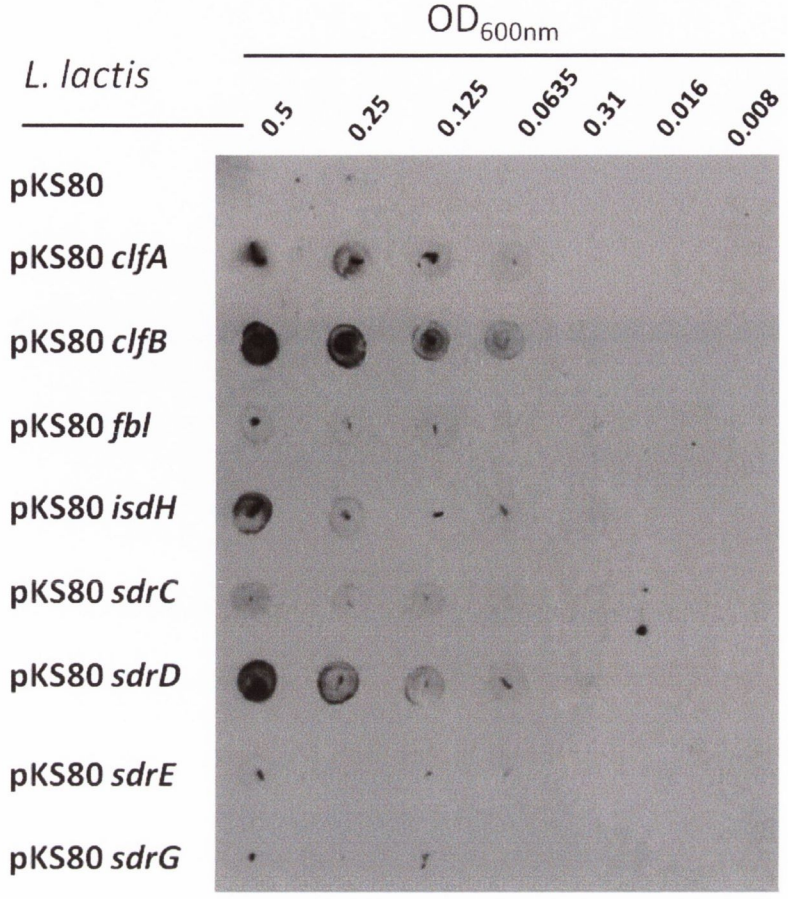


Figure 5.9 Whole cell dot ligand affinity blot of *L. lactis* expressing staphylococcal surface proteins binding factor I.

Whole cell dot ligand affinity blot of *L. lactis* expressing staphylococcal surface proteins ClfA, ClfB, Fbl, IsdH, SdrC, SdrD, SdrE or SdrG from pKS80. The strains are listed and the OD_{600} of the cells is indicated across the top of the blot. A suspension of cells (5 µl) at each concentration was spotted onto the membrane and probed with 10% normal human serum. Bound factor I was detected with murine monoclonal anti-factor I and HRP-conjugated rabbit anti-mouse IgG. *L. lactis* carrying pKS80 empty vector was used as a negative control.

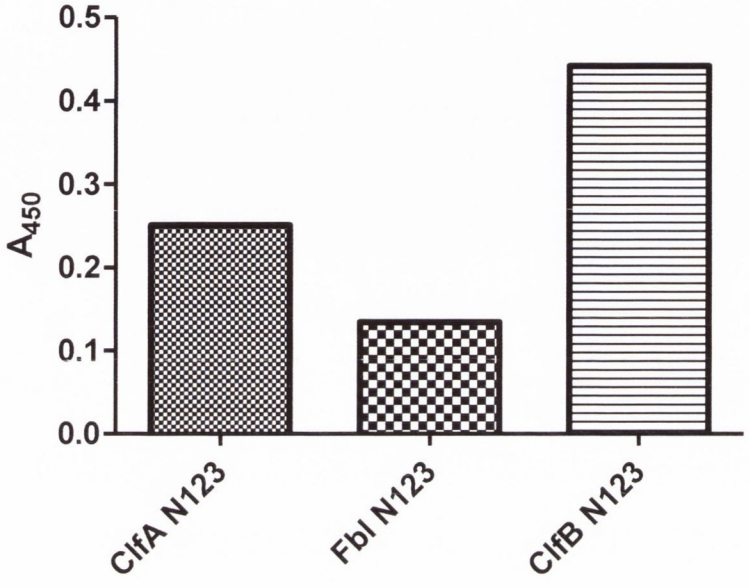


Figure 5.10 Capture of factor I by immobilised recombinant His-tagged ClfAN123$_{40-559}$, FblN123$_{40-533}$, and ClfBN23$_{201-542}$.

Wells of a microtitre plate were incubated overnight with 2 μM of each recombinant His-tagged protein. Coated wells were incubated with 10% normal human serum. Bound factor I was detected with monoclonal anti-factor I IgG and HRP-conjugated rabbit anti-mouse IgG. Wells were incubated with a chromogenic substrate and the A_{450} determined. Data points represent the mean of triplicate wells. The graph is representative of three independent experiments.

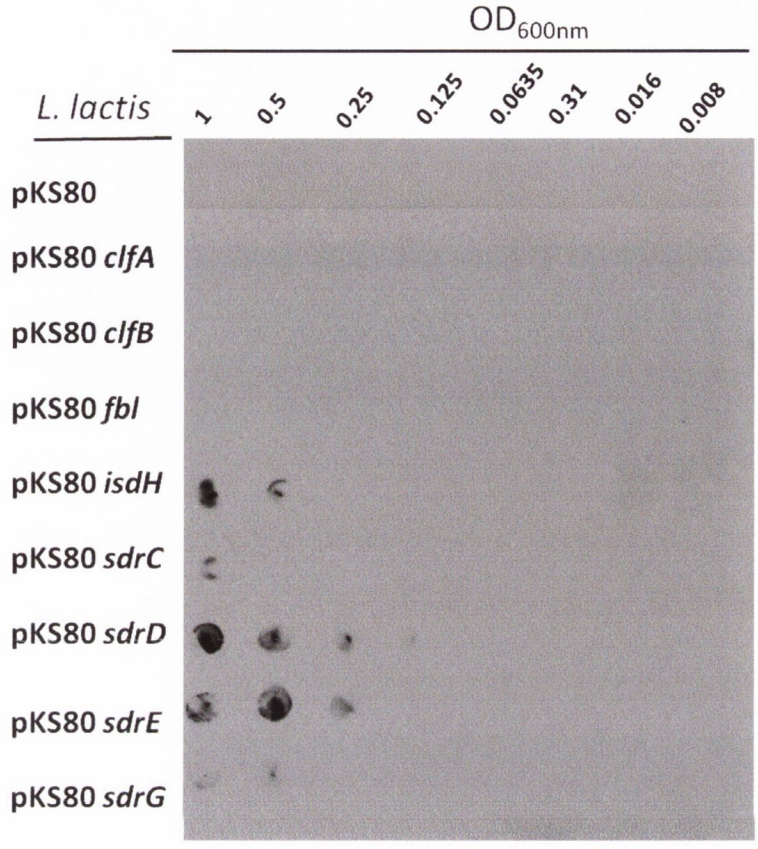


Figure 5.11 Whole cell dot ligand affinity blot of *L. lactis* expressing staphylococcal surface proteins binding factor H

Whole cell dot ligand affinity blot of *L. lactis* expressing ClfA, ClfB, Fbl, IsdH, SdrC, SdrD, SdrE or SdrG from pKS80. The strains are listed and the OD_{600} of the cells is indicated across the top of the blot. A suspension of cells (5 µl) at each concentration was spotted onto the membrane and probed with 10% normal human serum. Bound factor H was detected with murine monoclonal anti-factor H and HRP-conjugated rabbit anti-mouse IgG. *L. lactis* carrying pKS80 empty vector was used as a negative control.

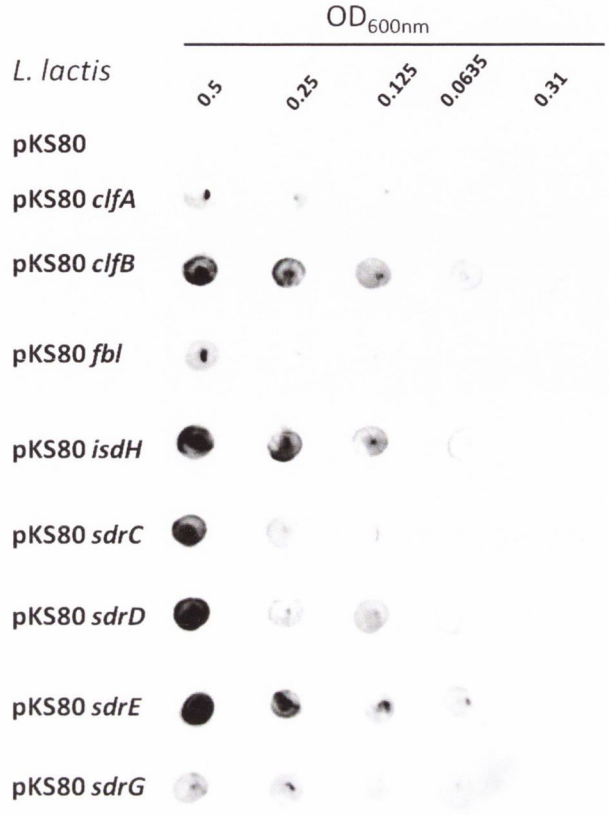


Figure 5.12 Whole cell dot ligand affinity blot of *L. lactis* expressing staphylococcal surface proteins binding C4BP

Whole cell dot ligand affinity blot of *L. lactis* expressing ClfA, ClfB, Fbl, IsdH, SdrC, SdrD, SdrE or SdrG from pKS80. The strains are listed and the OD_{600} of the cells is indicated across the top of the blot. A suspension of cells (5 µl) at each concentration was spotted onto the membrane and probed with 10% normal human serum. Bound C4-binding protein (C4BP) was detected with murine monoclonal anti- C4BP IgG and HRP-conjugated rabbit anti-mouse IgG. *L. lactis* carrying pKS80 empty vector was used as a negative control for C4BP binding by *L. lactis*.

surface proteins tested, IsdH, SdrD and SdrE were able to capture factor H from normal human serum. Of these proteins both IsdH and SdrD can interact with factor I. These results indicate that surface proteins may be capable of manipulating complement activation on the surface of the bacterial cells.

C4BP is another member of the RCA family. It contains multiple CCP repeats similar to factor H. Like factor H, C4BP also acts as a co-factor for factor I and can mediate breakdown of C4b to inhibit further complement activation. To determine whether staphylococcal surface proteins can bind C4BP whole cell dot ligand affinity blotting was performed on *L. lactis* expressing staphylococcal proteins ClfA, ClfB, Fbl, IsdH, SdrC, SdrD, SdrE or SdrG. Cells were spotted onto a membrane and incubated with 10% normal human serum. Bound C4BP was detected with monoclonal anti-C4BP IgG and HRP-conjugated rabbbit anti-mouse IgG (Fig 5.12). *L. lactis* expressing ClfB, IsdH, SdrC, SdrD and SdrE displayed an increase in C4BP binding above the level of the negative control. Of these proteins ClfB, IsdH and SdrD were also shown to interact with factor I. These results suggest that these proteins may be able modulate complement activation on the surface of the cell by manipulating factor I.

5.3 Discussion

ClfA of *Staphylococcus aureus* has been shown to bind to and activate complement factor I (Hair *et al.*, 2010b). This Chapter demonstrates that recombinant ClfA can capture factor I from normal human serum. This was initially illustrated by ligand affinity blotting where recombinant ClfAN123$_{40\text{-}559}$ could capture factor I from serum. A modified ELISA, where equimolar amounts of ClfAN123$_{40\text{-}559}$ and ClfAN23$_{221\text{-}559}$ were immobilized on the surface of microtitre plates demonstrated that subdomain N1 was not necessary for factor I binding. Intriguingly, immobilized His-tagged ClfAN23$_{221\text{-}559}$ did not mediate factor I binding. This is reminiscent of observations made in Chapter 3, that immobilized His-tagged ClfAN23$_{221\text{-}559}$ could not bind fibrinogen. It is tempting to speculate that factor I binding by ClfA is an active process which requires structural rearrangement similar to the 'dock, lock, latch' process of fibrinogen binding.

In order to determine which subdomains within the A region of ClfA were responsible for factor I binding, each of the individual GST-tagged ClfA subdomains were assayed for their ability to capture factor I from serum. Although the intact ClfAN123$_{40\text{-}559}$ region and the ClfAN23$_{221\text{-}559}$ regions can bind factor I, none of the individual subdomains were capable of promoting factor I binding. This result shows that the N23$_{221\text{-}559}$ region of ClfA must be intact for factor I binding.

Previously *S. aureus* espressing ClfAN23$_{221\text{-}559}$ P336A Y338S was shown to bind factor I more tightly and irreversibly than the wild type ClfA, but was unable to activate its serine protease activity. This Chapter demonstrates that recombinant ClfAN23$_{221\text{-}559}$ PYD binds factor I with the same affinity as wild type ClfA. However, the ability of ClfA PYD to activate factor I and the reversibility of the interaction was not examined. The observation that ClfA PYD binds factor I more tightly and irreversibly than the wild type ClfA suggests that fibrinogen binding may be important for factor I binding, and that the interaction may occur in the trench between the N2 and N3 region.

To determine if loss of fibrinogen binding by ClfA affects its ability to capture factor I, three ClfA variants with amino acid substitution in the second fibrinogen binding site at the top of the N3 subdomain were investigated. Although minor differences in the ability of ClfA variants to capture factor I at saturation were observed,

no difference in affinity was observed. These results demonstrate that fibrinogen binding is not necessary for factor I binding. This does not preclude the possiblity that fibrinogen binding is important in the interaction between ClfA and factor I. It may be that fibrinogen is required to activate factor I. Its is also possible that fibrinogen and factor I occupy similar or overlapping binding sites and that fibrinogen binding is necessary to allow dissociation of activated factor I.

This Chapter has demonstrated that ClfA can only interact with factor I in serum and not with purified factor I or which was pulled from serum using monoclonal anti-factor I IgG. These results strongly indicate that the interaction between ClfA and factor I cannot occur in the absence of one or more co-factors. Attempts to identify a cofactor for the ClfA-factor I interaction were unsucessful. Addition of fibrinogen, factor H, C4BP, C3b, C3, or factor H and fibrinogen at physiological concentrations was not sufficient to mediate the ClfA-factor I interaction. Interestingly, this Chapter also demonstrated that recombinant ClfA can interact with fibrinogen, factor H and C3 in serum. Although recombinant ClfA was capable of binding factor H, ClfA expressed on the surface of *L. lactis* was not. This suggests that the interaction is not of sufficient strength to promote bacterial attachement. Since *L. lactis* expressing ClfA could not promote factor H binding it is perhaps unsurprising that ClfA was not capable of promoting C4BP binding.These results demonstrate that the interaction between ClfA and factor I does not occur alone and may occur independently of the RCA proteins factor H and C4BP. The interaction between ClfA and factor I may be a multiprotein interaction, or require non-protein components to be reconstructed *in vitro*.

Functional redundancy is a common theme with staphylococcal surface proteins. This Chapter demonstrates that ClfA, ClfB, IsdH and SdrD were all capable of binding factor I when expressed on the surface of the surrogate host, *L. lactis*. Intriguingly although Fbl shares 60% sequence identity with ClfA it was not able to promote factor I binding on the surface of *L. lactis*. Homology modelling of Fbl on the crystal structure of ClfA shows that the majority of residues which differ between ClfA and Fbl are located on the surface of the protein. Since Fbl cannot promote factor I binding it is possible that factor I or cofactors required for the interaction may form contacts with ClfA on the surface of the protein. Chimeric ClfA-Fbl recombinant poteins could be used to investigate the regions within ClfA that are important for factor I binding. The factor I binding profiles of ClfB and Fbl were validated using recombinant ClfB and Fbl

confirming that lack of factor I binding by Fbl was not due to poor surface expression in *L. lactis*.

Although IsdH has previously been implicated as having a role in C3b degradation on the surface of the cell factor I binding was not studied and no mechanism was proposed. To date only the viral proteins Vaccinia complement control protein (VCP) and Kaposi's sarcoma associated herpesvirus complement control protein (KCP) have been shown to interact directly with factor I. These proteins mimic the CCP repeats found in members of the RCA family, enabling factor I recruitment. This Chapter demonstrates that IsdH can bind both RCA proteins factor H and C4BP. Based on these observations a mechanism for C3b degredation by IsdH can be proposed whereby IsdH first binds factor H or C4BP and the RCA protein in turn recruits factor I to mediate degradation of C3b or C4b, respectively. This ability to modulate the complement system at multiple stages of the cascade is frequently demonstrated by *S. aureus*. This ability to modulate complement activation explains why IsdH is a virulence factor (Visai *et al.*, 2009).

This is the first time that ClfB has been implicated in factor I binding and is primarily considerd to be a colonisation factor. As activation of factor I was not investigated in this Chapter it is unclear whether factor I binding by ClfB has any effect in immune evasion. However as ClfB also binds C4BP it seems unlikely that ClfB would be capable of interacting with two important complement regulatory proteins without modulating its response. It is possible that the role of ClfB in staphylococcal immune evasion has been heretofore masked by the pleothora of surface poteins capable of promoting immune evasion or it was tested with cells that do not express ClfB on their surface (eg stationary phase Newman). Although no active complement system has ever been demonstrated in the nares, it is possible that colonising staphylococci regularly encounter complement proteins in this location through micro-epistaxis events.

SdrD has never been implicated in modulating the complement immune response. In this Chapter SdrD has been shown to interact with the RCA proteins, factor H and C4BP in addition to factor I. This suggests that SdrD, like IsdH may be able modulate opsonin deposition on the surface of *S. aureus* at multiple stages of the complement cascade. This suggests SdrD may be an immunomodulatory factor worthy of further study.

Although SdrE demonstrated a clear ability to capture factor H and C4BP, it did not bind factor I. Recently a study was published highlighting a role for SdrE in factor H binding (Sharp *et al.*, 2012). Another study has demonstrated that *S. aureus* can capture C4BP from serum, but no binding partners have been identified (Hair *et al.*, 2012). This Chapter demonstrates that SdrE can promote C4BP binding in addition to factor H. It is unclear why factor I was not recruited, but it is possible that *S. aureus* can manipulate its RCA binding partners decay accelerating functions to facilitate premature dissociation of the C3 convertase to slow complement activation.

Mechanisms of immune evasion are crucial for survival of any sucessful pathogen. *Staphylococcus arueus* is well documented for its success as a human pathogen, and ability to evade the human immune system. This Chapter highlights the complex nature of the interaction between ClfA and factor I. Although it is clear from the data that an interaction between ClfA and factor I alone does not occur, it is unclear what additional factor or factors are required to faciliate this interaction. This Chapter demonstrates for the first time that multiple staphylococcal surface proteins are capable of binding members of the RCA family and factor I. These results indicate that these proteins may play important roles in survival of bacteria in human blood. Furthermore, although only two surface proteins were examined from coagulase negative staphylococci, Fbl and SdrG, neither were capable of binding the complement regulatory proteins tested. This is consistant with observations that coagulase negative bacteria are often less pathogenic than their coagulase positive counterparts.

Chapter 6
Discussion

6.1 Discussion

S. aureus is an important pathogen that causes serious and life threatening community acquired and nosocomial infections. Although MSCRAMMs play a pivotal role during colonization and establishment of *S. aureus* infection very little is known about how these proteins are directed to the secretion apparatus (Sibbald *et al.*, 2006, Schneewind and Missiakas, 2012). Members of the MSCRAMM family possess an N-terminal signal sequence, with a classic tripartite structure that is predicted to mediate their translocation across the cytoplasmic membrane by the general secretory (Sec) pathway (Sibbald *et al.*, 2006, DeDent *et al.*, 2008). The Sec system in *S. aureus* consists of homologues of the canonical SecYEG translocation channel and the SecA ATPase. However no homologue of the general secretory chaperone SecB has ever been identified. Studies have shown that MSCRAMM proteins are exported by the Sec system with the other major secretory pathways (twin-arginine and accessory Sec system) playing no role (DeDent *et al.*, 2008). However, virtually nothing is known about how proteins of the MSCRAMM family are trafficked to the Sec apparatus (Sibbald *et al.*, 2006, Schneewind and Missiakas, 2012). Previous studies identified a 'YSIRK-GS' motif in the signal sequence of MSCRAMMs and certain other surface proteins (DeDent *et al.*, 2008). This feature is required for the proteins to be targeted to the cross-wall of the cell but molecular details of the mechanism are lacking (Schneewind and Missiakas, 2012, DeDent *et al.*, 2008). Studies have demonstrated that surface display of *S. aureus* surface proteins is reduced in mutants lacking proteins with abortive infectivity domains encoded by the *spdA, spdB or spdC* genes but the molecular basis of this is not understood (Frankel *et al.*, 2010).

Clumping factor A displays the classical structural features associated with MSCRAMMs of *S. aureus*. Under the reclassification of staphylococcal cell wall-associated proteins the defining features of the Clf-Sdr group are (i) the A region and (ii) the long flexible unfolded region linking the A region to the cell wall spanning/anchoring domain. The A region comprises two independently folded subdomains N2 and N3, which adopt IgG-like folds. MSCRAMMs like ClfA which have a long flexible unfolded repeat region have an N-terminal subdomain N1 with no known function. This study set out to define a role for the N1 subdomain of ClfA as a prototypical N1 subdomain. Here, for the first time a crucial role for subdomain N1 in export and surface expression of MSCRAMMs is identified.

This study has demonstrated that residues 211-220 of N1 of ClfA are required for the protein to be translocated across the membrane so that it can be anchored by sortase to cell wall peptidoglycan. Deletion mutants lacking N1 were not exported but instead accumulated in the cytoplasm and the membrane. This could only be detected in bacteria where ClfA expression was tightly repressed and was only expressed after addition of an inducer. Constitutive expression of an N1 deletion mutant following transformation into *S. aureus* resulted in a very low yield of transformants. This was likely due to the failure of cells attempting to express the mutant constitutively to survive following the shock of electroporation. Those colonies that grew had plasmids with rearrangements which presumably prevented protein expression. Induction of the truncated *clfA* mutant in established *S. aureus* culture caused a significant loss of fitness compared to cells expressing wild-type ClfA. In an established culture of cells growing exponentially in rich medium the effect of inducing expression was less dramatic causing loss of fitness but not complete cessation of growth. Although the nature of the transformation and competitive growth assays is distinct both clearly demonstrate that attempting to express the protein lacking N1 impairs growth.

It is unclear why loss of subdomain N1 results in attenuated growth and failure to form colonies. No increase in cell size or morphology was observed by SEM or TEM in cells lacking subdomain N1. Furthermore improper localisation of ClfA variants lacking N1 did not affect the localisation of another MSCRAMM SdrE. It is possible that ClfA variants lacking N1 aggregate within the cytoplasm inhibiting their secretion and triggering a stress response (Bednarska *et al.*, 2013). Aggregation of recombinant cytoplasmic proteins has been shown to affect membranes in Gram-negative bacteria, resulting in rearrangements that modulate membrane trafficking, nutrients, metabolites and other small solutes (Ami *et al.*, 2009).

Dissection of the N1 subdomain of ClfA revealed that a stretch of 10 residues close to the boundary between N1 and N2 is necessary for surface expression, and that only this region plus the 8 residues that join it to the beginning of N2 as defined by the X-ray crystal structure is necessary. This is the first time a region which is necessary for surface expression of a pre-protein but is distinct from the signal sequence has been identified in *S. aureus*. It is possible that other surface proteins and secreted factors also contain previously unrecognised export motifs. This finding highlights the serious dearth in our understanding of the secretion process in Gram-positive organisms.

We have identified a new region of the MSCRAMM required for its export, but the mechanism involved is not yet understood. In the absence of a fundamental understanding of pre-protein trafficking in *S. aureus* it is difficult to speculate about the functions of N1. Figure 6.1 illustrates several potential mechanisms through which the N1 subdomain might facilitate export of a pre-protein. It is possible that residues 211-220 could engage a molecular chaperone that guides the long protein from the ribosome to the Sec translocation apparatus (Fig 6.1A). Recently ClfA has been implicated in an interaction with the ATP dependent ClpC chaperone (Graham *et al.*, 2013). It is possible that residues 211-228 form crucial contacts with ClpC, aiding in export of the pre-protein. This interaction is an interesting avenue for future work on elucidating a mechanism for residues 211-228. It is also possible that the N1 subdomain may contain residues necessary for interacting specifically with the Sec machinery (Fig 6.1B) Alternatively, the N1 subdomain may act as a cytoplasmic chaperone by directly binding to another region within the ClfA pre-protein helping to maintain it in an export competent state (Fig 6.1C,D).

As yet no function has been identified for residues 40–210 of ClfA. These residues are not required for fibrinogen or factor I binding by recombinant proteins, or for fibrinogen binding on the surface of *S. aureus*. Furthermore this region does not promote better survival of *S. aureus* in blood, nor does loss of this region affect antibody recognition of recombinant ClfA. Although no function for this region is evident, it is possible that it performs a function in *S. aureus* under conditions not examined in this study and the role of this region warrants further investigation.

In addition to ClfA, this study has demonstrated that FnBPB also requires its N1 subdomain for export and correct localisation of the pre-protein. However, when the N1 subdomains of ClfA and FnBPA were compared a common motif for export could not be located. Twenty-eight additional residues at the C-terminus of FnBPB are not sufficient to mediate correct localisation of the pre-protein. Furthermore, residues 211-220 of ClfA were not sufficient to mediate surface expression of FnBPB lacking its N1 subdomain. These results strongly suggest that a distinct region of N1 is necessary for correct localisation of FnBPB. This is perhaps not surprising when the likely mechanisms for N1 are considered. If subdomain N1 acts as a cytoplasmic chaperone by binding regions within the pre-protein, distinct motifs may be required for each MSCRAMM. If N1 acts as a recognition site for specific molecular chaperones,

different chaperones may require distinct binding motifs. Chimeric MSCRAMMs where the N1 subdomains of two MSCRAMMs are reciprocally replaced could be used to determine if N1 subdomains can functionally substitute for each other.

Truncates of ClfA and FnBPB lacking the long unstructured repeat region are exported and cell wall anchored in the absence of N1. This suggests that subdomain N1 is required to aid transport of the long flexible repetitive repeat regions. This is particularly intriguing considering that the nature of the repeat regions in ClfA and FnBPB is very different. The common feature between the two being that the region is long and unstructured. If N1 subdomains act as molecular chaperones specific for long unstructured regions then they will not be functionally interchangeable as the sequence of the Clf-Sdr repeat region is completely different from that of the fibronectin binding repeat region of FnBPs. Further work investigating the number of flexible repeats for which subdomain N1 is necessary to promote export may provide insights into the mechanism of action.

The only MSCRAMM of *S. aureus* which does not contain an N1 subdomain lacking a predicted secondary structure is collagen binding protein (CNA). CNA mediates binding to collagen by the 'collagen hug' mechanism. In this mechanism the N1 and N2 subdomains fold independently to form IgG-like folds. Collagen binding occurs in the trench at the intersection of the N1 and N2 subdomains. Unlike other members of the MSCRAMM family CNA also lacks a long unstructured repeat region, containing a B repeat region which adopts a secondary structure of an inverse IgG-like fold (Deivanayagam *et al.*, 2000). It is possible that a distinct region is not necessary in CNA to promote correct localisation of the pre-protein in the absence of a long unstructured region. Conversely a distinct region may be required but may not be located adjacent to the signal sequence. In CNA the N3 subdomain has no defined function, and a role in protein localisation has never been investigated.

The conserved architecture observed in all MSCRAMMs with the exception of CNA suggests that the N1 subdomain has a conserved role in surface expression of all MSCRAMMs. A diverse family of surface proteins, MSCRAMMs are also found in other staphylococcal species including Fbl of *S. lugdunensis* and SdrG of *S. epidermidis* and a role for the N1 subdomain in protein export has never been investigated in these organisms. This hypothesis could be tested by expressing variants of these proteins lacking subdomain N1 from pRMC2 and examining their cellular localisation profiles.

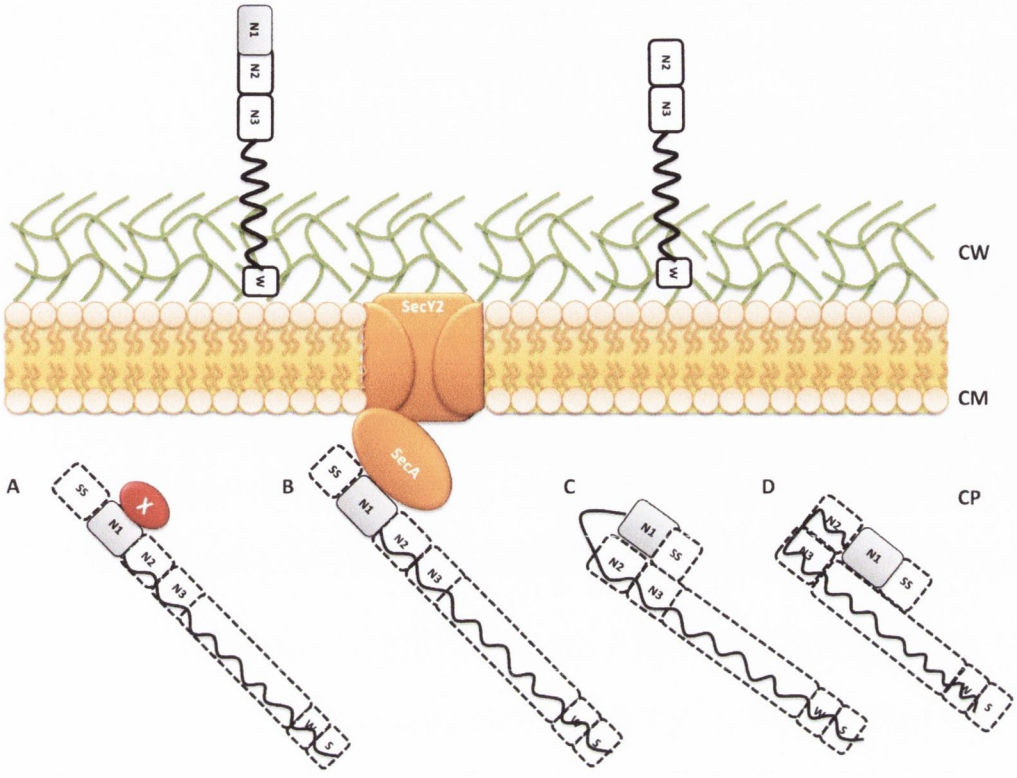


Figure 6.1 Schematic representation of the N1 subdomain dependent export of ClfA.

(A) The N1 subdomain may contain a binding site necessary for recognition of a specific or general secretory chaperone (X) represented by the red oval. **(B)** The N1 subdomain may interact directly with components of the Sec machinery to facilitate exprort of the pre-protein. **(C)** The N1 subdomain may act as a molecular chaperone maintaining the pre-protein in an export competent state by binding within the A region or **(D)** by binding within the long unstructured repeat region.

Further insight into the mechanism through which subdomain N1 mediates surface expression is necessary. Currently, the majority of antibiotics used in the treatment of MRSA infections target bacterial cell wall or protein synthesis. With increasing antibiotic resistance and no effective vaccination strategies novel therapeutic avenues need to be explored. Loss of residues 211-220 in the N1 subdomain of ClfA dramatically affects both fitness of *S. aureus* and expression of a conspicuous virulence factor. If similar regions can be identified in other MSCRAMMs it is possible that small molecule inhibitors could be utilised to help attenuate virulence of *S. aureus in vivo.* Such inhibitors could simultaneously target multiple virulence factors and could potentially be used in concert with conventional therapies. Alternatively if key MSCRAMM chaperones could be identified these could be targeted to disregulate normal MSCRAMM expression. This could represent a viable alternative therapeutic strategy simultaneously targeting factors involved in colonisation, immune evasion and pathogenesis.

The use of anti-virulence and anti-immunomodulatory strategies has been previously suggested for the treatment of MRSA infections. Sortase A inhibitors have been developed which inhibit surface expression of cell wall associated proteins which contain an LPXTG motif *in vitro* (Fitzgerald-Hughes *et al.*, 2012). However, these proteins would be secreted into the extracellular milieu, where certain proteins may still function as virulence factors. Currently, pharmaceutical companies still favour the development of next generation classical antibiotics. Minor changes in drugs where the safety and efficacy is well established *in vivo* and which are amenable to pharmaceutical preparation represent a more favourable risk:benefit ratio than the more risky venture of an entirely novel class of anti-infective. However, in the long term it seems likely that pharmaceutical companies will have to explore alternative anti-staphylococcal agents with alternative and multifaceted modes of antibacterial activity.

Although subdomain N1 is necessary for surface expression of ClfA no role could be attributed to the N1 subdomain on the surface of *S. aureus*. Investigation into the interaction between ClfA and factor I revealed that the N1 subdomain was not involved, but that an intact N23 region is required to promote factor I binding. Amino acid substitutions which abrogate fibrinogen binding, located both in the ligand binding trench and at the top of the N3 subdomain, do not significantly reduce factor I binding. These residues may still form contacts with factor I but are not essential for the

interaction to occur. Furthermore Fbl, a homologue of ClfA with ~60% identity in the N23 region does not bind factor I. Since the majority of different residues between ClfA and Fbl are located on the surface exposed regions of Fbl, it is likely that factor I makes contact with ClfA on surface exposed regions of N23. Previous studies have shown that the ClfAPY variant which cannot bind fibrinogen binds factor I but cannot activate or release it. Factor I is a large multi-domain protein which may make contact at multiple regions in ClfA both on the surface and within the trench region. It is possible that although fibrinogen binding is not necessary for factor I binding it may be necessary to displace the activated factor I molecule.

Although binding of complement regulatory proteins by surface proteins and their contribution to immune evasion has been well documented in invasive pathogens such as meningococci and streptococci relatively little is known in *S. aureus* (Laarman *et al.*, 2010). The mechanisms through which staphylococcal surface proteins interact with complement regulatory proteins is summarised in Fig 6.2. Surface proteins can bind members of the RCA family directly, possibly facilitating the premature decay of C3 and C5 convertases. Acceleration of the decay of the complement convertases inhibits further opsonisation of bacteria and inhibits the release of the anaphylatoxins C3a and C5a (Fig 6.2i). Surface proteins which recruit members of the RCA family may also recruit factor I. Factor I may then promote the degradation of C3b to iC3b and reduce phagocytosis (Fig 6.2ii). The ClfA-factor I interaction was originally reported to be a direct interaction. However, no experimental evidence of a direct ClfA-factor I interaction has ever been demonstrated. Attempts to reconstitute the ClfA-factor I interaction with purified proteins were unsuccessful. To date only viral proteins which express molecular mimics of the RCA family have been shown to bind factor I directly (Mullick *et al.*, 2005, Mark *et al.*, 2007). It is clear that ClfA does not interact with factor I in the absence of a co-factor, but binding to factor H or C4-binding protein was not observed demonstrating that ClfA requires an undiscovered co-factor to bind and activate factor I. If correct, this represents an entirely new mechanism of circumventing complement activation by recruiting complement factor I through an undiscovered intermediate.

Functional redundancy is a common feature observed in staphylococcal surface proteins. This is now exemplified by the multitude of *S. aureus* surface proteins capable of binding proteins which modulate complement activation. In *S. aureus* all of the

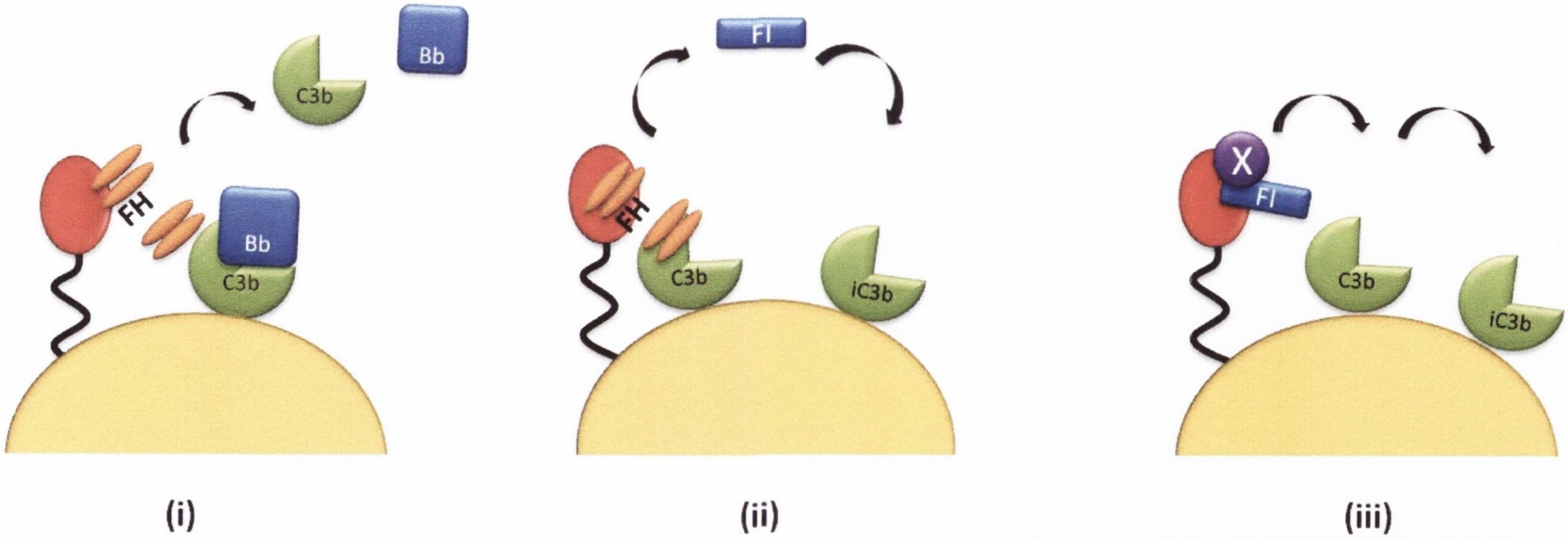


Figure 6.2 Mechanisms for modulating the activity of complement regulatory proteins by staphylococcal surface proteins.

Staphylococcal surface proteins can bind directly to members of the regulators of complement activation (RCA) family such as factor H (FH, orange ovals) and C4 binding protein (C4BP) and **(i)** accelerate the decay of complement C3 and C5 covertases or **(ii)** recruit factor I (FI) to facilitate the degradation of C3b or C4b on the surface of the cell. **(iii)** Alternatively staphylococcal surface proteins can interact with FI in the absence of FH or C4BP.

surface proteins tested displayed interactions with either a member of the RCA family, or factor I. However only SdrD and IsdH displayed interactions with all three complement regulatory proteins tested. This level of functional redundancy and the expression of surface proteins which can modulate complement activation through-out all stages of growth suggests that evasion of complement activation is crucial for bacterial survival. To examine the biological significance of staphylococcal surface proteins binding to members of the RCA family it will be necessary to test their ability to protect *L. lactis* from complement mediated opsonophagocytosis in human blood.

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